Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 10643–10647, November 1992
Evolution

Extreme clonal diversity and divergence in populations of a selfing hermaphroditic fish

(*Rivulus marmoratus*/DNA fingerprinting/microsatellites/genetic variation)

BRUCE J. TURNER*†, JOHN F. ELDER, JR.*, THOMAS F. LAUGHLIN*, WILLIAM P. DAVIS‡, AND D. SCOTT TAYLOR§

*Department of Biology, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061; ‡U.S. Environmental Protection Agency Environmental Research Laboratory, Gulf Breeze, FL 32561; and §Brevard Mosquito Control District, Palm Bay, FL 32905

Communicated by Bruce Wallace, July 31, 1992

ABSTRACT **Recombination is unknown in natural populations of *Rivulus marmoratus*, a selfing hermaphrodite, and genetic variation is likely due to mutation alone. DNA fingerprinting with an array of microsatellite [e.g., $(CT)_9$] and minisatellite (e.g., the 33.15 core sequence) probes reveals very high clonal diversity within samples of seven Floridian populations, of which five contain about as many clones as there are individuals. There are 42 clones among 58 individuals surveyed (mean, 1.4 individuals per clone), a level of genetic diversity unprecedented among clonal animals. Moreover, all of the probes recognize the same clones even though, at high hybridization stringencies, there is little overlap in the fingerprint patterns they generate. This suggests that most sympatric clones differ by multiple and independent mutational steps. In one population studied in detail, the average number of mutational steps separating two clones is estimated at 9 or 10 and may be substantially higher. The mutational discontinuities among sympatric clones make it unlikely that they evolved by accumulation of neutral mutations in populations that are otherwise genetically uniform. The data argue that the mixing of unrelated individuals from different local populations occurs to an extent previously unappreciated and/or that divergence of clones is mediated by natural selection. If confirmed, the latter would be a serious challenge to current ideas on the predominant role of recombination in promoting the evolution of biological novelty.**

We recently applied the technique of DNA fingerprinting to the problem of measuring genetic variation in natural populations of clonal organisms (1). In the clonal killifish *Rivulus marmoratus*, the only vertebrate known to be a selfing hermaphrodite, intrapopulation allozyme variation is unknown. Genetic variation was evident only with histocompatibility surveys based on organ transplant experiments; it was frequently presumed to be nearly negligible in magnitude. Microsatellite (or simple sequence) probes $(CAC)_5$ and $(GACA)_4$ detected high levels of clonal diversity in several populations of this species. Here we report the results of extending our surveys to samples of additional Floridian populations and to a battery of different microsatellite and minisatellite probes. The data reveal levels of genetic diversity and mutational discontinuity that have previously not been reported among clonal animals. They challenge the widely held notion that clonal populations are necessarily limited in genetic variation and evolutionary potential. A preliminary account of some of our findings has appeared in a symposium volume (2).

MATERIALS AND METHODS

Organism. *R. marmoratus* (*Rivulus ocellatus* in some publications) (family Cyprinodontidae or Rivulidae) is a synchronous hermaphroditic fish species with highly efficient internal self-fertilization (3). So far as is known, most populations consist exclusively of obligate, automatic selfers with a breeding system equivalent to cleistogamous plants (see ref. 4). Population structure is clonal, and individuals are apparently homozygous (5). Outcrossing and heterozygosity are thus far unknown in natural populations. True females have never been collected, but functional males can be induced in the laboratory and are known in nature (6). It has been suggested that these males may promote outcrossing by mating with hermaphrodites and fertilizing viable ova that have escaped self-fertilization (7). However, while males are common on some Caribbean islands (8), they have been absent from or are exceedingly rare in all Floridian collections made thus far (9). It is doubtful that they affect significantly the genetics of these populations even if outcrossing does sometimes occur.

Specimens. Field-caught material (all from Florida): Lagoon on Indian River, 5 km north of Vero Beach, January 1989 (n = 9); No Name Key, Key Deer National Wildlife Refuge, March 1986 (n = 10), May 1989 (n = 12); Lower Matecumbe Key, March 1989 (n = 7); Rookery Bay, Collier County, April 1989 (n = 8), March 1990 (n = 8). All specimens were hermaphrodites, and all were taken from marine mangals, generally from the burrows of the crab *Cardisoma guanhumi* and most frequently with small funnel traps or miniature light tackle. Laboratory lines listed in our previous report (1) were also used. These included three long-term clonal lines originally founded by R. W. Harrington and six shorter-term lines derived from individual hermaphrodites collected on Marco Island in April 1986 (n = 3 lines); No Name Key, March 1986 (n = 2); and Rookery Bay, May 1981 (n = 1).

Initial DNA Fingerprinting. Methods of DNA preparation, restriction digestion, gel electrophoresis, probe preparation and radiolabeling, and hybridization directly in dried agarose gels were as described (10) with minor modifications. Analyses were generally replicated with the restriction enzymes *Alu* I and *Hin*fI; some were also replicated with *Hae* III. Clones were identified by fingerprinting with probes $(CAC)_5$ or $(GACA)_4$.

Analyses with Multiple Probes. After initial fingerprinting, gels were stripped and rehybridized with up to 6 additional oligonucleotides. In early work, probe hybridization and stringency rinses were at 48°C, the temperature originally used for $(CAC)_5$. In subsequent experiments, these steps were done at 20°C below the calculated melting temperature (T_m) of each probe sequence [15°C below T_m for $(GT)_9$]. Most survey gels were hybridized with the microsatellite sequence $(CT)_9$ and the minisatellite sequences AGAGGTGGGCTG-GAGGGC, the 33.15 core sequence (11), and $(AGAGGC)_4$, a repeat in the *Per* locus of *Drosophila* (12). In addition, some

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

†To whom reprint requests should be addressed.

gels were also hybridized with $(GT)_9$, $(GGCAGG)_4$, a minisatellite sequence motif with a high mutation rate in the mouse genome (13), and/or GGCTGGTGGCTGGTG, a dimer of the prokaryote recombination signal Chi (14).

RESULTS

Clonal Diversity. The number of clones per locality and individuals per clone are documented in Table 1. In five of eight population samples, the number of clones detected approached or equaled the number of individuals surveyed (Fig. 1). With the exception of a pair of apparent sister clones from the Vero Beach sample, all clones could be differentiated with both *Hin*fI and *Alu* I, although the number and sizes of bands generated by each of these enzymes were usually different. There was no detectable overlap in clonal composition among localities nor within localities surveyed in two different years.

Multiple Probes. At moderate stringency, overlap in banding patterns detected by each probe varied with both sample and sequence. Often, the same bands were detected with several probes but at very different intensities. Average banding overlap among probes varied from ≈20% to ≈70%. All probes except $(CT)_9$ detected some bands that were not detected by any of the others; bands detected with $(CT)_9$ were a subset of those visualized with $(CAC)_5$ or $(GACA)_4$. In all but one case, clones that could be differentiated with one probe could be differentiated with all that were tried, and very often each probe revealed additional divergent bands (Fig. 2). The two sister clones in the Vero Beach sample could be differentiated only with $(CAC)_5$. Individuals that were scored as coclonal with one probe were always coclonal with other probe sequences (up to a sample size of five, the largest number of coclonal individuals we have detected thus far).

Hybridization at high stringency sharply reduced or virtually eliminated band overlap in the fingerprints generated by different probes. Nonetheless, all clones assayed could be distinguished by all of the probes used. The 1989 Lower Matecumbe Key sample was analyzed in detail: all seven individuals (presumptive clones) could be resolved readily with probes $(CT)_9$, $(GT)_9$, and 33.15 although the fingerprint patterns showed little or no band overlap (Fig. 3). The pairwise fingerprint band distances for this sample—i.e., the number of bands for each probe—by which the fingerprints of each pair of clones differ are given in Table 2. These pairwise distances almost certainly underestimate mutational divergence, for there is no reason to suggest that the three probes, chosen arbitrarily, exhausted the repertoire of divergent bands that separate the clones. The number of divergent bands and the total number of bands in each pairwise comparison (Table 2) are strongly correlated, suggesting that adding new bands to the sample by surveying additional microsatellite and minisatellite sequences would uncover yet additional divergence. Four of the clones were subsequently fingerprinted with the *Per* sequence and $(GGCAGG)_4$, and several additional divergent bands could be detected.

Table 1. Clonal diversity in Floridian samples of *R. marmoratus*

Locality	Year	*n*	No. of clones	Individuals per clone Max	Individuals per clone Avg
Indian River (Vero Beach)	1989	9	5*	3	1.8
No Name Key	1986	6	5	2	1.2
No Name Key	1989	12†	4	5	3.0
Lower Matecumbe Key	1989	7	7	1	1.0
Rookery Bay	1989	8	8	1	1.0
Rookery Bay	1990	8†	7	2	1.1
Everglades	1990	8	6	3	1.3
	Total	58	42		10.4

There is no overlap in clonal composition between localities.
*Includes two sister clones distinguishable only with *Hin*fI and with probe $(CAC)_5$. See figure 5 in ref. 1.
†No overlap in clonal composition in repetitive samples from the same localities.

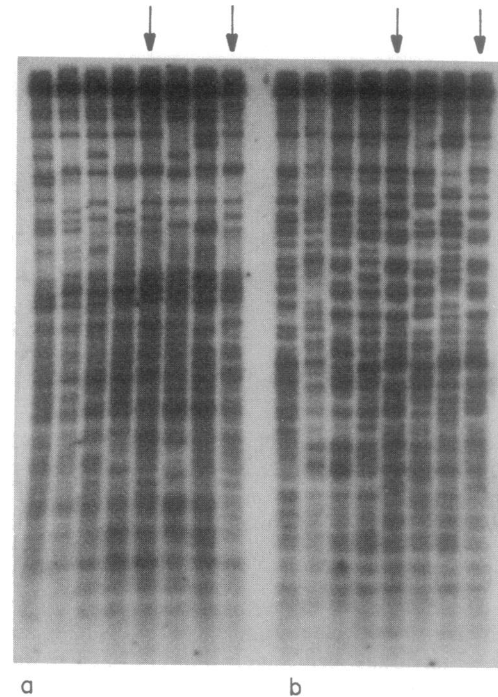

FIG. 1. DNA fingerprinting of a population sample of eight individuals of *R. marmoratus* from Rookery Bay, Collier County, FL, April 1990. (*a*) DNAs digested with *Hin*fI. (*b*) DNAs from the same individuals digested with *Alu* I and loaded in the same order as in *a*. Arrows indicate two coclonal individuals with identical fingerprints with both restriction enzymes; i.e., this sample contains seven clones. Probe, $(GACA)_4$. This 18-cm 1% agarose gel was run until a 1-kilobase marker had reached the end.

Similarity indices (pairwise proportional band sharing) among the clones in the Lower Matecumbe sample, for each of three probes, are plotted in Fig. 4. There is no meaningful correlation among the patterns of similarity measured by each of the probes. Proportional band sharing has been suggested as a general measure of genetic or genealogical relationship for fingerprint data (15), but, at least in the present case, it is obvious that "relationship" is primarily a function of the probe used.

DISCUSSION

In a widely cited analogy, Williams (ref. 16, p. 15) suggested that ". . . sexually produced offspring may be analogous to lottery tickets, and those asexually produced analogous to redundant copies of the same ticket." Our data imply that, despite its demonstrably clonal method of reproduction, most individuals in typical populations of *R. marmoratus* have unique genotypic "lottery tickets," and, moreover, that most of the tickets bear rather different numbers.

In all but two population samples, the number of distinguishable genotypes (clones) was very close to or equaled the number of individuals, essentially a one fish/one clone distribution. This level of genetic variation seems to approach that expected of individuals in a sexual population. It is apparently completely unprecedented among clonal animals (partial review in ref. 17). For example, the existence of seven electromorph clones in a single population sample of 47 obligate parthenogenetic *Daphnia* was referred to by its

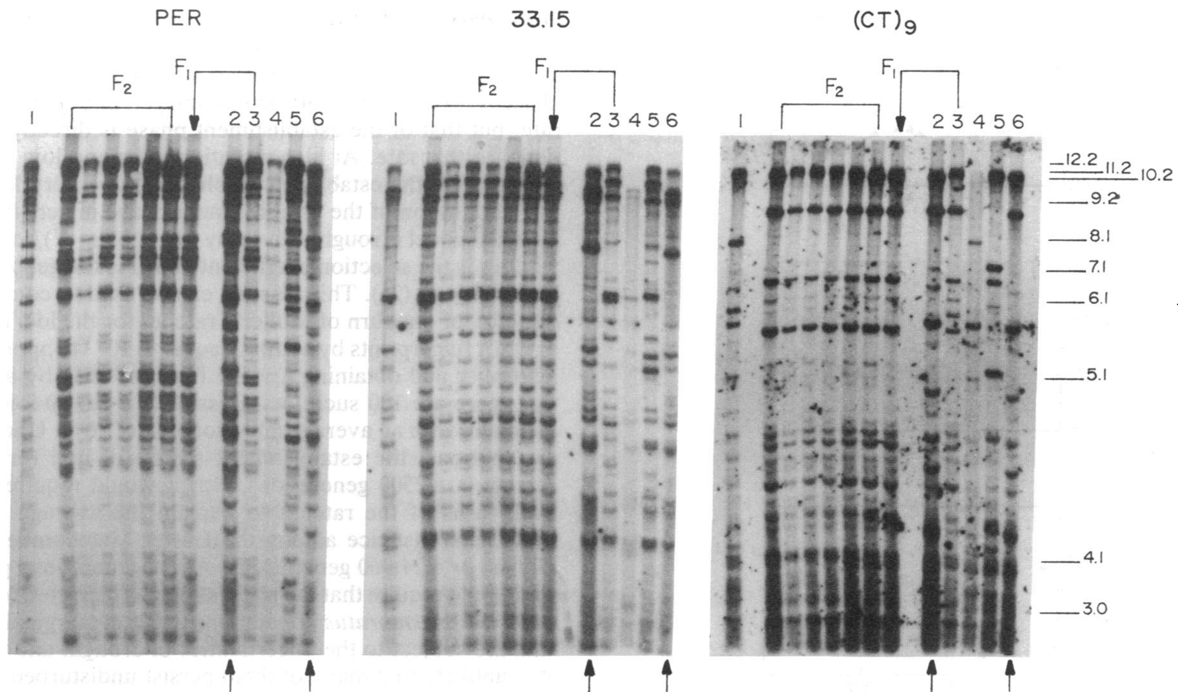

FIG. 2. Three sequential multiple probe hybridizations at moderate stringencies. Sample, six field-caught individuals from No Name Key, March 1986 (arrows indicate specimens 2 and 6 with identical fingerprints), and F_1 and F_2 laboratory-raised progeny of one of them. Restriction enzyme, *Hin*fI. Molecular size standards are given in kilobases. Note that although each probe detects specific bands, genetic identities within the field-caught sample and among the laboratory progeny are stable. This 0.9% agarose gel is identical to that shown in figure 1*B* of ref. 1 but probed with $(GACA)_4$.

discoverers as a "planktonic paradox" (18). Among clonal plants, the level of genetic diversity characteristic of *Rivulus* populations appears to be known only in certain grasses [e.g., *Festuca* (19, 20), *Agrostis* (20), and *Trifolium* (21)].

The current perception that the genetic diversity of clonal animals is rather limited is derived almost entirely from allozyme (enzyme electrophoresis) surveys. Such surveys fail to resolve any variation at all within and among Floridian

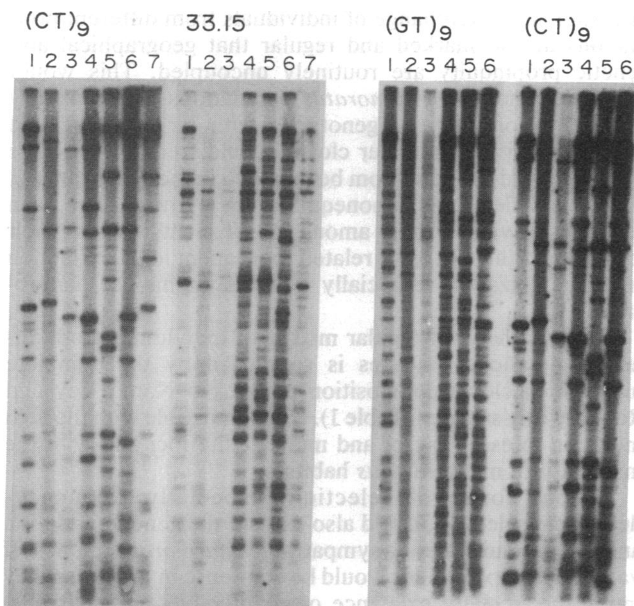

FIG. 3. Sequential multiple probe hybridizations at high stringencies. Sample, Lower Matecumbe Key, March 1989 ($n = 7$, *Left*; $n = 6$, *Right*). Restriction enzyme, *Alu* I. Note divergences among individuals with all three probes and repeatability of $(CT)_9$ pattern on two different gels. These 18-cm 0.9% agarose gels were run until a 2-kilobase marker had reached the end.

R. marmoratus populations (1). Typical mutation rates reported for the minisatellite loci that comprise many DNA fingerprints are at least 100 times greater than those usually quoted for protein-encoding genes (roughly 10^{-4} vs. 10^{-6}) (11, 22). Therefore, it is not clear whether *R. marmoratus* populations are really more variable than those of other clonal animals or whether the variation of the latter has been underestimated by a relatively insensitive technique.

However, one might turn the last suggestion around and argue instead that the fingerprint variation in our samples is simply evidence of the inevitable decay of genetic similarity by the accumulation of neutral mutations, possibly accompanied by sampling error, in a population whose members are otherwise virtually identical. The high mutation rates of minisatellite sequences, which in at least one case (in the

Table 2. Pairwise genetic divergence among six clones of *R. marmoratus* from Lower Matecumbe Key, FL, estimated with three probes

	1	2	3	4	5	6
1		6,6,9	4,5,7	9,9,18	3,9,10	8,11,10
		21	16	36	22	29
2	31,61,61		3,2,9	4,9,6	6,10,9	5,7,16
	153		14	19	25	28
3	27,52,52	26,45,33		6,8,9	2,5,7	4,6,6
	131	104		23	14	16
4	40,77,88	39,70,66	35,61,57		11,9,12	9,17,19
	202	175	153		32	45
5	39,75,83	38,68,64	34,59,55	47,84,88		4,7,16
	189	110	148	135		27
6	36,74,89	35,67,70	31,58,61	43,83,94	43,81,92	
	199	172	150	220	135	

Upper right of diagonal, no. of divergent bands with $(CT)_9$, $(GT)_9$, and 33.15 (top numbers); total divergent bands (bottom number). Lower left of diagonal, total bands detected with each probe (top numbers); grand totals (bottom number). Divergent bands and total bands are correlated ($r_p = 0.62$; $P = 0.006$, one-tailed).

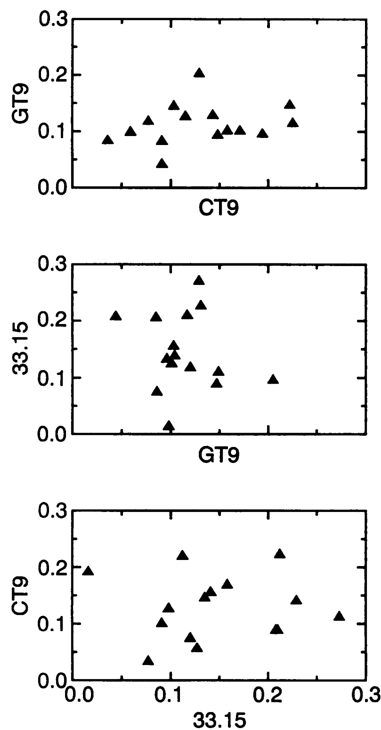

FIG. 4. Comparison of similarity indices among six or seven clones of *R. marmoratus* from Lower Matecumbe Key (March 1989) computed from DNA fingerprints generated by three different probes. Similarity indices are plotted for all pairwise comparisons for each probe. Of the three comparisons, only $(GT)_9$ vs. $(CT)_9$ is significantly correlated ($P = 0.045$; product-moment, one-tailed) but the relationship is weak ($r^2 = 0.2$).

mouse genome) approaches 0.1 per gamete-generation (13), seem to lend credence to this argument.

Intuitively, most of the genotypes produced by mutation in an otherwise genetically uniform clonal population should differ by one or a few mutational steps. These sister or near-sister clones should be divergent for one or a few fingerprint bands and the differences should be evident only with the use of one or a small number of different probes; they would otherwise be indistinguishable. More divergent clones can only evolve stepwise from less divergent (more intermediate) ancestors.

With the exception of two from Vero Beach, sister clones are conspicuously absent in our samples. Most sympatric clones, although often taken from the same or from closely adjacent crab holes, clearly differ by several independent mutations. For example, in the Lower Matecumbe Key sample (Table 2), the minimum number of total bands separating two clones is 14 (mean, 24; maximum, 45). Even if one adjusts this by 20% to allow for band overlap among probes, and allows a genetic distance of two bands between clones per homozygous mutation, the average clones differ by at least 9 or 10 mutational steps. Since the survey clearly did not detect all bands by which the clones differ, this estimate is obviously minimal and is likely markedly so.

Thus, to explain the *Rivulus* data by the gradual accumulation of neutral mutations in an otherwise uniform population, one must postulate the regular disappearance of less divergent ancestral clones in favor of more divergent descendants. This replacement process, in a context in which mutations are assumed to be slightly deleterious, has been studied by population biologists as "Muller's ratchet" (23). Haigh's analysis (24) suggests that every turn of the ratchet has an extinction phase, during which the least mutationally loaded class disappears stochastically, and an establishment phase, during which the size of the next-least-loaded mutational class shrinks from a previous deterministic level to one whose dynamics are governed by the extinction phase. At any given level of selection, the duration of the extinction phase is inversely proportional to the genome-wide mutation rate, but that of the establishment phase is directly proportional to that rate. At high mutation rates and low selection coefficients, the establishment phase can be very long.

The duration of the establishment phase in generations in Haigh's model is roughly given by $(\log s - \log U)/\log(1 - s)$, where s is the selection coefficient and U is the genome-wide mutation rate (25). This formula can be used to compute the duration of one turn of Muller's ratchet for the loci involved in DNA fingerprints by setting s equal to 10^{-3} to approximate neutrality and obtaining a value for U of 10^{-2} by assuming that there are 100 such loci (possibly a 5- to 10-fold underestimate) with an average mutation rate of 10^{-4}. Under these assumptions, the establishment phase for one turn of the ratchet is ≈2500 generations. But it would require 9 or 10 such turns of the ratchet to explain the average pairwise mutational distance among the Lower Matecumbe *Rivulus* clones, or ≈24,000 generations. At 1.5 generations per year, this would require that the population has persisted for 15,800 years. *R. marmoratus* populations occur in marginal habitats that are subject to the uncertainties of drought and tide, and it is unlikely that many of them persist undisturbed for even 1% of this time.

The genetic discontinuities among sympatric clones therefore imply that gradual accumulation of neutral mutations in otherwise genetically uniform populations is not a convincing explanation for DNA fingerprint variation within populations of *R. marmoratus*, even if mutation rates of the loci involved are very high and even if mutational distances among clones have been overestimated by a factor of two.

However, if high mutation rates are combined with regular mixing of individuals from different populations, a potential explanation of both genetic variation and discontinuity emerges. The vagility, at least over short distances, of individuals of *R. marmoratus* and congeneric species is well known and includes well-documented terrestrial excursions (see ref. 9 for a partial review). It is conceivable that migration and interchange of individuals from different populations are so marked and regular that geographical and genetic propinquity are routinely uncoupled. This would imply that most *R. marmoratus* populations are transitory admixtures of different genotypes with largely unrelated mutational histories. Sister clones would frequently not be sympatric and would seldom be included in the same sample. To our knowledge, such nonequilibrium mixtures have been reported previously only among clonal plants, where their formation is thought to be related to frequent local extinction and recolonization, especially after major habitat disturbances (26).

The hypothesis of regular mixing of individuals from different mutational lineages is supported by the complete turnover in clonal composition of the No Name Key and Rookery Bay samples (Table 1). It is compatible with the high mutation rates of micro- and minisatellite loci and with the instability of many *Rivulus* habitats.

Various forms of selection, especially frequency-dependent selection, could also explain the genetic variation and discontinuity among sympatric *R. marmoratus* clones. If valid, such explanations would be of intense interest, for they imply the frequent existence of adaptively significant phenotypic differences among clones. Since recombination is unknown in natural populations of this species, these differences could arise only by mutation. If mutation without recombination could generate large amounts of such variation, a major theoretical explanation for the perceived "evolutionary sluggishness" (27) of clonal organisms and of the

general predominance of sexual reproduction would be challenged.

However, DNA fingerprint variation *per se* is generally thought to be phenotypically inconsequential (but see refs. 28 and 29). The three *R. marmoratus* clones originally identified by Harrington and Kallman by histocompatibility experiments are divergent in the laboratory in several life history traits, which could be adaptively significant in nature (30). These clones also have distinctive DNA fingerprints (1), but the extent of the correlation among immunogenetic, DNA fingerprint, and other classes of genetic variation is unknown. Hypotheses based primarily on clonal selection are therefore tantalizing but premature.

We thank C. Grizzle, J. Grizzle, S. Ritchie, D. Schultz, A. Stark, and T. Stark for field assistance; D. Stanley for help in the laboratory; J. Tyson for useful discussions; and R. Andrews and an anonymous referee for comments on the manuscript. B.J.T. especially thanks B. Clarke and R. K. Selander for independent and spirited presentations of the mutation *in situ* hypothesis discussed above. This work was supported by National Science Foundation Grant BSR8506417 and by various VPISU Department of Biology research monies.

1. Turner, B. J., Elder, J. F., Jr., Laughlin, T. F. & Davis, W. P. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 5653–5657.
2. Turner, B. J., Elder, J. F., Jr., & Laughlin, T. F. (1991) *J. Fish Biol.* **39** Suppl. A, 131–142.
3. Harrington, R. W., Jr. (1963) *Physiol. Zool.* **36,** 325–431.
4. Lloyd, D. G. (1988) in *The Evolution of Sex*, eds. Michod, R. E. & Levin, B. R. (Sinauer, Sunderland, MA), pp. 233–252.
5. Kallman, K. D. & Harrington, R. W., Jr. (1964) *Biol. Bull.* **126,** 101–114.
6. Harrington, R. W., Jr. (1975) in *Intersexuality in the Animal Kingdom*, ed. Reinboth, R. (Springer, Heidelberg), pp. 249–262.
7. Atz, J. W. (1965) *Science* **150,** 789–797.
8. Turner, B. J., Davis, W. P. & Taylor, D. S. (1992) *J. Fish Biol.* **40,** 307–310.
9. Davis, W. D., Taylor, D. S. & Turner, B. J. (1990) *Ichthyol. Explor. Freshw.* **1,** 123–134.
10. Turner, B. J., Elder, J. F., Jr., & Laughlin, T. F. (1989) *Fingerprint News (Cambridge)* **1,** 15–16.
11. Jeffreys, A. J., Wilson, V. & Thein, S. L. (1985) *Nature (London)* **314,** 67–73.
12. Shin, H., Bargiello, T. A., Clark, B. T., Jackson, F. R. & Young, M. W. (1985) *Nature (London)* **317,** 445–448.
13. Mitani, K., Takahashi, Y. & Kominami, R. (1990) *J. Biol. Chem.* **265,** 15203–15210.
14. Ledwith, B. J., Warren, S. M., Nichols, W. & Bradley, M. O. (1990) *Biotechniques* **9,** 149–152.
15. Lynch, M. (1990) *Mol. Biol. Evol.* **7,** 478–484.
16. Williams, G. C. (1975) *Sex and Evolution* (Princeton Univ. Press, Princeton).
17. Hughes, R. N. (1989) *A Functional Biology of Clonal Animals* (Chapman & Hall, London).
18. Hebert, P. D. N. & Crease, T. J. (1980) *Science* **207,** 1363–1365.
19. Harberd, D. J. & Owen, M. (1969) *New Phytol.* **68,** 93–104.
20. Smith, A. (1972) *New Phytol.* **71,** 937–945.
21. Burdon, J. J. (1980) *J. Ecol.* **68,** 717–735.
22. Jeffreys, A. J., Royle, N. J., Wilson, V. & Wong, Z. (1988) *Nature (London)* **332,** 278–281.
23. Felsenstein, J. (1974) *Genetics* **78,** 737–756.
24. Haigh, J. (1978) *Theoret. Pop. Biol.* **14,** 251–267.
25. Bell, G. (1988) *Sex and Death in Protozoa* (Cambridge Univ. Press, Cambridge).
26. Sebens, K. P. & Thorne, B. L. (1985) in *Population Biology and Evolution of Clonal Organisms*, eds. Jackscon, J. B. C., Buss, L. W. & Cook, R. E. (Yale Univ. Press, New Haven), pp. 357–398.
27. Hughes, R. N. (1987) *Functional Ecol.* **1,** 63–69.
28. Chandley, A. C. & Mitchell, A. R. (1988) *Cytogenet. Cell Genet.* **48,** 152–155.
29. Caskey, G. T., Pizzuti, A., Fu, Y.-H., Fenwick, R. G., Jr., & Nelson, D. L. (1992) *Science* **256,** 784–789.
30. Massaro, E. J., Massaro, J. C. & Harrington, R. W., Jr. (1975) in *Isozymes*, ed. Markert, C. L. (Academic, New York), Vol. 3, pp. 439–453.