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Long-term retention of self-fertilization in a fish clade

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Among vertebrate animals, only the mangrove rivulus (*Kryptolebias marmoratus*) was known to self-fertilize. Here, we use microsatellite analyses to document a high selfing rate (97%) in a related nominal species, *Kryptolebias ocellatus*, which likewise is androdioecious (populations consist of males and hermaphrodites). In contrast, we find no evidence of self-fertilization in *Kryptolebias caudomarginatus* (an androdioecious species closely related to the *marmoratus-ocellatus* clade) or in *Kryptolebias brasiliensis* (a dioecious outgroup). These findings indicate that the initiation of self-fertilization predated the origin of the *marmoratus-ocellatus* clade. From mitochondrial DNA sequences and microsatellite data, we document a substantial genetic distance between *Kryptolebias marmoratus* and *K. ocellatus*, implying that the selfing capacity has persisted in these fishes for at least several hundred thousand years.

androdioecy | hermaphroditism | mangrove killifish | mating systems | reproductive modes

Hermaphroditism is not uncommon in fishes (1, 2), but self-fertilization is rare; among all vertebrate animals, only the mangrove rivulus (*Kryptolebias marmoratus*) offers a well-confirmed case (3, 4). Even among plants and invertebrates, where monoecy and hermaphroditism are widespread, outcrossing often remains the primary reproductive mode (5). Selfing entails intense inbreeding, and its general rarity probably reflects negative selection via inbreeding depression (6, 7). Exceptions are thus of evolutionary interest. Simultaneous hermaphroditism and self-fertilization in *K. marmoratus* were discovered in the 1960s (3). Later, researchers used molecular markers to confirm self-fertilization in this species (8) and to derive the first quantitative estimates of selfing and outcrossing rates (9–12). The latter vary geographically; outcrossing rates are high ($\approx 50\%$) in some Belize islands where males are common but low ($< 3\%$) in Florida and the Bahamas where males are rare (11–14).

The genus *Kryptolebias* (15) contains four to eight named species (depending on the degree of taxonomic splitting) that constitute a distinct clade of killifishes, Rivulidae. To evaluate the presence or absence and the rate of selfing, we screened microsatellite loci in populations of four nominal *Kryptolebias* species (Table 1): the sister taxa *K. marmoratus* and *Kryptolebias ocellatus*; *Kryptolebias caudomarginatus*, the closest phylogenetic outlier to that clade; and a more distant relative, *Kryptolebias brasiliensis* (16, 17). These taxa are deemed valid species in recent taxonomical evaluations (18), although *K. marmoratus* and *K. ocellatus* have been synonymized (19, 20). For current purposes (estimating the antiquity of the self-fertilization capacity), the taxonomic status of *K. marmoratus* and *K. ocellatus* is much less important than the elapsed time since these evolutionary entities separated. All other cyprinodontiform species are known or suspected to be gonochoristic (separate sexes). Thus, hermaphroditism and self-fertilization are derived rather than ancestral conditions in these fishes.

Results

Histologically, all of our specimens of *K. ocellatus* were simultaneous hermaphrodites, whereas all *K. brasiliensis* were gonochoristic. Previously, *K. caudomarginatus* was considered a gono-

chorist (e.g., ref. 18), but our histological appraisal identified putative females as hermaphrodites. The PCR primers for the 33 microsatellite loci that we used were developed for *K. marmoratus* (9). Our success with these primers varied: 31, 28, and 14 loci from *K. marmoratus* cross-amplified successfully with *K. ocellatus*, *K. caudomarginatus*, and *K. brasiliensis*, respectively. Polymorphism levels were high, although *K. ocellatus* displayed less genetic variation than the other species (Table 1).

With regard to departures from Hardy–Weinberg equilibrium, selfing should affect all loci equally, whereas several other evolutionary factors often tend to be locus-specific. Analysis of Hardy–Weinberg proportions revealed strikingly different patterns in *K. marmoratus* and *K. ocellatus* versus those in *K. caudomarginatus* and *K. brasiliensis* (Fig. 1). *K. ocellatus* and the Floridian populations of *K. marmoratus* boasted high positive values of F_{IS} at most loci, indicating substantial heterozygote deficits. At many polymorphic loci, F_{IS} reached its maximum possible value of 1.0 (no heterozygotes). The mean (across-loci) F_{IS} values in *K. ocellatus* and in Floridian *K. marmoratus* were > 0.95 and highly significant. The Belize *K. marmoratus* sample showed a similar but less pronounced pattern of heterozygote deficiency, with F_{IS} values ranging from 0.07 to 0.65 (mean 0.30).

In sharp contrast, *K. brasiliensis* and *K. caudomarginatus* displayed little tendency toward heterozygote deficiency. Although mean F_{IS} was statistically significant in *K. caudomarginatus* (Table 1), single-locus F_{IS} values in this species and in *K. brasiliensis* were typically low and mostly nonsignificant and showed approximately equal numbers of negative and positive values (Fig. 1). Several factors can influence departures from Hardy–Weinberg equilibrium within a population, including inbreeding, selection, and null alleles, and we do not interpret these locus-specific departures as evidence for selfing. Instead, the larger departures at a few exceptional loci in *K. caudomarginatus* probably register the presence of null alleles, a possibility also consistent with the output from Micro-Checker (21) (suggesting that null alleles were present at loci R30 and R92 in the Rio Iriri sample and at loci R9 and R38 in the Rio Piracão sample). Excluding these atypical loci from the analysis rendered mean F_{IS} values nonsignificant.

Different calculation methods yielded quantitatively similar mean estimates of selfing rates (Table 2): consistently near or above 0.90 for *K. ocellatus* from Brazil and for *K. marmoratus* from all Florida localities, ≈ 0.40 for *K. marmoratus* from Belize, and not significantly different from zero in *K. caudomarginatus* and *K. brasiliensis*.

Discussion

In the current study, we have genetically documented high selfing rates in a natural population of *K. ocellatus* in Brazil. The capacity for self-fertilization in this species was previously sus-

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The authors declare no conflict of interest.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. GQ389232–GQ389616).

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Table 1. Genetic variation in four species of *Kryptolebias*

Species (locality)	Sample size	No. loci	Percent loci polymorphic, 99% criterion	Mean no. alleles per locus	Expected heterozygosity, random mating	Observed heterozygosity	Inbreeding coefficient, F_{IS}
<i>K. ocellatus</i> (Rio Piracão, Guaratiba, Brazil)	10	31	12.9	1.29	0.060	0.003	0.95*
<i>K. marmoratus</i> (Long Key, Florida)	6	33	75.8	2.73	0.463	0.020	0.96*
<i>K. marmoratus</i> (No Name Key, Florida)	10	33	81.8	3.46	0.495	0.024	0.95*
<i>K. marmoratus</i> (Big Pine Key, Florida)	40	33	87.9	4.24	0.474	0.015	0.97*
<i>K. marmoratus</i> (Twin Cays, Belize)	40	33	93.9	8.39	0.665	0.467	0.30*
<i>K. caudomarginatus</i> (Rio Piracão, Guaratiba, Brazil)	24	28	78.6	7.75	0.522	0.486	0.07*†
<i>K. caudomarginatus</i> (Rio Iriri, Magé, Brazil)	51	28	82.1	10.71	0.501	0.480	0.04*†
<i>K. brasiliensis</i> (Ribeirão Imbariê, Magé, Brazil)	8	14	42.9	2.5	0.262	0.259	0.01

*Significantly larger than zero ($P < 0.001$).

†Nonsignificant ($P > 0.05$) after excluding two loci with inferred null alleles.

pected from aquarium observations wherein individuals produce progeny in isolation, although parthenogenesis (apomixis) could not be ruled out. Lubinski et al. (22) DNA-fingerprinted a brood from a single progenitor and found identical genotypes among the offspring, also consistent with either self-fertilization or apomixis. Our microsatellite data unequivocally document selfing in *K. ocellatus* and demonstrate that this reproductive mode prevails ($S > 0.90$) in a natural population. The high selfing rate for *K. ocellatus* is similar to estimates for *K. marmoratus* in the Florida Keys (current study) and from several other localities in Florida and the Bahamas (10–12).

Our genetic findings suggest that selfing is absent in *K. caudomarginatus* and *K. brasiliensis*. *K. caudomarginatus* formerly was regarded as dioecious, but the disclosure that at least some “females” are hermaphrodites implies that this species tends toward androdioecy (mixtures of males and hermaphrodites). The comparison of *K. caudomarginatus* with *K. marmoratus* and *K. ocellatus* shows that populations with similar reproductive morphologies can have different mating systems (rates of outcrossing). Thus, as is well known for many plants and invertebrates, sexual systems defined by reproductive anatomy

often differ from those defined by reproductive function (e.g., ref. 23).

These findings now can be placed in evolutionary context. On the basis of a single specimen from each species, Murphy et al. (16) reported that *K. ocellatus* and *K. marmoratus* differ at 1.3% of 1,691 nucleotide positions across four mtDNA loci: COI, 12S and 16S rRNA, and cytB. From our current mtDNA data across three other mtDNA regions, net nucleotide sequence divergence (after correction for within-species variation) between *K. ocellatus* and *K. marmoratus* is even greater: 3.2–4.3% (mean $3.8 \pm 0.4\%$). Furthermore, from the coding regions of our dataset (1,881 bp), 10 fixed amino acid differences distinguished *K. ocellatus* and *K. marmoratus*, whereas no fixed differences were found among populations of *K. marmoratus* in Florida, Bahamas, and Belize. Mean genetic distance within *K. marmoratus* was 0.26% (i.e., 14 times smaller than the mean genetic distance between *K. ocellatus* and *K. marmoratus*). The much larger genetic distances between versus within *K. marmoratus* and *K. ocellatus* are also obvious in a mitochondrial phylogeny in which individual fish are treated as units of analysis (Fig. 2), and they are consistent with separate-species status for these two taxa (24). A qualitatively similar pattern of relationships is apparent in a microsatellite phenogram of populations (Fig. 2).

All of these genetic data imply a considerable antiquity for the population split between *K. marmoratus* and *K. ocellatus*. If we provisionally use a conventional mtDNA clock calibration for vertebrate mtDNA [1% sequence change per lineage per million years (25)], then “speciation” probably took place nearly 2 million years ago. However, our general conclusion that selfing has been long-retained in a *Kryptolebias* clade does not rely unduly on a specific mtDNA clock. Even if mtDNA in *Kryptolebias* evolves 10 times faster than the vertebrate norm, then the estimated speciation date would still be $\approx 200,000$ years ago, a substantial length of evolutionary time. Nor does our conclusion rest unduly on correction factors for estimating net sequence divergence between these species. For example, if we subtract the maximum observed mtDNA distance within *K. marmoratus* ($0.69 \pm 0.14\%$ between fish from Belize and the Bahamas) from the minimum mtDNA distance between *K. ocellatus* and *K. marmoratus* ($3.59 \pm 0.35\%$), we still estimate a net sequence divergence of $\approx 2.9\%$, which would imply much more than 100,000 years of population separation even under a 10X-accelerated mtDNA clock. Nor does our broad conclusion depend upon separate-species status for *K. marmoratus* and *K. ocellatus*. Even if these fish are deemed conspecific [as they are in a current formal classification (20)], the empirical case for the long-term retention of self-fertilization would remain. Thus, even under extremely conservative assumptions, the data forcefully argue that the capacity for self-fertilization is a long-term reproductive mode in *Kryptolebias*.

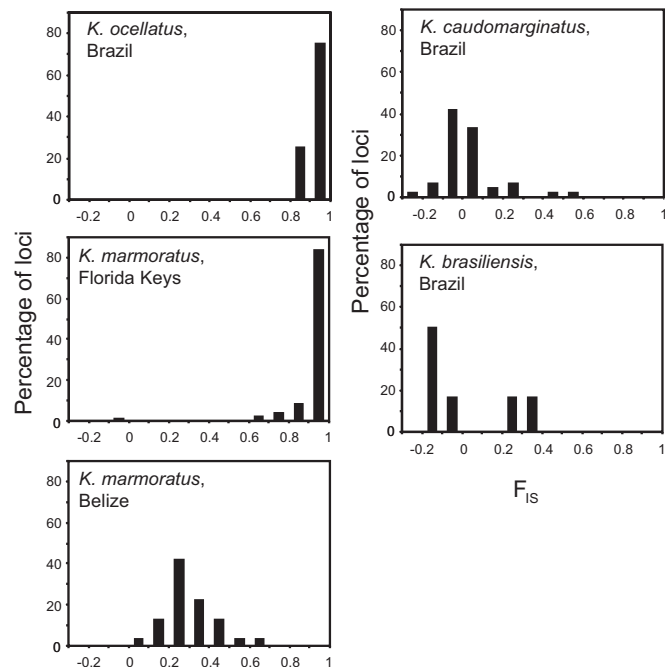


Fig. 1. Frequency distributions of single-locus inbreeding coefficients (F_{IS}) in various *Kryptolebias* populations.

Table 2. Selfing rates (S) in *Kryptolebias*. $S(F_{IS})$, estimated from F_{IS} values (after excluding loci with null alleles); $S(g_2)$, estimated from g_2 values; and $S(ML)$, a maximum likelihood estimate

Species (locality)	No. loci polymorphic	$S(F_{IS})$	No. loci		$S(ML)$
			useful	$S(g_2)$	
<i>K. ocellatus</i> (Rio Piracão, Guaratiba, Brazil)	4	0.974 (0)	1	NA	NA
<i>K. marmoratus</i> (Long Key, Florida)	25	0.980 (0)	4	0.925 (0.01)	0.878 (0.03)
<i>K. marmoratus</i> (No Name Key, Florida)	27	0.976 (0)	7	0.969 (0)	0.919 (0)
<i>K. marmoratus</i> (Big Pine Key, Florida)	29	0.984 (0)	11	0.971 (0)	0.961 (0)
<i>K. marmoratus</i> (Twin Cays, Belize)	31	0.459 (0)	31	0.398 (0)	0.383 (0)
<i>K. caudomarginatus</i> (Rio Piracão, Guaratiba, Brazil)	22	0.064 (0.07)	21	0.018 (0.2)	0.002 (1)
<i>K. caudomarginatus</i> (Rio Iriri, Magé, Brazil)	23	0.020 (0.21)	23	0.009 (0.25)	0.003 (1)
<i>K. brasiliensis</i> (Ribeirão Imbariê, Magé, Brazil)	6	0.024 (0.52)	5	0.120 (0.26)	0.013 (1)

Probabilities of the null hypothesis that there is no selfing ($S = 0$) are shown in parentheses. "Useful" loci are those with both homozygotes and heterozygotes (only such loci could be used in the g_2 -based and maximum likelihood methods).

Self-fertilization is often considered a poor reproductive tactic because of intense inbreeding. Another vertebrate limitation on selfing comes from inherent physiological and hormonal conflicts in producing eggs and sperm simultaneously. Nevertheless, we present a vertebrate lineage in which simultaneous hermaphroditism has arisen and persisted for at least hundreds of thousands of years. However, self-fertilization is merely a component of a mixed-mating system in *Kryptolebias* (9–12), where various populations self-fertilize and outcross at different rates. Although *Kryptolebias* fishes provide the only known examples of mixed-mating systems in vertebrate animals, such systems are relatively common in many plants and invertebrate taxa (4).

In some respects, a mixed-mating system can convert a generally maladaptive strategy of pure selfing to a combined strategy with favorable elements of both selfing and outcrossing (4). The many advantages of outcrossing mostly relate to genetic recombination and resultant adaptive flexibility. The potential benefits of selfing are twofold: the "clonal" perpetuation of homozygous multilocus genotypes that might be selectively advantageous in a particular environment (26) and assured fertilization with no need for a mate (27). We strongly suspect that the latter is the primary selective advantage of selfing in *Kryptolebias*, enabling these fish to reproduce and colonize even at low population densities. (*K. marmoratus* is distributed widely in the Americas but is often locally rare.) If this argument has merit, then it suggests that the broader rarity of vertebrate selfing may reflect mechanistic difficulties of evolutionary origin rather than inherent problems in maintaining self-fertilization, once present, as part of a mixed-mating system.

Materials and Methods

Table 1 shows sample sizes and collection locales for fish used in the microsatellite analyses. The PCR amplifications and genotyping of 33 microsatellite loci were carried out as described in ref. 9, except that in the current study we fractionated alleles on a capillary instrument (GA3100) and sized them by using software GeneMapper (both from Applied Biosystems). For the mtDNA analyses, we used samples from our previous studies (10–12) plus newly collected specimens. We sequenced a total of 2,946 nucleotide positions from three mitochondrial regions in 10 specimens of *K. ocellatus* and from 136 or more fish (depending on the gene) of *K. marmoratus*. Region ND6 spans 873 bp and

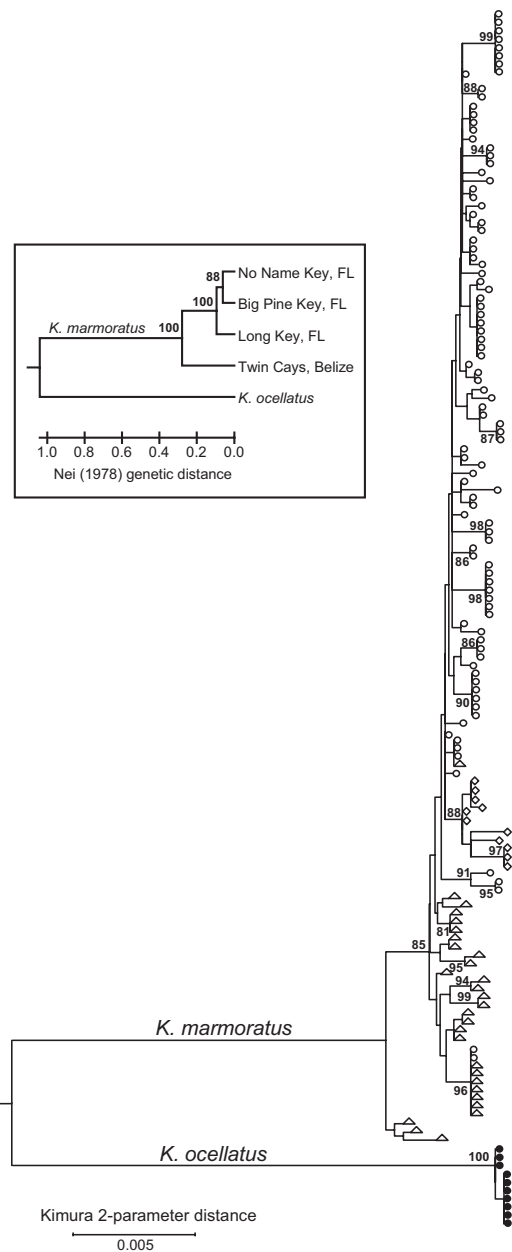


Fig. 2. Genealogy for 136 individuals of *K. marmoratus* and 10 individuals of *K. ocellatus* based on 2,946-bp mtDNA sequences. Each circle, triangle, or rhombus represents an individual. In the *K. marmoratus* clade, triangles, open circles, and rhombi designate fish from Belize, various locations in Florida, and the Bahamas, respectively. Bootstrap values above 80% are shown. (Inset) Population phenogram for these species based on a cluster analysis of Nei's genetic distances from 31 microsatellite loci.

includes the full NADH dehydrogenase (ND)-6 gene and adjacent tRNA-Glu and portions of ND-5 and cytochrome (cyt) B (positions 14383–15255 on the complete mitochondrial genome of *K. marmoratus*; GenBank accession AF283503). Region cytB-CR1 spans 1246 bp of aligned sequences (positions 16060–17300) and includes the 3' end of cytB, all of tRNA-Thr and tRNA-Pro, and most of the control region (CR) I, except for its terminus (which we could not resolve due to a long stretch of C repeat). Region ATP6 spans 827 bp (positions 8848–9674) that includes ATP6 plus portions of ATP8 and cytochrome oxidase 3. Histological analysis of the Brazilian material followed standard procedures with hematoxylin and eosin staining. Gender assignments were done as defined in ref. 28.

Observed and expected heterozygosities and inbreeding coefficient F_{IS} were calculated in FSTAT (29). This program also was used to assess the

significance of F_{IS} in each population for each locus and across all loci by randomization tests. Sequential Bonferroni corrections were applied (30). In cases where departures from Hardy–Weinberg equilibrium were inconsistent across loci, tests for the presence of null alleles were performed using Micro-Checker (21). Selfing rates (S) were estimated by three approaches: from the inbreeding coefficient (F_{IS}) using the relationship $S = 2F_{IS}/(1 + F_{IS})$ (31), from two-locus heterozygosity disequilibrium values (g_2) using the software RMES (32), and by maximizing the log-likelihood of the multilocus heterozygosity structure of the sample, also using RMES. For the microsatellite data, genetic distances were calculated according to Nei (33) and summarized in a phenogram using Microsatellite Analyser (34) and Phylip (35). The number of surveyed loci varied among species, so we calculated genetic distances in two

ways: from the maximum possible number of loci for each pair of species and from a subset of 31 loci scored in *K. ocellatus* and *K. marmoratus*. For the aligned mtDNA sequences, genetic distances [Kimura's two-parameter method (36)] were calculated using Mega3 (37).

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