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Extreme clonal uniformity of *Phoxinus eos/neogaeus* gynogens (Pisces: Cyprinidae) among variable habitats in northern Minnesota beaver ponds

(clonal vertebrates/population structure/genetic variation/DNA fingerprinting)

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ABSTRACT **Genetic surveys of parthenogenetic vertebrate populations have demonstrated a common pattern of relatively high degrees of clonal variation and the coexistence of numerous clones. In striking contrast, the *Phoxinus eos/Phoxinus neogaeus*/hybrid gynogen complex of cyprinid fishes exhibits no clonal variation within a northern Minnesota drainage characterized by successional beaver ponds. Gynogens were sampled from three habitats in each of four different pond types in a single drainage in Voyageurs National Park, Minnesota. The abundance of gynogens relative to sexual dace varied with pond type, being least common in deep upland ponds and most common in shallow, collapsed, lowland ponds (13.4% and 48.6%, respectively). Simple-sequence multilocus DNA fingerprinting of 464 individual gynogens detected one, and only one, clone. DNA fingerprints, generated sequentially by using three oligonucleotide probes, $(CAC)_5$, $(GACA)_4$, and the Jeffreys' 33.15 probe, all revealed the same unprecedented lack of variation. The extreme lack of clonal diversity in these gynogens across a range of habitat types does not fit the general pattern of high clonal diversity found within populations of other vertebrate parthenogens.**

Since the first reported clonal vertebrate was described (1), these organisms have received a large amount of attention due to their usefulness as models for evolutionary and ecological studies. These studies have covered topics ranging from resource partitioning (2) to the age of unisexual lineages (3, 4) and the persistence of sexual reproduction (5). Dawley (6) has identified two main approaches used in research involving clonal vertebrates: studies which investigate the mechanisms of clonal reproduction and studies which assay clonal variation within and among populations. Studies which focus on assaying genetic variation have, contrary to prior expectations, revealed relatively high levels of variation within populations of a given taxon. The coexistence of numerous clonal lineages seems to be the general rule among unisexual vertebrates (see ref. 6 for a review covering the taxonomic range of unisexual vertebrates and refs. 2, 7, and 8 for specific examples concerning parthenogenetic fishes). Natural populations of gynogenetic cyprinid fishes of the *Phoxinus eos/Phoxinus neogaeus*/hybrid complex appear to represent an example where this general rule is not the case. Mitochondrial DNA analysis of limited numbers of individuals from geographically disjunct hybrid populations suggests relatively recent, repeated originations (9). Despite repeated origination events occurring over a broad geographic area, techniques which are intractable to large-scale surveys, such as fin graft histocompatibility analysis, detect only very limited or no clonal variation within any given local population. These immunological data led Goddard *et al.* (9) to postulate that local populations of *Phoxinus* gynogens were composed of only a single clonal lineage. The possibility existed, however, that these immunological groups actually consisted of numerous clonal lineages, having diverged at other than histocompatibility loci subsequent to common origination. For instance, in the case of *Poecilia formosa*, another gynogenetic fish, presumed clonal groupings based on allozyme data actually prove to be assemblages of numerous, distinct, and highly variable clonal lineages when hypervariable variable-number tandem repeat (VNTR) loci are surveyed (7). Furthermore, no such assessment of the fine-scale resolving power of fin graft histocompatibility analysis has been done (7). Here we report the definitive demonstration of an extreme lack of population genetic variation in this species/gynogen complex in a northern Minnesota stream drainage. By assaying hypervariable, simple-sequence, VNTR loci, using multilocus DNA fingerprinting and distributional frequency for both sexual progenitor species and gynogens across a number of habitats, we demonstrate that *Phoxinus* gynogens do not exhibit the population structure predicted by the commonly accepted "frozen niche" model (2). These findings support the hypothesis that local populations of *Phoxinus* gynogens are composed of a single genetic clone and provide a counterpoint to the commonly held view that unisexual vertebrate populations are composed of numerous coexisting clones.

THE HYBRID COMPLEX

P. eos and *P. neogaeus* are common cyprinid dace that range over most of continental North America. Both species are commonly found alone and in sympatry, and gynogens may or may not be present in both cases (9). Where it occurs, this species/hybrid complex consists of the two progenitor species, *P. eos* and *P. neogaeus*, and a semiclonal gynogenetic hybrid form.

Usually, among gynogenetic fishes, as typified by *Poecilia formosa* (7, 10–15), reproduction is accomplished by all-female populations, of hybrid origin. Gynogens are perpetuated by mating with males of their progenitor species. In such cases sperm acts solely as a trigger for development of diploid eggs, no syngamy takes place, and the new paternal chromosome complement is rejected (6).

Because gynogenesis is not 100% efficient in the *Phoxinus* complex, hybrids exist as diploid clonal lineages and triploid and triploid somatic mosaic classes (9). Some triploid biotypes are capable of breeding back into the sexual populations of *P. eos* and *P. neogaeus*, and one instance of a triploid regenerating diploid *P. eos* offspring is known (16). The clonal gynogenetic lineages, however, are reproductively dependent upon either *P. eos* or *P. neogaeus* males, as is typical of other gynogens (17), and persist through time.

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Abbreviation: VNTR, variable-number tandem repeat.

THE STUDY AREA

Both *Phoxinus* species and the gynogenetic hybrid are found in the drainages of Voyageurs National Park in northern Minnesota. These drainages are of relatively young geological age because of glaciation approximately 8–10 thousand years ago. The drainages in this area are characterized by considerable spatial and temporal environmental heterogeneity due to the historical influence of beaver (*Castor canadensis*) activity (18–20). Spatial environmental heterogeneity in beaver ponds includes lateral habitat complexity associated with littoral vs. open water areas, vertical habitat complexity associated with upper aerobic and lower anaerobic regions, and longitudinal habitat complexity associated with differences in deep upland vs. shallow lowland ponds (20). Temporal environmental variability in beaver ponds includes short-term fluctuations in temperature and oxygen (I.J.S., unpublished data) and long-term successional changes associated with the creation, aging, and collapse of ponds (18). This temporal and spatial environmental heterogeneity is likely to affect both the ecological and the genetic structure of fish populations (21, 22).

MATERIALS AND METHODS

Spatial Variation in Biotype Distribution and Collection of Hybrids for Genetic Analysis. Fish were sampled with 5-mm-mesh minnow traps from June 2 to 13, 1994, in four different types of beaver ponds in the Lost Ponds drainage of Voyageurs National Park (18). These ponds included a deep upland pond, the shallow meadow of a collapsed lowland pond, the dam region of a collapsed lowland pond, and a newly created lowland pond. A total of 24 minnow traps were set in three different habitats in each of the four ponds; 12 littoral, 6 upper pelagic, and 6 lower pelagic. Traps were run for 3–4 days in each pond and catch per unit effort (number of dace captured per trap per day^{-1}) was determined. A random subsample of fish from each pond type was collected, anesthetized, and categorized to species/biotypes (*P. eos, P. neogaeus*, and hybrids) on the basis of pharyngeal tooth counts and intestine morphology (17). Once biotype was determined, hybrid specimens were transported on dry ice and stored at −80°C until used for DNA isolation. A total of 464 hybrids were collected from the full range of habitat types in the four different ponds (Table 1).

DNA Isolation and Fingerprinting. *Phoxinus* genomic DNA samples used in the DNA fingerprinting were isolated by a chaotropic salt (guanidinium isothiocyanate) procedure (23). Recovered nucleic acid pellets were air dried and redissolved in 1.5 ml of distilled deionized water. Generally, milligram quantities were obtained from each fish.

DNA fingerprinting using radiolabeled oligonucleotide probes (24, 25) has proven an ideal tool for assaying genetic variation in a number of clonal and semiclonal fishes (7, 8, 15, 23). Sample concentrations were measured with a Pharmacia Genequant dedicated spectrophotometer. Five micrograms of each sample was then digested with an excess of *Hae* III restriction endonuclease at 37°C overnight, in the presence of 1 mM spermidine (26) to ensure complete digestion. Electrophoresis of digested samples was then done in 25-cm, 0.9% agarose gels in 1× TBE buffer (27) at 30 mA until the 1.6-kb marker fragment of BRL's 1-kb commercial ladder was within 2 cm of the gel end. Gels were dried at 55°C under vacuum and subsequently used directly in an in-gel hybridization protocol (23). Oligonucleotide probes were radiolabeled by using [α-^{32}P]dCTP and terminal deoxynucleotidyltransferase according to the United States Biochemical protocol. The 15-bp oligonucleotide $(CAC)_5$ (24), which produces hypervariable, multilocus VNTR DNA fingerprints, was used for the large-scale survey of this study. A subsample was also fingerprinted with $(GACA)_4$ (28) and a 16-bp oligonucleotide derived from the Jeffrey's 33.15 probe (29). Hybridization was carried out overnight at 20°C below the calculated midpoint of the annealing curve of each oligonucleotide probe in 5× standard saline citrate/0.1% SDS. For those samples assayed with more than a single oligonucleotide probe, gels were sequentially stripped and rehybridized with multiple probes.

Table 1. Numbers of hybrid gynogens fingerprinted from the Lost Ponds drainage in Voyageurs National Park

Pond type	Littoral	Open-water pelagic	Open-water benthic	Total
Upland	93	24	18	135
Collapsed lowland				
Meadow	37	76	64	176
Dam	102	6	0	108
Lowland	44	0	0	44
Total	276	106	82	464

Samples required differing amounts of catch effort and do not reflect relative frequencies of gynogens among habitat types.

RESULTS

Spatial Variation in *Phoxinus* Abundance and Hybrid Frequency. Strong spatial variation occurred in the absolute abundance of dace and the relative frequency of hybrids in the different pond environments (Table 2). Total dace abundance and abundance of all three biotypes was highest in the deep upland pond. Total dace abundance was lowest, while relative frequency of hybrids was highest, in the shallow meadow area of the collapsed pond. Overall relative abundances for the entire drainage (Table 2) further reaffirmed the increased use of collapsed ponds by the hybrids compared with the other taxa: *P. eos*, 28.3%; *P. neogaeus*, 5.9%; and hybrids, 45.3%.

Clonal Variation. Analysis of the multilocus DNA fingerprint data for these clonal organisms was greatly simplified (and unequivocal) over that for sexual organisms, since entire banding patterns could be compared for identity, requiring no subjective procedure for scoring matches on a band-by-band basis. $(CAC)_5$ fingerprints of 464 individual diploid hybrids definitively demonstrated that all were descended from a single, common gynogenetic lineage (Fig. 1). $(CAC)_5$ DNA fingerprints of individuals from this clonal diploid line were all identical (averaging 25 bands per individual scorable) and were clonal with the exception of 4 individuals differing by a fragment length of a single band (Fig. 1). Of these 4 individuals, no two matched for the variant bands. $(GACA)_4$ fingerprints of a subsample of individuals revealed the same invariant overall patterns and detected rare variant bands in the same 4 individuals mentioned above (data not presented). Because these individuals differed at only a single fragment and because no other matching individuals were found in the relatively large number of individuals sampled, it was unlikely that they represented distinct clonal lineages having originated as a result of mutation. That two distinct probes detected rare variant bands in the same individuals is also inconsistent with their origination by mutation, since mutation at a given VNTR locus should produce variant bands detectable only with a probe for that VNTR sequence. The most likely explanation was that they were somatic mosaic triploids, which have very low numbers of triploid cells. In such a case only fragments present at very high copy number from the new, variable, paternal complement may be visible in the final DNA fingerprint. Varying intensity among bands was common with this technique, as can be seen even in the DNA fingerprints of the clonal diploids. Such variation in band intensity could produce the above observation in somatic mosaic individuals.

A smaller-scale survey of samples collected in 1992 from the Sucker Creek drainage in Voyageurs National Park, which is located ≈4 km from the Lost Ponds drainage, found all diploid

Table 2. Catch per unit effort (CPUE, no. per minnow trap per day) of *P. eos*, *P. neogaeus*, and *P. eos–neogaeus* hybrids in the Lost Ponds drainage of Voyageurs National Park

Pond type	*P. eos*	*P. neogaeus*	Hybrids	Total
Upland	22.8 (58.6) [55.8]	10.9 (28.0) [91.6]	5.2 (13.4) [44.4]	38.9
Collapsed lowland				
Meadow	3.4 (46.0) [8.3]	0.4 (5.4) [3.4]	3.6 (48.6) [30.8]	7.4
Dam	8.2 (80.4) [20.0]	0.3 (2.9) [2.5]	1.7 (16.7) [14.5]	10.2
Lowland	6.5 (81.2) [15.9]	0.3 (3.8) [2.5]	1.2 (15.0) [10.3]	8.0
Total	40.9	11.9	11.7	

Number in parentheses immediately following each CPUE value is the relative abundance (%) within a given beaver-pond type. Number in brackets following each CPUE entry is the proportion (%) of the total sample for each species or hybrid taken from each of the four different types of ponds. Taxonomic identifications were based on gut morphology and pharyngeal tooth count.

gynogens to be identical to those of the Lost Ponds sample. A subsample of 24 individuals from the three habitat types within Lost Ponds was also fingerprinted with the $(GACA)_4$ and 33.15 oligonucleotides in order to verify the $(CAC)_5$ results. These probes also detected no variation among diploid gynogens (Fig. 2), yielding further evidence that only a single clonal genotype was present in the Lost Ponds drainage.

DISCUSSION

The presence of only a single clonal lineage throughout the Lost Ponds drainage stands in marked contrast to the clonal diversity typically found among other unisexual vertebrates. For instance, among the unisexual fishes, populations of both *Rivulus marmoratus* (8) and *Poecilia formosa* (7) were found to exhibit very high levels of clonal diversity when surveyed by simple-sequence fingerprinting (42 clones out of 58 individuals and 16 clones out of 19 individuals, respectively). Even relatively low-resolution techniques such as allozyme analysis have detected as many as 3 apparently clonal phenotypes within the above-mentioned population of *Poecilia formosa* (30), although histocompatibility analysis has revealed these three allomorphs to be actually composed of at least 12 distinct clonal lineages. Similar levels of clonal diversity have been

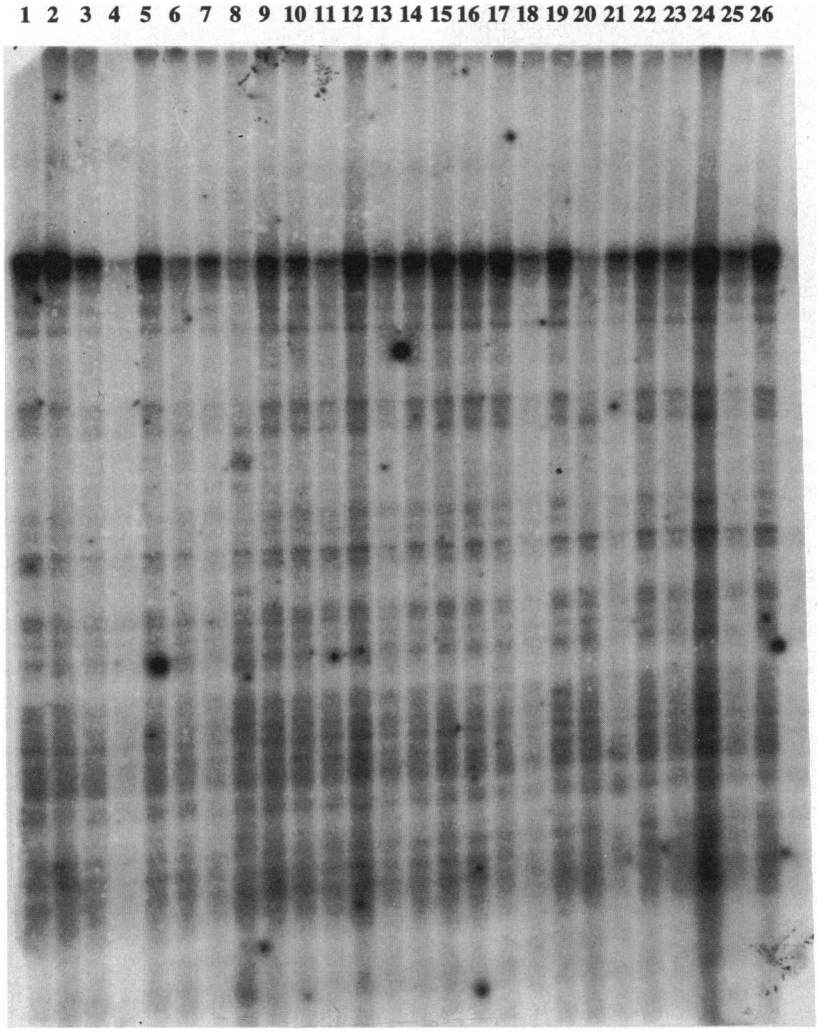

FIG. 1. In-gel DNA fingerprint of 26 individual *Phoxinus* gynogens sampled from one location in the Lost Ponds drainage. Genomic DNAs were digested to completion with *Hae* III and hybridized with ^{32}P-labeled $(CAC)_5$ oligonucleotide probe. Note that all patterns are identical, except for the individual represented in lane 8, which differs by a single band.

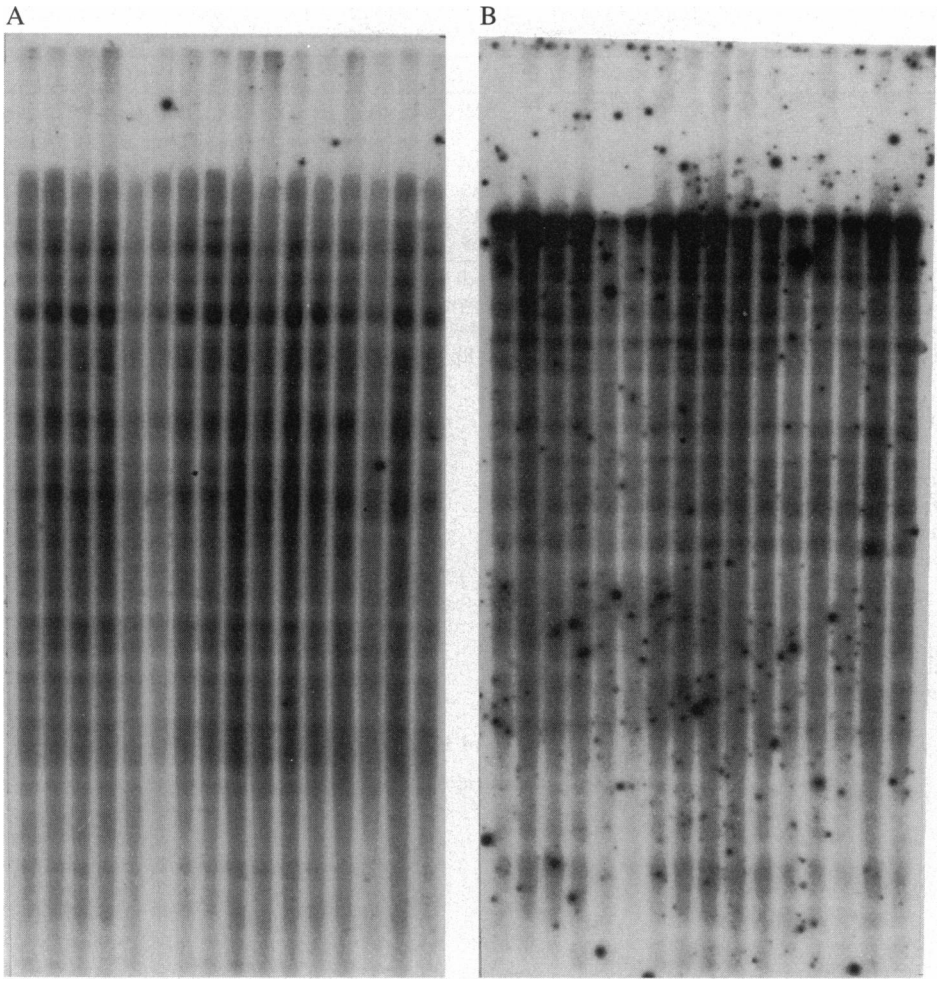

FIG. 2. (*A*) DNA fingerprints of 16 individual *Phoxinus* gynogens from a Lost Ponds littoral sample, hybridized with $(CAC)_5$. Note the identity of all patterns. (*B*) Same gel stripped and reprobed with $(GACA)_4$, showing that identification of only one clone in the Lost Ponds system is not probe specific. Identical patterns were also obtained with the Jeffreys' 33.15 oligonucleotide probe (data not shown).

found in other unisexual fishes, as well as unisexual amphibians and reptiles (see ref. 6 for a convenient compilation of examples from numerous authors).

The strong spatial variation in frequencies of the sexual *Phoxinus* and the gynogen in this system suggests that the biotypes differ in their tolerance of marginal habitat conditions. In marginal habitats, such as meadows in collapsed ponds, it appears that the gynogenetic clone is relatively more successful than the sexual forms, to the point of nearly becoming the predominant dace in the system. Because of the ecological complexity of the landscape in these drainages, it is possible that a single gynogenetic clone has become established in spite of significant overlap of its ecological niche with those of its progenitor species. It is unlikely that any parthenogenetic organism can become coestablished with its progenitor species if their ecological niches completely overlap (2). However, small differences in habitat utilization should be sufficient to allow the establishment and persistence of parthenogens, if such differences restrict access of sexual progenitors to some habitats within a complex landscape. Such restricted habitats may act as refugia for gynogens, with reproduction still occurring via movement of sexual progenitors and parthenogens among habitat types. These dace move between pond types within drainages, especially during periods of high water flow (31). Such broad tolerances, relative to progenitor species, for environmental variables have been demonstrated for some hybridogenic strains of *Poeciliopsis* (32). Abiotic components of the environment, such as oxygen availability, have been shown to alter species composition of fish communities in northern temperate environments (33) and oxygen level is spatially and temporally quite variable among habitat types within these drainages (I.J.S., unpublished data).

The "frozen niche variation" model (34, 35), which predicts structured, relatively stable communities of variable clones, each specialized to utilize a specific subcomponent of a broader habitat, does not appear to apply to the current state of the *Phoxinus*/gynogen system as it exists in Voyageurs National Park. While only a single, abundant clone now exists in this system, we cannot rule out the possibility that a more complex community existed in the past. The current presence of only a single clone could be due to either selective or stochastic forces. If a complex community of clones did historically exist, selection among clones for a single highly successful "generalist clone" (36, 37) may well have occurred. It is also equally likely that stochastic processes, such as rapid clonal extinction due to unpredictable disappearance of microniches (intimately linked to the above generalist model) (37, 38) or the stochastic "turnover" of ecologically equivalent clones (8) could have produced the current clonal homogeneity. Stochastic turnover, in this case, would be interpreted as a scenario in which only a single clone originated *de novo* or fortuitously ever managed to colonize this system. At this point, the nature of the data does not allow a clear choice among the models.

What is unique about this species/gynogen complex is its unprecedented genetic homogeneity and the fact that it coex-

ists with its apparently ecologically similar sexual progenitors by utilizing a broader range of habitats without either displacing the sexual species or going extinct itself.

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