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Genetic variation in clonal vertebrates detected by simple-sequence DNA fingerprinting

(gynogenesis/hermaphroditism/*Poecilia formosa*/*Rivulus marmoratus*)

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ABSTRACT **The measurement of clonal heterogeneity is central to understanding the evolutionary and population genetics of the roughly 50 species of vertebrates that lack effective genetic recombination. Simple-sequence DNA fingerprinting with oligonucleotide probes $(CAC)_5$ and $(GACA)_4$ is a sensitive and efficient means of detecting this heterogeneity in natural populations of two clonal fishes, *Poecilia formosa*, an apomictic unisexual, and *Rivulus marmoratus*, a selfing hermaphrodite. The fingerprints are clonally stable for at least three generations. The technique clearly differentiates allozymically identical laboratory lines of *R. marmoratus* that were previously distinguishable only by histocompatibility analysis. The technique also reveals apparent cases of shifts in clonal composition of a natural population of each species. Clonal variation in most natural populations is quite high. For example, a sample of 19 specimens of *P. formosa* from one station on the Rio Soto la Marina contained 16 clones (average clonal frequency = 0.07). This level of clonal diversity implies that mutation, subsequent to the founding of clonal lineages, is an important source of variation in these populations. It also suggests that chance (sampling error) has a previously unappreciated role in determining the clonal composition of populations even though some of the clones may be divergent in biologically significant features.**

About 50 species of vertebrates have reproductive systems that exclude effective genetic recombination (1). Natural populations of these organisms consist of arrays of clonal lineages. Immunological techniques [i.e., histocompatibility analyses (2, 3)], electrophoretic "allozyme surveys" (4, 5), and surveys of restriction site variation in mitochondrial DNAs (6, 7) have been used to detect and analyze clonal variation in these populations. Each technique has limitations in sensitivity and/or utility.

We have used DNA fingerprinting, based on ubiquitous simple sequences (8, 9), to assess genetic variation in samples of two clonal fish species. One of these, *Poecilia formosa* (Poeciliidae), is an all-female, ameiotic, gynogenetic, matroclinous fixed heterozygote, ultimately of hybrid origin (10). The other, *Rivulus marmoratus* (Cyprinodontidae), is the only known selfing hermaphroditic vertebrate (11); natural populations apparently consist entirely of homozygous clones (12, 13). Genetic recombination among clones is unknown in natural populations of either species. Clonal population structure results from an apomictic breeding system in *P. formosa* and from an essentially monoecious one in *R. marmoratus*. Though breeding structures and possibly the origins of variation differ, the problems of measuring clonal heterogeneity are similar in each.

DNA fingerprinting demonstrates very high levels of genetic variation in natural populations of both species. The data provide insights into population divergence and clonal dynamics that were unavailable with other techniques and emphasize the hitherto unappreciated significance of mutation as a source of clonal heterogeneity.

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MATERIALS AND METHODS

Specimens. Material from various laboratory lines and field collections was used. Laboratory lines allowed us to assess the stability of DNA fingerprint phenotypes and to directly compare our results with previous measurements of clonal diversity.

P. formosa. Laboratory lines: (*i*) Ditch at airport, Madero (Tampico), Tamaulipas, Mexico, September 1982; three generations (one mother, four of her progeny, and four progeny of one of the latter sibs). (*ii*) Cade's Cove, San Marcos River, San Marcos, Texas, May 1988; two generations (one mother and four progeny). (*iii*) Rio Tigre at Highway 180 crossing, Aldama, Tamaulipas, Mexico, February 1989 (Travis clone 3); 14 sibs from a single brood. (*iv*) Rio Tigre at Highway 180 crossing, Aldama, Tamaulipas, Mexico, February 1989 (Travis clone 4); 16 sibs from a single brood. Field collections: (*i*) Thompson's Island, San Marcos River, San Marcos, Texas, October 1988 ($n = 4$). (*ii*) Stream at Lulu Sam's State Fish Hatchery, Olmito, Texas (Rio Grande drainage), July 1989 ($n = 8$). (*iii*) Rio Purificacíon (Rio Soto la Marina drainage), Nuevo Padilla, Tamaulipas, Mexico, May/June 1988 ($n = 19$); this sample also included four triploids [ascertained by parvalbumin phenotype (14)], which were excluded from fingerprinting surveys.

R. marmoratus. Laboratory lines: (*i*) Three of the clones originally identified by R. W. Harrington and K. D. Kallman (12, 13) are maintained by one of us (W.P.D.); samples were as follows: clone DS ($n = 3$), clone NA ($n = 3$), clone M ($n = 6$). (*ii*) A line derived from an hermaphrodite collected at Rookery Bay, Collier County, Florida, May 1981; WPD 219 ($n = 3$). (*iii*) Lines derived from individual hermaphrodites collected on Marco Island, Collier County, Florida, April 1986; clones WPD 264 ($n = 9$; one second generation parent and eight progeny), WPD 268 ($n = 6$), and WPD 270 ($n = 5$). (*iv*) Lines derived from hermaphrodites collected on No Name Key, Key Deer National Wildlife Refuge, Florida, March 1986; clone NNK1 (one field-caught adult, one second generation parent, and six progeny) and clone NNK12 (one field-caught parent and three progeny). Field collections: (*i*) Lagoon on Indian River, 5 km north of Vero Beach, Florida, January 1989 ($n = 9$). (*ii*) No Name Key, Key Deer National Wildlife Refuge, Florida, March 1986 ($n = 10$). (*iii*) No Name Key, Key Deer National Wildlife Refuge, Florida, April 1989 ($n = 12$). (*iv*) Rookery Bay, Collier County, Florida, April 1989 ($n = 8$).

DNA Preparation. DNA was isolated from homogenates of whole fish (degutted or starved prior to sacrifice) with

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guanidinium isothiocyanate and phenol/chloroform as described (15).

Fingerprinting. Restriction digestion, agarose gel electrophoresis, and fingerprint development were also as previously described (15). The most useful enzymes for fingerprint determination were *Hae* III (*Poecilia*), *Hin*fI (*Poecilia* and *Rivulus*), and *Alu* I (*Rivulus*). ^{32}P-labeled oligonucleotides $(CAC)_5$ and/or $(GACA)_4$ were used as fingerprinting probes in a dried gel hybridization procedure.

RESULTS

The oligonucleotide probes yielded multibanded fingerprints from the genomes of both species. In general, patterns generated by both probes were very similar, though $(CAC)_5$ frequently produced some high molecular weight bands that were absent with the $(GACA)_4$ probe. Genetic heterogeneity detected by one probe was always detected by the other. The number of scorable bands in a fingerprint phenotype varied with the individual, population sample, species, and restriction enzyme: *Hae* III yielded 16–33 bands (mean = 24) in *Poecilia*, *Alu* I yielded 24–30 bands in *Rivulus* (mean = 29), and *Hin*fI produced 19–24 bands (mean = 23) in *Poecilia* and 28–34 bands (mean = 33) in *Rivulus*.

All fingerprint phenotypes tested appeared to be clonally stable. Thus, mother, progeny, and second generation progeny of a *P. formosa* line from Tampico had identical fingerprints with both *Hae* III and *Hin*fI, as did mother and progeny from Cade's Cove and all members of both Rio Tigre broods (Fig. 1). There was no variation among members of each individual *Rivulus* line from Marco Island and No Name Key, even though each line had a unique fingerprint. Each of the long-established Harrington–Kallman lines could be distinguished from the others (Fig. 2).

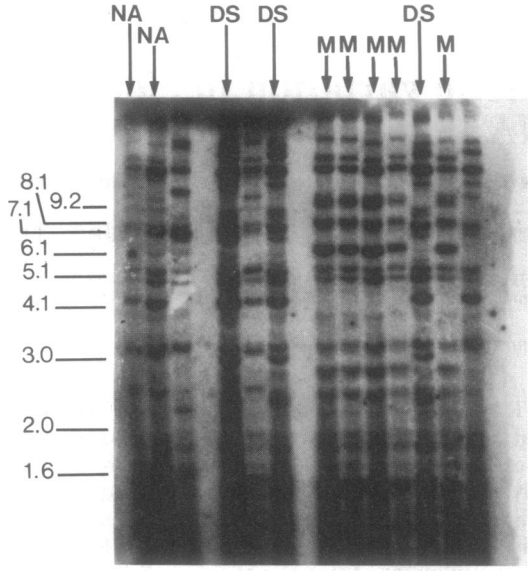

FIG. 2. *R. marmoratus*. Specimens labeled NA, DS, and M are from laboratory-maintained clones originally identified with histocompatibility analyses by R. W. Harrington, Jr. and K. D. Kallman (see refs. 12 and 13). Unlabeled specimens are from other laboratory clones [*Hin*fI digests; $(CAC)_5$ probe].

Substantial fingerprint variation was evident in most field samples of each species (Figs. 3 and 4). We interpret this as clonal heterogeneity (Table 1). The maximum number of clones detected at a single locality was 16 (out of 19 individuals surveyed) in a sample of *P. formosa* collected at Nuevo Padilla, Mexico. The minimum was 3 in a sample of the same species from a Rio Grande locality in Texas. In each species, clones were usually distinguishable with both restriction

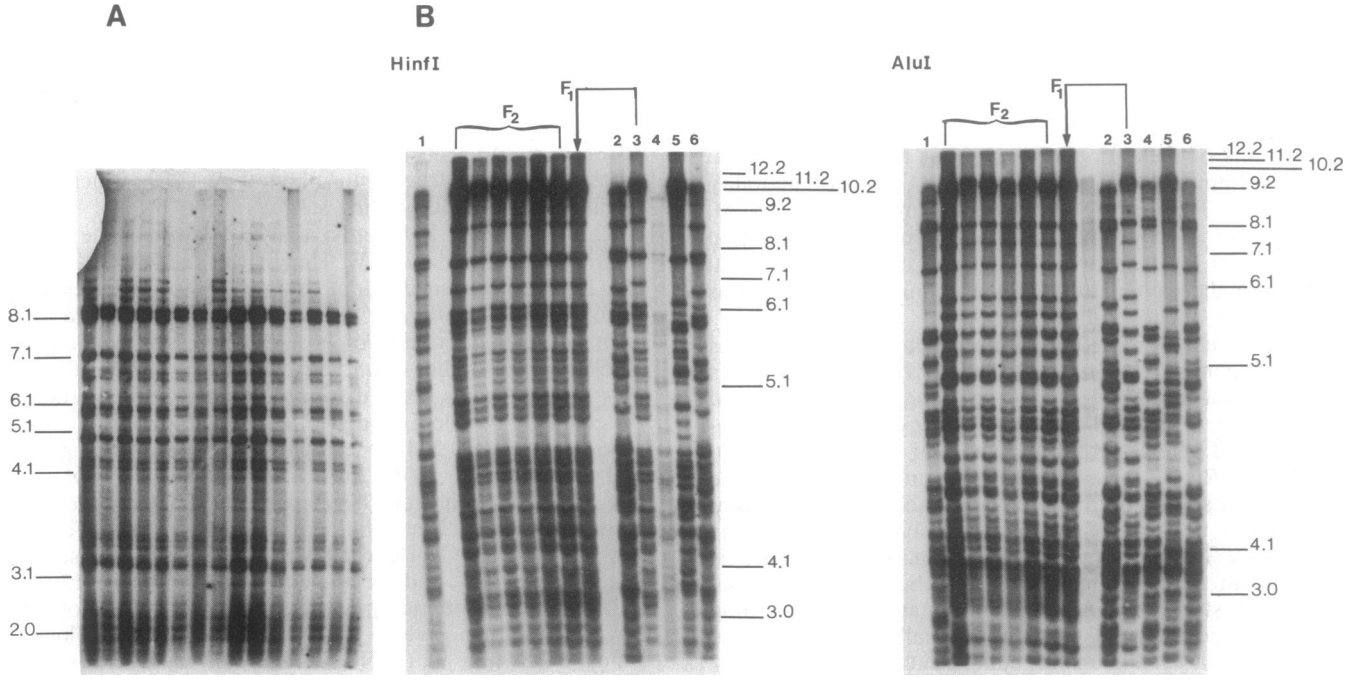

FIG. 1. Clonal stability of simple-sequence DNA fingerprints in *P. formosa* and *R. marmoratus*. (*A*) *P. formosa*: 15 individuals from a single brood from a female collected from the Rio Tigre, Tamaulipas, Mexico. With the exception of differences in sample concentration, the variation seen among individuals is equivalent to that noted in replicate analyses of the same individual [*Hae* III digests; $(GACA)_4$ probe]. (*B*) *R. marmoratus*: six field-caught specimens collected at No Name Key in 1986 (numbered 1–6; note that specimens 2 and 6 have identical fingerprints), an F_1 progeny of specimen 3, and 6 of its F_2 progeny. The analysis was performed with the two restriction enzymes indicated [$(GACA)_4$ probe]. In this and following figures, sizes of molecular weight standards are given in kilobases; their positions have been derived from the locations of bands (from various commercial marker mixtures) on ethidium bromide-stained gels photographed prior to probe hybridization.

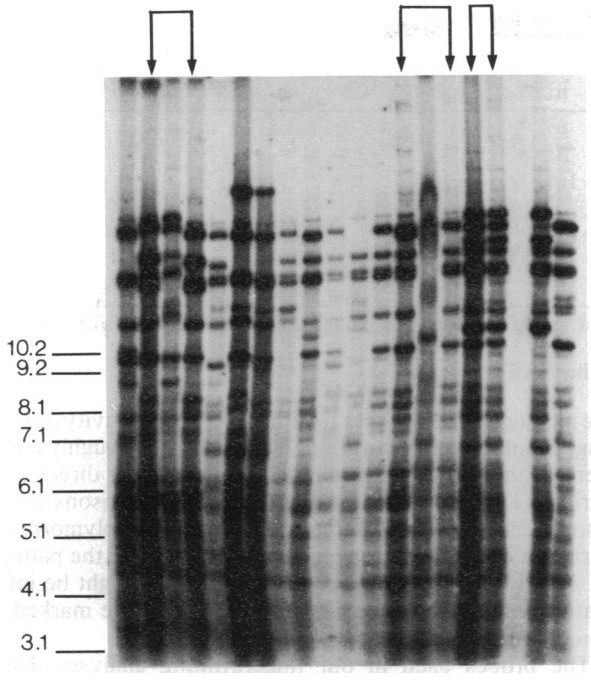

FIG. 3. Clonal differentiation in *P. formosa*. Fingerprints of 19 diploid individuals collected at a single station on the Rio Purificacíon (Rio Soto la Marina drainage), Nuevo Padilla, Tamaulipas, Mexico. Arrows identify specimens with identical fingerprints [*Hin*fI digests; $(GACA)_4$ probe].

enzymes used, but in some cases (Table 1), clones had identical phenotypes with one of the two enzymes. Two *R. marmoratus* clones from north of Vero Beach differed by only a single band detected in *Hin*fI digests only (Fig. 5), but this subtle difference was quite reproducible. No clone was found at more than a single locality in samples of either species. There was no overlap in the 1986 and 1989 samples of *R. marmoratus* from No Name Key.

The fingerprint divergence among clones varied with species and locality. In the 1989 *R. marmoratus* sample from No Name Key, the average pairwise distance among clones was 28 bands (range, 14–38), or roughly 50% of the average number of scorable bands (29 per individual), while in the Rookery Bay sample this difference was only 16 bands (range, 13–24), or roughly 24% of the average number of scorable bands (33 per individual). In the *P. formosa* sample from Nuevo Padilla, the average pairwise distance among clones was 10 bands (range, 3–14), or roughly 21% of the average number of scorable bands (24 per individual), while the three clones in the Rio Grande sample differed on average by 3 bands, or about 7% of the average number of scorable bands (22 per individual).

DISCUSSION

Clonal organisms are of general interest because their clones are, in effect, multilocus genotypes that are stable from generation to generation. Such genotypes are not available in organisms with regular recombination. What factors govern the diversity of these genotypes? What is the relationship between clonal diversity and variation in fitness or among the clones of one population and those of others? Nearly all clonal vertebrate species are unisexuals of hybrid ancestry (1, 16); in these, how much diversity stems from multiple hybrid origin and how much from subsequent mutations?

Implicit in these questions is the ability to recognize and measure clonal diversity. Among clonal vertebrates, histocompatibility analyses and allozyme surveys have been the techniques most widely used to assess genetic heterogeneity. Of the two, histocompatibility analyses, presumably based on systems homologous to the mammalian major histocompatibility complex (17), are clearly more sensitive. For example, Kallman (2) could detect as many as 12 clones in a

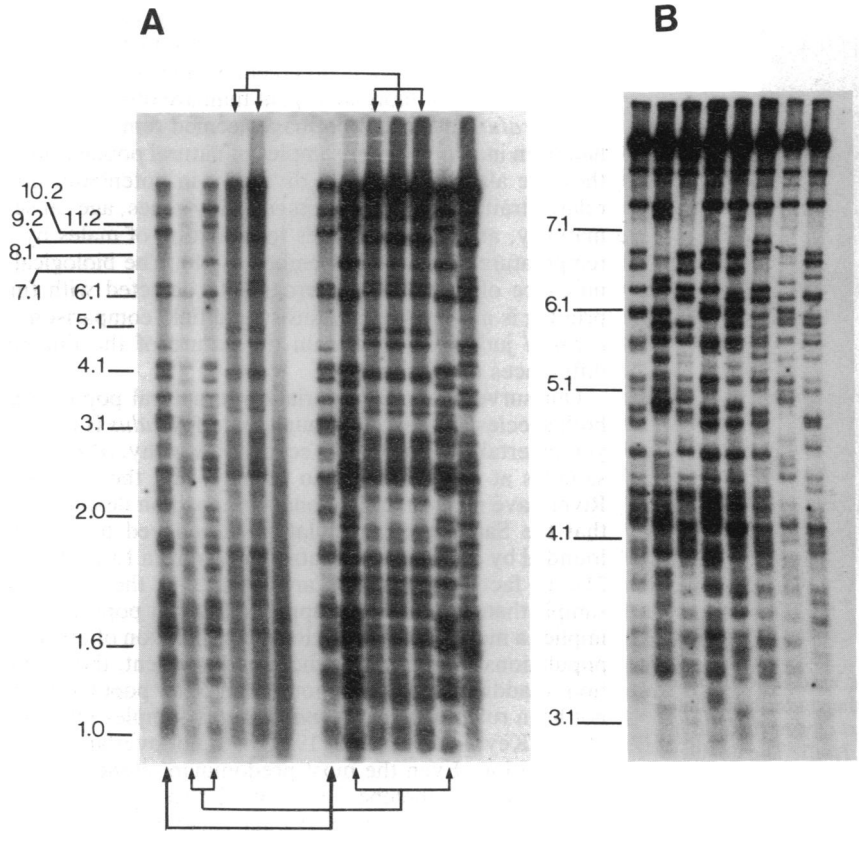

FIG. 4. *R. marmoratus*: contrasting patterns of clonal variation. (*A*) No Name Key, 1989 (*Alu* I digests). Arrows identify specimens with identical fingerprints. Note that this sample of 12 individuals contains only four clones. (*B*) Rookery Bay (*Hin*fI digests). The 8 individuals in this sample are members of eight different clones. [$(GACA)_4$ probe.]

Table 1. Clonal distributions in field-collected samples of *P. formosa* and *R. marmoratus*

Species	Locality	Sample, *n*	Clones, *n*	*n* clones with *j* members					
				j = 1	*j* = 2	*j* = 3	*j* = 4	*j* = 5	*j* = 6
P. formosa	San Marcos River, Thompson's Island	13	6	4		1			1
	Rio Grande, Lulu Sam's	8	3		2		1		
	Rio Purificacíon, Nuevo Padilla	19	16*	13	3				
R. marmoratus	Indian River, Vero Beach	9	5†	2	2	1			
	No Name Key, 1986	6	5	4	1				
	No Name Key, 1989	12	4	1	1		1	1	
	Rookery Bay	8	8†	8					

*The total includes two clones that were identical with *Hae* III but differed with *Hin*fI and two clones that were identical with *Hin*fI but distinguishable with *Hae* III.
†The total includes two clones that were identical with *Alu* I but different with *Hin*fI.

single population sample of *P. formosa* with histocompatibility analysis, while only two allozyme "clones" could be detected from the same locality (5). However, in its present form, the histocompatibility technique is difficult to use on a survey scale. Consequently, allozyme surveys have been emphasized in the literature. In some cases distinct histocompatibility clones with the same allozyme phenotype have been dismissed as "minor" variants, though there is no compelling evidence that their overall genetic differentiation is less than that of allozymically distinct "clones" or that it is less significant biologically.

The sensitivity of DNA fingerprinting as a means of detecting clonal variation is apparent from the observation that all three of the Harrington–Kallman clones of *R. marmoratus* have different fingerprints (Fig. 2). These clones were previously separable only by histocompatibility tests; allozyme comparisons made in two laboratories (18, 19) failed to detect any differences among them. Similarly, only three clones of the diploid *P. formosa* could be discerned in the entire Rio Soto la Marina drainage system by allozyme survey (14); fingerprinting detects 16 clones in a sample of 19 individuals collected at a single station in one tributary, the Rio Purificacíon. The increment in sensitivity available from the technique is obviously significant. The sensitivity of DNA fingerprinting to clonal variation appears to be roughly equivalent to that of histocompatibility analysis, but direct comparisons have not yet been made. Such comparisons should also be made with restriction fragment length polymorphism variation in mitochondrial genomes (6), because the patterns of variation revealed by the two techniques might be informative, especially if different hybrid origins are marked by unique mtDNA variants.

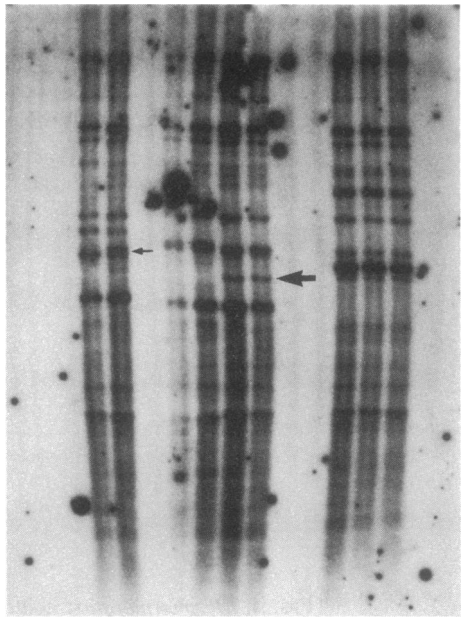

FIG. 5. *R. marmoratus* from Indian River near Vero Beach [*Hin*fI digests; $(CAC)_5$ probe]. The four individuals in the center lanes had identical fingerprints with *Alu* I but fall into two clones that differ consistently by a single band (large arrow) with *Hin*fI. The doublet of bands resolved in only one of two samples on the left (small arrow) was not seen on subsequent or prior runs of the same specimens, and these specimens are regarded as members of the same clone.

The probes used in our fingerprinting analyses detect stretches of simple DNA sequences, which occur as highly repetitive, dispersed elements in eukaryotic genomes. These stretches are hypervariable in length; slippage during DNA replication or repair is believed to be the source of the variation (9). Such slippage apparently occurs at a rate higher than that of point mutations in structural genes (20), and this higher mutation rate may account for the greatly increased ability of the fingerprinting technique to detect clonal variation.

However, though we have used seemingly superficial genetic differences to delineate clones, it would be incorrect to assume that all of them differ solely in the lengths of some simple-sequence DNA stretches. The Harrington–Kallman *R. marmoratus* clones are a convincing example. These clones were originally collected at random from natural populations. They have divergent simple-sequence fingerprints but are not more divergent than are other clones of *R. marmoratus* and would not have seemed remarkable if they had been included in our samples of natural populations. Yet they are also known to be divergent in potentially fitness-related traits such as histocompatibility genes, ages at sexual maturity, and susceptibilities to induction of males by low-temperature incubation of embryos (18). The biological significance of the clonal heterogeneity detected with fingerprinting is a matter of laboratory and field comparisons, not *a priori* judgments based on the nature of the fingerprint differences themselves.

Our surveys of clonal variation in natural populations of both species are limited, though that of *Rivulus* is the largest yet undertaken. Two points seem noteworthy. (*i*) *P. formosa* samples at Lulu Sam's (Rio Grande) and the San Marcos River have no overlap in clonal composition despite the fact that the San Marcos population is believed to have been founded by a documented introduction from LuLu Sam's (2, 21). In fact, more clones are present in the San Marcos sample than in that of the supposed founder population. This implies a major shift in the clonal composition of one or both populations subsequent to the founding event, though mutation or additional introductions from other populations have not been ruled out. (*ii*) *R. marmoratus* samples taken at No Name Key in 1986 and 1989 had no overlap in clonal composition. Even the most predominant clone in the 1989 sample, with 5 members (out of 11 fish surveyed), is not present in the 1986 sample. Though sampling error cannot be

completely discounted, the most straightforward interpretation of these data is that a substantial turnover has occurred in the clonal composition of the population. A change of this magnitude would be consistent with the documented vagility and colonizing ability of this species (22).

A potentially significant aspect of our data is the large number of clones that comprise several samples. This is readily apparent in the Rookery Bay and 1986 No Name Key *Rivulus* samples (8 clones from a sample of 8 individuals and 5 from a sample of 6, respectively) and is especially striking in the Nuevo Padilla sample of *P. formosa* (16 clones out of 19 individuals surveyed: an average clonal frequency of ≈0.07). In *P. formosa*, clonal diversity on this scale immediately suggests that mutation and not recurrent hybridization is the source of much of the variation. Biochemical genetic evidence (14) indicates that the *P. formosa* in the Rio Soto la Marina drainage are of exogenous origin. Moreover, *Poecilia latipinna*, one of the gonochoristic ancestors of *P. formosa*, does not regularly occur in the river system, so that new hybrid origins are most unlikely. Much of the current literature emphasizes recurrent hybridization as the source of genetic variation in unisexual vertebrates (e.g., ref. 23). This emphasis may require modification. Ultimately, comparisons of variance in clonal adaptations in *Poecilia* and *Rivulus* may prove quite informative, because new mutations, presumably usually recessive, are available in *Rivulus* in homozygous form within a single generation, whereas those in *P. formosa* will persist as heterozygotes indefinitely.

High levels of clonal heterogeneity in these species have another implication, especially since several of our samples consist of many clones at low frequencies. Current ecological models of genetic diversity in clonal vertebrates are deterministic: clones in a particular population are believed to have specializations (trophic adaptations, diet, thermal tolerance ranges, etc.) that enable them to exist in distinct microhabitats or subniches (see refs. 24 and 25 for examples and reviews of the literature). As the number of clones becomes larger, however, the role of chance variation (sampling error) must increase, especially for small populations. Clonal specializations almost certainly exist, but their role in determining the genetic structure of populations may have been overestimated. Our data suggest that chance variation is a significant factor in determining the genetic composition of all but the largest populations of clonal vertebrates. We suspect that the clonal composition of these natural populations may be more strongly determined by stochastic factors, such as migration, founder effects, population size, and fluctuation, than by particular clonal adaptations.

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