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Brief Communication

A Genetic Test for Whether Pairs of Hermaphrodites Can Cross-Fertilize in a Selfing Killifish

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Abstract

Kryptolebias marmoratus, a small killifish that lives in mangrove habitat from southern Florida to Brazil, is one of the planet's only known self-fertilizing hermaphroditic vertebrates. Generation after generation, hermaphroditic individuals simultaneously produce sperm and eggs and internally self-fertilize to produce what are, in effect, highly inbred clones of themselves. Although populations are composed primarily of hermaphrodites, they also contain some true males. The frequency of males in a population varies geographically, from <2% in Florida to as high as 25% in Belize. Males are known to mate occasionally with hermaphrodites, thereby releasing genetic variation that has profound consequences for population genetic structure. However, it is unknown whether hermaphrodites can or do sporadically mate with each other also. Here, we test whether hermaphroditic individuals of the killifish *Kryptolebias marmoratus* are capable of crossing with one another, in addition to their much more common habits of self-fertilization and occasional outcrossing with pure males. We employ an experimental design in which replicate hermaphrodite pairs were housed together and allowed to reproduce naturally. Among 173 embryos screened at diagnostic microsatellite loci, all were found to result from selfing (i.e., no embryos were the product of a hermaphrodite cross). We thus conclude that hermaphrodite pairs are unlikely to cross, or do so exceedingly rarely.

The mangrove killifish, *Kryptolebias marmoratus* and its congener *K. ocellatus*, are unique among vertebrates in consisting primarily of hermaphroditic individuals that are capable of internal self-fertilization (Costa et al. 2010; Harrington 1961). When continued across successive generations, such selfing produces what in effect are near-clonal lineages of isogenic progeny. Many populations of *K. marmoratus* also exhibit a small but variable number of "true" males. Population genetic analyses and laboratory experiments indicate that selfing is the predominant mode of reproduction, with occasional outcrossing

(Mackiewicz et al. 2006b, 2006c). The outcross events that produce genetically diverse progeny are thought to result from hermaphrodites mating with true males. This is because outcrosses between males and hermaphrodites have been documented in the laboratory (Harrington and Kallman 1968; Mackiewicz et al. 2006a), and in the wild there is a positive correlation between the frequency of males in a population and the inferred frequency of outcrossing (based on levels of genetic variation/homozygosity) (Tatarenkov et al. 2015). In general, the population genetic structure of *K. marmoratus* is characterized

by a high level of homozygosity owing to predominant selfing (i.e., extreme inbreeding), with occasional releases of large amounts of genetic variation owing to outcross events between hermaphrodites and males. This androdioecious mating system and its resulting population genetic architectures are highly analogous to those displayed by some plants and invertebrate animals with mixed-mating systems, and thus represent a remarkable case of convergent evolution across distantly related taxa (Mackiewicz et al. 2006b, 2006c).

Hermaphroditic individuals in *K. marmoratus* contain an ovotestis that produces both eggs and sperm; self-fertilization typically occurs internally in the posterior portion of the gonadal lumen, soon after which the embryos are deposited externally (Sakakura et al. 2006). As indicated above, in *K. marmoratus* occasional outcrossing between hermaphrodites and true males has been documented in the laboratory (Harrington and Kallman 1968; Mackiewicz et al. 2006a), when unfertilized eggs are released by a hermaphrodite and fertilized externally by a male. Hermaphrodite–hermaphrodite matings have been considered unlikely owing to a lack of observed mating behavior in the laboratory (Harrington 1963), but this hypothetical phenomenon has not been subjected to experimental tests and it remains theoretically plausible that hermaphrodites are capable of crossing with one another. Crosses between hermaphrodites might be expected for the same reason that crosses between hermaphrodites and males are observed. Such crosses increase the genetic diversity of progeny, and this may be adaptive. Alternatively, occasional outcrossing may happen simply because males exist in small numbers and are able to induce hermaphrodites to mate with them. If we assume that occasional outcrossing provides some fitness advantage, then presumably it should not matter whether the source of sperm is from a true male or another hermaphrodite. Furthermore, in some populations males are rare or have not been observed (Tatarenkov et al. 2012). Yet such populations exhibit the genetic signature of occasional outcrossing. In circumstances where males are hard to find owing to rarity, hermaphrodites could presumably serve the function of a male and occasionally outcross with other hermaphrodites. Here, we provide a critical test of this possibility by genotyping many progeny at diagnostic microsatellite loci.

Methods

A total of 6 hermaphrodite pairs were placed into individual 4-quart plastic Tupperware containers that contained 25 parts-per-thousand (ppt) seawater and a spawning mop made of brown yarn. Fish were fed newly hatched brine shrimp nauplii *ad libitum*. Embryos were collected every 2–3 days over a period of 60 days and incubated in plastic Solo™ cups containing 25 ppt seawater.

Hermaphrodites used in the experiment had previously been genotyped at multiple microsatellite loci (Tatarenkov et al. 2012,

2015) and found to be fully homozygous. Thus, we were able to set up crosses (i.e., place 2 hermaphrodites together) in which each individual was derived from 1 of 7 isogenic lines known to differ from other such clonal lineages at 1 or more microsatellite loci (Table 1; Figure 1). This circumstance allowed for a relatively quick and simple parentage screen of numerous embryos. Specifically, embryos produced by self-fertilization would be homozygous at the chosen microsatellite loci; whereas in contrast, any embryos produced by a cross between hermaphrodites would be heterozygous at the diagnostic microsatellite loci. Our 6 crosses represented both within-population and between-population pairings of individuals. This was important because there could potentially be a preference for outcrossing with either genetically similar (i.e., within population) or genetically divergent (i.e., different population) individuals.

Eggs were washed in freshwater, distributed individually into the wells of 96-well PCR plates, frozen at –20° Celsius for 10 min, suspended in 50 uL of 10 mM Tris pH 7.6, 0.1 mM EDTA buffer, and then physically broken with a toothpick. We then performed isolation of total DNA by boiling (Milligan 1998) to extract genomic DNA from each embryo. Cellular debris was pelleted by centrifugation and the resulting lysate was used in the PCR reactions. Sequences for PCR primers used in this study have been previously published and include those for microsatellite loci R3, R10, and R18 (Mackiewicz et al. 2006a). The 10 uL PCR reaction mix was composed of 1X GoTaq reaction buffer (which included 1.5 mM $MgCl_2$), 0.25 mg BSA, 0.2 mM each dNTP, 0.25 mM each primer, 0.4 units GoTaq DNA polymerase (Promega), and 1 uL genomic DNA. PCRs were carried out using the following protocol: initial denaturation at 95 °C for 5 min; 32 cycles of 40 s at 95 °C (denaturation), 40 s at 53 °C (annealing), and 60 s at 72 °C (extension); with a final extension time of 7 min at 72 °C. Diagnostic loci were amplified separately, diluted 10–15-fold and pooled for fragment analysis. About 1 μL of the resulting multiplex was mixed with 9.6 μL of deionized formamide and 0.4 μL size standard GS500 (ROX labeled; Applied Biosystems), denatured for 4 min at 95 °C, and electrophoresed on an GA 3100 instrument using 50 cm capillaries filled with Pop4 (Applied Biosystems). Alleles were scored using Genemapper 4.0 (Applied Biosystems). In fulfillment of data archiving guidelines (Baker 2013), we have deposited the primary data in the online supporting information.

Results

A total of 208 embryos were produced by the 6 different hermaphrodite pairs over the 60-day period of observation. About 191 of these embryos (91.8%) remained viable and were subjected to molecular parentage analyses. DNA extraction and subsequent PCR were successful for 173 embryos. All embryos proved to be homozygous at

Table 1. Lineage details for *K. marmoratus* used in the present study

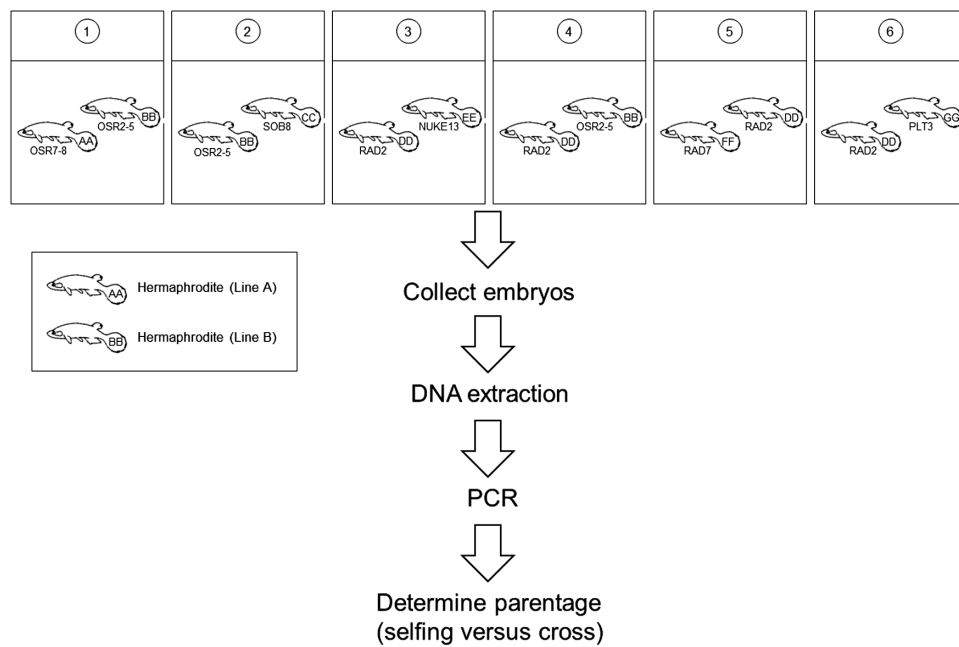| Lineage ID	Location	GPS coordinates	Microsatellite length (bp)		
			R3	R10	R18
PLT3	Plantation Key, FL	N24°59′24.8″, W080°33′04.7″	159	267	216
OSR2-5	Sugarloaf Key, FL	N24°36′49.4″, W081°33′06.7″	159	228	196
OSR7-8	Sugarloaf Key, FL	N24°36′49.4″, W081°33′06.7″	135	222	204
SOB8	Sugarloaf Key, FL	N24°36′05.2″, W081°34′34.1″	159	234	220
RAD2	St. Lucie Co., FL	N27°20′48.4″, W080°14′16.9″	147	228	216
RAD7	St. Lucie Co., FL	N27°20′48.4″, W080°14′16.9″	147	219	216
NUKE13	St. Lucie Co., FL	N27°21′00.2″, W080°14′22.5″	155	219	216

Figure 1. Illustration of the experimental design used to test whether *K. marmoratus* hermaphrodites can successfully mate with one another. Two hermaphrodites that were homozygous at a given microsatellite locus (but differed in alleles between the inbred strains) were set up in each of 6 tanks and provided with spawning substrate. Homozygous embryos are the product of self-fertilization, whereas any heterozygous embryos would have been produced by a cross between clonal lineages.

Table 2. Summary of experimental results testing whether hermaphroditic individuals of *K. marmoratus* will cross with each other, in addition to self-fertilizing

Tank	Hermaphrodite		Diagnostic microsatellite(s)	Eggs (viable/total)	No. of PCRs that worked	Embryos produced by hermaphrodite cross	Embryos produced by hermaphrodite 1 selfing	Embryos produced by hermaphrodite 2 selfing
	Individual 1	Individual 2						
1	OSR7-8	OSR2-5	R3, R10, R18	59/63	49	0	8	41
2	OSR2-5	SOB8	R10, R18	8/14	7	0	7	0
3	RAD2	NUKE13	R3, R10	67/69	65	0	53	12
4	RAD2	OSR2-5	R3, R18	47/50	45	0	7	38
5	RAD7	RAD2	R10	8/8	5	0	2	3
6	RAD2	PLT3	R3, R10	2/4	2	0	2	0

the screened microsatellite loci (Table 2), indicating that they were produced by self-fertilization of a homozygous hermaphrodite. No embryos were the product of a hermaphrodite-by-hermaphrodite mating (which otherwise would have been apparent as heterozygous progeny at the screened microsatellite loci).

Discussion

Owing to its unique mating system, *K. marmoratus* is of considerable interest from a genetic perspective (Avise and Tatarenkov 2015). The experiment performed here was designed to test whether hermaphrodites of *K. marmoratus* might be capable of mating with one another and producing genetically diverse progeny. Such a phenomenon would have significant consequences on the population genetic architecture of this species. The possibility of hermaphrodite × hermaphrodite crosses was considered unlikely by previous authors (Harrington 1963; Turner et al. 2006), but had not been ruled out on empirical grounds. Here, we explicitly test this

possibility and demonstrate that hermaphrodite × hermaphrodite crosses are unlikely to be a regular feature of the mating system of *K. marmoratus*.

Several observations are consistent with the lack of hermaphrodite × hermaphrodite crosses observed here. Harrington and Kallman (1968) and later Mackiewicz et al. (2006a) demonstrated that unfertilized eggs occasionally emitted by hermaphrodites can be fertilized by primary males. However, Harrington (1963) p. 338 states: "Supplementary observations on pairs of hermaphrodites in the same aquarium…failed so far to disclose any obvious reciprocity between ovipositing hermaphrodites…" Likewise, Turner et al. (2006) p. 1478 concluded: "Matings between hermaphrodites are unknown in this species and are unlikely to be productive, for sperm is believed to be in limiting supply and probably does not leave the ovotestis (Harrington, 1963). Therefore, outcrossing (and consequent heterozygosity) most likely stems from matings between hermaphrodites … and rare gonochoristic males. Unlike hermaphrodites, males produce abundant sperm."

Of course, it is impossible to categorically prove the null hypothesis (in this case that pairs of hermaphrodites never cross). Nevertheless, all available evidence currently suggests that crosses between hermaphrodites are likely to be rare in *K. marmoratus*, if they occur at all.

Supplementary Material

Supplementary material can be found at http://www.jhered.oxford-journals.org/.

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