Hybrids of the killifish species *Fundulus diaphanus* and *F. heteroclitus* are diploid unisexual gynogens producing genetically identical, all-female offspring without a genetic contribution from males. We used isozymes to assess clonal diversity at the two localities in Nova Scotia where the unisexual hybrids are known to occur. Four isozyme loci, fixed for different alleles in the two parental species and heterozygous in the hybrids, served to distinguish the three taxa. In addition to these four loci, *ADA* was polymorphic in *F. diaphanus* but monomorphic in nearly all of the hybrids, which suggests that clonal diversity cannot be high among the hybrids. And *sIDHP*, fixed for different alleles in *F. diaphanus* and *F. heteroclitus*, appeared homozygous in nearly all hybrids for the allele characteristic of *F. heteroclitus*. This homozygosity may be the result of gene regulation, a null allele, a rare recombinational event, or undetected variability in *F. diaphanus*. Some of these explanations suggest a rare event in the history of these homozygous unisexuals, indicating that they comprise a single clone, although other explanations for this homozygosity do not rule out the presence of multiple clones.
Three individuals identified as triploid by flow cytometry; see text.

Table 1. Presumptive isozyme genotypes observed in F. heteroclitus, F. diaphanus, and diaphanus × heteroclitus hybrids from Porter’s Lake and the St. Mary’s River estuary, Nova Scotia

<table>
<thead>
<tr>
<th></th>
<th>N Porter’s</th>
<th>N St. Mary’s</th>
<th>ADA* (4, M)</th>
<th>GPA* (1, EM)</th>
<th>sIDHP* (1, ELM)</th>
<th>LDHA* (1, M)</th>
<th>sMDHA* (1, M)</th>
<th>MPI* (4, M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F. heteroclitus</td>
<td>51</td>
<td>65</td>
<td>100/100</td>
<td>64/64</td>
<td>77/77</td>
<td>100/100</td>
<td>100/100</td>
<td>90/90</td>
</tr>
<tr>
<td>F. diaphanus</td>
<td>16</td>
<td>0</td>
<td>70/70</td>
<td>100/100</td>
<td>100/100</td>
<td>41/41</td>
<td>50/50</td>
<td>100/100</td>
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<tr>
<td></td>
<td>56</td>
<td>10</td>
<td>70/64</td>
<td>100/100</td>
<td>100/100</td>
<td>41/41</td>
<td>50/50</td>
<td>100/100</td>
</tr>
<tr>
<td></td>
<td>64</td>
<td>140</td>
<td>64/64</td>
<td>100/100</td>
<td>100/100</td>
<td>41/41</td>
<td>50/50</td>
<td>100/100</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>3</td>
<td>70/64</td>
<td>120/100</td>
<td>100/100</td>
<td>41/41</td>
<td>50/50</td>
<td>100/100</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>7</td>
<td>64/64</td>
<td>120/100</td>
<td>100/100</td>
<td>41/41</td>
<td>50/50</td>
<td>100/100</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0</td>
<td>70/64</td>
<td>100/100</td>
<td>100/100</td>
<td>41/41</td>
<td>50/50</td>
<td>100/100</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0</td>
<td>64/64</td>
<td>100/100</td>
<td>100/100</td>
<td>41/41</td>
<td>50/50</td>
<td>100/100</td>
</tr>
<tr>
<td>Female hybrids</td>
<td>139</td>
<td>108</td>
<td>100/64</td>
<td>100/64</td>
<td>77/77</td>
<td>100/41</td>
<td>100/50</td>
<td>100/100/90</td>
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<tr>
<td></td>
<td>0</td>
<td>2</td>
<td>100/64</td>
<td>100/64</td>
<td>100/100</td>
<td>41/41</td>
<td>50/50</td>
<td>100/100</td>
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<tr>
<td></td>
<td>1</td>
<td>0</td>
<td>100/70</td>
<td>100/70</td>
<td>100/70</td>
<td>41/41</td>
<td>50/50</td>
<td>100/100</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0</td>
<td>100/100</td>
<td>100/100</td>
<td>100/100</td>
<td>41/41</td>
<td>50/50</td>
<td>100/100</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>1</td>
<td>100/100</td>
<td>100/100</td>
<td>100/100</td>
<td>41/41</td>
<td>50/50</td>
<td>100/100</td>
</tr>
<tr>
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<td>0</td>
<td>100/100/64</td>
<td>100/100/64</td>
<td>100/100/77</td>
<td>100/41/41</td>
<td>100/50/50</td>
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<tr>
<td></td>
<td>3*</td>
<td>0</td>
<td>100/100/64</td>
<td>100/100/64</td>
<td>100/100/77</td>
<td>100/41/41</td>
<td>100/50/50</td>
<td>100/100/90</td>
</tr>
</tbody>
</table>

Allelic variants at each locus were resolved from homogenates of eye (E), liver (L), or muscle (M) using buffer 1 or 4 of Turner (1983) and were named by their relative mobility.

* Three individuals identified as triploid by flow cytometry; see text.
is exceptionally polymorphic further south (Powers et al. 1986).

The remainder of this study, involving a much larger sample of fish and summarized in Table 1, focused on the two isozyme loci among the 20 that provided information on clonal diversity (\(ADA^*\) and \(sIDHP^*\)), plus four others (\(GPI\), \(LDH\), \(sMDHA\), and \(MPI\)) that were diagnostic (or nearly so) for the parental species and their hybrids.

Of the 20 loci examined initially in fish from Porter’s Lake and the St. Mary’s River, only \(ADA^*\) in \(F. diaphanus\) exhibited a second allele with a frequency greater than 2%. In this study, \(ADA^*\) was highly polymorphic among \(F. diaphanus\) in Porter’s Lake (Table 1; frequency of \(ADA^* \geq 0.68\); frequency of \(ADA^* \geq 0.32\), \(N = 139\)), but much less polymorphic among \(F. diaphanus\) in the St. Mary’s River (Table 1; frequency of \(ADA^* \geq 0.96\); frequency of \(ADA^* \geq 0.77\)).

At Porter’s Lake nearly all hybrids possessed an \(ADA^*100/64\) genotype, the \(ADA^*64\) allele presumably inherited from \(F. diaphanus\) and the \(ADA^*100\) allele inherited from \(F. heteroclitus\). The \(ADA^*70\) allele, also common in \(F. diaphanus\) at this site, was seen in only 2 of 146 hybrids examined (Table 1; Figure 2). All St. Mary’s River hybrids possessed an \(ADA^*100/64\) genotype.

The isozyme locus \(sIDHP^*\) provided a different type of evidence for clonal diversity. At both localities \(F. diaphanus\) is fixed for an \(sIDHP^*100\) allele and \(F. heteroclitus\) is fixed for an \(sIDHP^*77\) allele. We expected the hybrids, therefore, to be fixed heterozygotes with an \(sIDHP^*100/77\) genotype, as is true for the other diagnostic loci. Instead, most wild hybrids examined at both localities (\(N = 250\)) appear to be \(sIDHP^*77/77\) homozygotes, identical to \(F. heteroclitus\) (Table 1; Figure 3). In contrast, offspring of reciprocal \(F. diaphanus \times F. heteroclitus\), \(N = 3\), and \(F. diaphanus \times F. heteroclitus\), \(N = 3\) exhibited at \(sIDHP^*\) the three bands characteristic of heterozygotes for a dimeric enzyme (Figure 3). We will refer to the 247 wild hybrids that share the \(ADA^*100/64\) genotype and the (apparent) \(sIDHP^*77/77\) genotype as the “major allozyme clone,” bearing in mind that it may represent more than one true clone.

Triploids are found in low frequency among the wild hybrids (Dawley 1992). Of 51 hybrids examined in this study for genome size by flow cytometry (all from Porter’s Lake), 48 were diploid and three were triploid (Table 1). The three triploids showed the allozyme genotypes of the “major allozyme clone,” but with dosage effects consistent with a double dose of the \(heteroclitus\) genome (Figure 4). Among those hybrids not examined by flow cytometry, one showed allozymic evidence of triploidy, exhibiting one dose each of the \(ADA^*64, ADA^*70,\) and \(ADA^*100\) alleles and, at other loci (except \(sDHP^*\)), showing dosage effects consistent with a double dose of the \(diaphanus\) genome (Table 1).

Seven other wild diploid hybrids showed various allozyme phenotypes (Table 1) that could have resulted from \(F. diaphanus\) hybridization, backcrossing by sexually reproducing \(F. diaphanus\) hybrids, or occasional recombinational or mutational events within the unisexuals. Two hybrids (St. Mary’s River) had the same allozyme phe-
notypes as the "major allozyme clone" except that they showed the heterozygous sIDHP 100/77 phenotype seen in the laboratory-raised F₁ hybrids. A third hybrid (Porter's Lake) differed from the "major allozyme clone" at both sIDHP* and ADA*, exhibiting an sIDHP 100/77 phenotype instead of sIDHP 77/77 and an ADA 100/70 phenotype instead of ADA 100/64. One hybrid (Porter's Lake) showed the heterozygous sIDHP 100/77 phenotype, but was homozygous for the ADA*100 allele that is characteristic of F. heteroclitus. Two other hybrids (Porter's and St. Mary's) showed the sIDHP 77/77 phenotype characteristic of the "major allozyme clone" but also were homozygous for the ADA*100 allele that is characteristic of F. heteroclitus. Finally, one hybrid (St. Mary's River) exhibited the phenotype of the "major allozyme clone" except that it was heterozygous for an ADA 120 allozyme, observed nowhere else in this study.

Discussion

The great majority of diaphanous × heteroclitus hybrids examined from the two Nova Scotia localities shared an ADA*100/64 genotype and an (apparent) sIDHP*77/77 genotype. We have referred to hybrids possessing this genotype as members of the "major allozyme clone." Does this allozyme "clone" represent a true clone, a single lineage derived from one original hybridization event? Or might it represent a collection of clones that happen to share a genotype at these two loci?

Among Porter's Lake (but not St. Mary's River) hybrids, two ADA* alleles were found in sufficiently high frequency (0.68 for ADA*64 and 0.32 for ADA*70) that one would expect to find both represented among multiple clones of separate hybrid origin (Figure 1). Instead, nearly all hybrids from that locality possessed the ADA*64 allele. The most we can conclude from this, however, is that the diversity of true clones within the "major allozyme clone" at Porter's Lake is not high. Several clones, distinct at other loci, by coincidence could have inherited the same ADA*64 allele from the diaphanus gene pool.

The isozyme locus sIDHP* provides somewhat stronger evidence that the "major allozyme clone" may represent one true clone. Although the laboratory-raised F₁ hybrids examined here showed the heterozygous sIDHP 100/77 phenotype expected of hybrids at this diagnostic locus, the wild hybrids that comprise the "major allozyme clone" appeared homozygous for the sIDHP*77 allele characteristic of F. heteroclitus. Such unexpected homozygosity has been observed in clones of the urodele lizard Cnemidophorus lemniscatus, which are homozygous at several loci where one would expect heterozygosity based on the genotypes of their presumptive parental species (Sites et al. 1990). Sites et al. attributed homozygosity at these loci to rare recombinational events, null alleles, or undetected variability within the parental species.

If apparent homozygosity at sIDHP* is the result of some rare event in the hybrids' past, this suggests that all hybrids exhibiting this trait share the same ancestry, a rare event being one that is unlikely to have happened twice. Recombination in an ancestor of the current hybrids, for example, could have converted the original sIDHP*100/77 genotype to sIDHP*77/77. Experimental crosses of the female hybrids show no evidence that such recombination occurs; reproduction appears to be purely clonal (Dawley 1992). Several hybrids were observed in this study to be homozygous for the ADA*100 allele characteristic of F. heteroclitus, suggesting that recombination had occurred in their ancestry. But it is unclear whether these hybrids were the recombined descendants of the clonal gynogens or simply backcrossed progeny of recently derived, sexually reproducing hybrids.

Alternatively, the sIDHP*100 allele may be present in hybrids of the "major allozyme clone" as a null allele (Spinella and Vrijenhoek 1982), inactivated by a mutation that prevents the formation of an active heterodimer as well as the sIDHP 100/100 homodimer. Again, the rarity of such an event would argue for a single origin of...
the putative clone. Unique or null alleles have been interpreted similarly in other unisexual systems. The diploid gyenogen
Menidia clarkhubbsi, for example, possesses a unique allele, PGM*Ad, unknown in its putative parental species (Echelle and Mosier 1981). Either this allele arose by mutation in the common ancestor of all currently known clones, or it is derived from a parental population yet to be detected despite some searching (Echelle et al. 1983, 1989). A number of clones of Poeciliopsis are characterized by unique or null alleles at isozyme and mitochondrial loci (Quattro 1992a), as are clones of the Iberian Tropidophoxinellus albunoides complex (Carmona et al. 1997).

A different class of explanation for apparent homozygosity at sIDHP* among most wild hybrids is that a second s-IDHP*77 allele actually was inherited from their F. diaphanus parent(s), either because this allele currently exists, but rarely, among F. diaphanus in the localities under study, or because it was more common in the past and has since disappeared by drift or under selection, or because the hybrids originated elsewhere, at a site where the sIDHP*77 allele does occur within the gene pool of F. diaphanus. The first of these possible explanations again argues for a rare event and a single origin for the “major allozyme clone,” the latter two argue at least that this phenotype, however many true clones it represents, is not a recent creation by in situ hybridization.

Several wild hybrids, whose multilocus genotypes exclude them from membership in the “major allozyme clone,” suggest yet another possible explanation for apparent homozygosity at sIDHP*. One individual, a triploid exhibiting one dose each of the ADA*64, ADA*70, and ADA*100 alleles and dosage effects at other loci indicating that it had a double dose of the diaphanus genome, nevertheless exhibited only the s-IDHP 100/100 homodimer. If this triploid was derived from the “major allozyme clone” by the fertilization of a diploid clonal egg (containing ADA*100/64) by a diaphanus sperm (containing ADA*70), it also should have been homozygous at sIDHP*.

That it was not suggests that repression of expression of the sIDHP*100 allele occurred in this hybrid. Such repression of maternal or paternal alleles is observed occasionally in interspecific hybrids (Castro-Sierra and Ohno, 1968; Danzmann and Down 1982; Whitt et al. 1972). If repression occurs routinely at this locus in diaphanus × heteroclitus hybrids, then the single-banded s-IDHP 77/77 phenotype that we see in the hybrids of the “major allozyme clone” provides no information on clonal diversity; it merely confirms that these hybrids are hybrids. However, because the F₁ hybrids examined here showed no such repression at sIDHP*, we tentatively conclude that repression of sIDHP*100 is not routine, that it is possibly a characteristic of the “major allozyme clone” but not of diaphanus × heteroclitus hybrids in general.

Three other hybrids, not members of the “major allozyme clone,” exhibited the sIDHP 77/77 phenotype along with apparent recombination or mutation of alleles at ADA*. If these three individuals represent recombinant or mutant derivatives of the “major allozyme clone,” then their homozygosity at sIDHP* could still be the result of a single rare event. If, however, they represent backcrossed descendents of recently derived, sexually reproducing hybrids, with separate origins of homozygosity at sIDHP*, then sIDHP* loses its value as a marker for a single hybrid origin.

We had expected that clonal diversity might be high among the unisexual hybrids found in Porter’s Lake and the St. Mary’s River because there appeared to be ample opportunities for hybridization to occur between their sexual progenitors, F. heteroclitus and F. diaphanus. The little information that exists suggests that these species may hyrbidize when sympatric: their spawning times and habits are similar (Scott and Crossman 1973), and hybrids are readily obtained from artificial crosses (Newman 1914). That at least some hybrids have formed in situ at these two sites is suggested by the four wild hybrids that exhibited the heterozygous s-IDHP 100/77 phenotype seen in F₁ crosses. Of these four, two possessed the more common (among F. diaphanus) ADA*64 allele, one the less common (among F. diaphanus) ADA*70 allele, and one (a result of backcrossing?) was homozygous for the ADA*64 allele characteristic of F. heteroclitus. That sexually reproducing F₁s may occasionally arise in situ is also suggested by evidence of introgression into F. heteroclitus of alleles, at ADA* and MPI*, normally found in F. diaphanus.

Recurrent hybridization, of course, provides no guarantee of high clonal diversity. The F₁ female hybrids that arise from such hybridizations must surmount several barriers to found successful clones (Vrijenhoek 1989). The parental genomes of the hybrids must be sufficiently incompatable to disrupt meiosis and trigger some form of asexual reproduction, but sufficiently coadapted that the hybrids are vigorous and fertile. In addition, the parental genomes must combine to yield a phenotype that allows the hybrids to fill an available niche or outcompete others for a niche that is already filled. In Poeciliopsis, for example, unisexuals can be re-created in the laboratory by hybridizing the appropriate sexual parental species (Schultz 1973), but few of these F₁ females have the viability or fertility of successful clones from nature (Wetherington et al. 1987). And even vigorous new clones might have difficulty inserting themselves into a natural community where already-established clones and hemiclones of Poeciliopsis occupy distinct niches (Schenck and Vrijenhoek 1989). Nevertheless, the genetic evidence is clear that recurrent hybridization has led to high clonal diversity among unisexual biotypes of Poeciliopsis (Quattro et al. 1991, 1992a; Vrijenhoek et al. 1978), as well as in Menidia clarkhubbsi (Echelle et al. 1989), triploid (but not diploid) Poecilia formosa (Turner et al. 1983), and many of the complexes of unisexual Cnemidophorus (Dessauer and Cole 1989; Sites et al. 1990).

Why is clonal diversity so much lower among the Fundulus hybrids? Part of the explanation may be that the range of the hybrids, as well as the genetic diversity within local gene pools of the parental species that give rise to clones, is much more restricted than that of many other unisexuals like Poeciliopsis or the highly diverse unisexual lizard Heteronotia binoei (Moritz et al. 1989b). The Fundulus hybrids are known only from the two localities along the Atlantic coast of Nova Scotia. If other populations exist elsewhere in Nova Scotia, they have yet to be identified in surveys by Nova Scotia ichthyologists (Gilhen J, personal communication).

In addition, it is uncertain whether F. heteroclitus and F. diaphanus at these two localities, Porter’s Lake and the St. Mary’s River, are capable of generating the unisexuals by hybridization. Attempts to recreate the gyenogenetic fish P. formosa by hybridization have failed repeatedly; the hybrids that result from crosses of the putative parental forms reproduce sexually, showing random segregation upon backcrossing (Hubbs 1955). Although allozyme (Turner et al. 1983) and mtDNA analyses (Avise et al. 1991) have shown that P. formosa arose from one or several crosses involving female “P. mexicana” and male P. latipinnia, the specific parental strains that gave rise to the cross have yet to be
identified (Turner 1982). If unisexual Fundulus of the “major allozyme clone” did not arise in situ (and an allogenic origin is one possible explanation for apparent homozygosity at $sDHFP^*$), they too may require very specific parental genotypes to be re-created.

For the Fundulus clone, like many unisexuals, it may prove “impossible to determine whether novel alleles . . . are a product of post-formational mutations and hence evolutionary longevity or just a consequence of incomplete sampling of the putative sexual ancestors” (Vrijenhoek 1994). However, recent efforts to date the origin of unisexual clones by analyses of mitochondrial genes have estimated the age of a hemisphere of Poeciliopsis monacha-occidentalis at 60,000–150,000 years (Quattro 1992b) and the age of unisexual Ambystoma lineages at an astonishing 4 to 5 million years (Hedges et al. 1992; Spolsky et al. 1992). If the Fundulus unisexual clone(s) is just a fraction of the age of the Poeciliopsis or Ambystoma hemi-clones, then its origin must have been far south of its current known range, since Nova Scotia was glaciated just 13,000 years ago (Gilton 1974). A direct analysis of the nuclear and mitochondrial genomes of the Fundulus unisexuals and their putative parental species is the obvious next step.

References


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