

A mixed-mating strategy in a hermaphroditic vertebrate

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Mixed-mating systems, in which hermaphrodites can either self-fertilize or outcross, are common in many species of plants and invertebrates and have been informative models for studying the selective forces that can maintain both inbreeding and outbreeding in populations. Here, we document a remarkable instance of evolutionary convergence to an analogous mixed mating system by a vertebrate, the mangrove killifish (*Kryptolebias marmoratus*). In this androdioecious species, most individuals are simultaneous hermaphrodites that characteristically self-fertilize, resulting in local populations that consist of (nearly) homozygous lines. Most demes are also genetically diverse, an observation traditionally attributed to de novo mutation coupled with high levels of inter-site migration. However, data presented here, from a survey of 35 microsatellite loci in Floridian populations, show that genotypic diversity also stems proximally from occasional outcross events that release ‘explosions’ of transient recombinant variation. The result is a local population genetic pattern (of extensive genotypic variety despite low but highly heterogeneous intra-individual heterozygosities) that differs qualitatively from the genetic architectures known in any other vertebrate species. Advantages of a mixed-mating strategy in *K. marmoratus* probably relate to this fish’s solitary lifestyle and its ability to colonize new habitats.

Keywords: outcrossing; selfing; heterozygosity; mangrove killifish; *Kryptolebias marmoratus*

1. INTRODUCTION

Three categories of ‘clonal’ reproduction in vertebrates each yield a characteristic genetic footprint with respect to intra-individual heterozygosities. Parthenogenesis and related reproductive modes (gynogenesis and hybridogenesis) that are characteristic of several unisexual fishes, amphibians and reptiles (Vrijenhoek *et al.* 1989) originated in all known cases via hybridization between related sexual species, so specimens within each resulting lineage typically display high levels of heterozygosity (Balloux *et al.* 2003). Polyembryony or ‘twinning’, characteristic of armadillos in the genus *Dasypus* (Loughry *et al.* 1998) and occurring sporadically in many other species, produces intra-generational clonemates from outcrossed zygotes, so such individuals show moderate or normal heterozygosities. Self-fertilization in vertebrates, known only in *Kryptolebias* (formerly *Rivulus*) *marmoratus* (Harrington 1961), is an intense form of inbreeding yielding strains that traditionally have been referred to as ‘clones’ and that normally show extremely low heterozygosities (Harrington & Kalman 1968; Turner *et al.* 1990; Laughlin *et al.* 1995).

The mangrove killifish *Kryptolebias marmoratus* lives in and around red mangrove forests along the eastern and northern coasts of South America, throughout most of the

Caribbean region and in the Bahamas and southern Florida (Huber 1992). Earlier DNA fingerprinting studies using multi-locus probes revealed high genetic diversity among lineages of wild-caught hermaphrodites (Turner *et al.* 1990, 1992), a result attributed to the accumulation of mutations coupled with high rates of migration among populations (Turner *et al.* 1990; Laughlin *et al.* 1995). In this study, we genotyped wild-caught individuals representing six geographic regions in Florida at 35 microsatellite loci. The data provide unequivocal evidence for occasional outcrossing and they also reveal a qualitatively unique population genetic structure (never before reported in any vertebrate species) reflective of a mixed-mating strategy for this species.

2. MATERIAL AND METHODS

(a) Collections

Samples were from frozen collections of fish from the following Florida locales (with abbreviations): Long Key (TLK) near the middle of the Florida Keys; Charlotte Co. (CC) near Venice; St Lucie Co. (StL); Marco Island (MI) in Collier Co.; Monroe Co. near Flamingo in Everglades National Park (ENP) and Shark River (SR), also in Monroe Co. (figure 1). Specimens were captured using tiny traps inserted into crab holes, or by dipnetting, seining or by miniature hook and line.

(b) Microsatellite genotyping

High-molecular weight genomic DNA was extracted and purified from whole fish by standard phenol/chloroform

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The electronic supplementary material is available at <http://dx.doi.org/10.1098/rspb.2006.3594> or via <http://www.journals.royalsoc.ac.uk>.

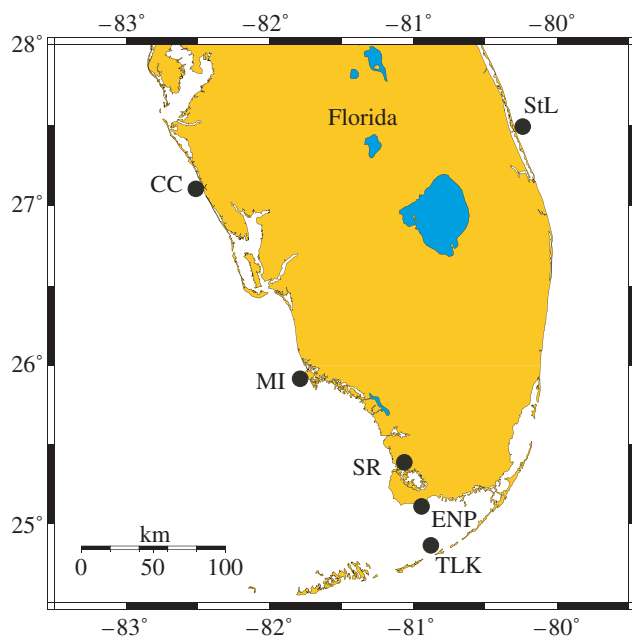


Figure 1. Map of collection locales in southern Florida. The map was produced using online map creator available at <http://www.aquarius.geomar.de/omc>.

protocols. PCR amplifications, genotyping gel runs and analyses were carried out (as described by Mackiewicz *et al.* submitted) using 35 microsatellite loci. GenBank accession numbers for the microsatellite loci analysed in this study are DQ335413–DQ335447.

3. RESULTS

Figure 2 shows the genotypes of all 78 specimens surveyed (see also electronic supplementary material). Consider first the seven specimens from Long Key (TLK; figure 2a). Individuals TLK2 and TLK4–TLK7 represent one highly homozygous lineage and individual TLK3 represents another highly homozygous line that differs from the first at 20 of 35 loci (57%). By contrast, specimen TLK1 was heterozygous at 20 loci, 18 of which were precise combinations of alleles that distinguished the two homozygous lineages observed at that locale. (At the other two heterozygous loci (R27 and R30), one allele differed from those observed in the homozygous lines but it could be attributed in each case to a single-step germline mutation in recent generations.) We interpret this concentration of heterozygosity in TLK1 to be conclusive documentation of a natural outcross event ‘caught in the act.’

Consider next the 17 hermaphroditic individuals from Charlotte County (CC; figure 2b). Individuals CC1, CC2, CC13 and CC14 were identically homozygous across all 35 microsatellite loci and thus constitute one inbred lineage, whereas individuals CC3, CC10, CC15 and CC17 each differ from that lineage by single-locus changes that could plausibly be explained by germline mutations in ancestors followed by selfing that re-established homozygosity at most loci. Similarly, individuals CC11, CC12 and CC16 are closely related to a second inbred line composed of specimens CC5–CC9. However, CC4 was heterozygous at 7 of 35 loci (20%), and at 12 loci it also displayed alleles not otherwise observed in this population. In principle, this individual could represent a third

lineage that had diverged from the others by multiple mutations and had not yet reconverted to homozygosity by selfing, but this seems very improbable given the number of heterozygous loci observed. Indeed, assuming an average mutation rate $\mu = 10^{-3}$ typical for microsatellite loci (Goldstein & Schlötterer 1999), we expect that a new mutation will strike one of 35 loci once in about 29 generations ($1000/35 = 28.6$). Since the probability that a locus remains heterozygous decreases by 50% per generation in an obligate selfer, by the time a second mutation strikes it is highly unlikely ($P = 1.9 \times 10^{-9}$) that the previously mutated locus would still be heterozygous. Thus, almost certainly, CC4 is the product of a recent outcross event (involving lineages not otherwise detected in our genetic survey).

A more complex picture of genetic population structure emerged from the 24 specimens from StL, MI and ENP (figure 2c). Although some nearly perfect multi-locus genotypic matches were present (e.g. individuals ENP3 and ENP10 from ENP and MI3 and MI4–MI8 from MI), fewer specimens shared identical multi-locus genotypes and numbers of heterozygous loci were somewhat higher overall. Nonetheless, 41 of the 51 heterozygous genotypes (80.4%) were concentrated in just four among the 24 specimens surveyed (StL5, StL7, StL12 and MI6). Finally, in our SR collection, nearly all individuals showed complete multi-locus homozygosity yet few shared multi-locus genotypes (figure 2d). Again, this type of local population genetic pattern (extensive inter-specimen genetic variation but negligible heterozygosity within most individuals) has not before been reported in any vertebrate species.

4. DISCUSSION

Formerly, *K. marmoratus* in Florida had been assumed to be an obligate self-fertilizer, so variation among strains was attributed to mutation, migration and genetic drift in small populations (Turner *et al.* 1992; Laughlin *et al.* 1995). However, in light of the current evidence we now propose that the genetic architecture of natural populations reflects a mixed mating strategy of predominant selfing with occasional outcrossing. From fixation indices (Gillespie 2004) calculated from our microsatellite data, provisional estimates of outcrossing rate range from 0.0 to 0.22 (mean 0.06) across the locales surveyed. Each outcross event between distinct inbred lines releases a sudden burst of genic heterozygosity that then is available upon a resumption of self-fertilization for rapid conversion into a plethora of new recombinant inbred lines. The discovery that outcrossing occurs in local Florida populations also means that researchers can no longer simply assume full homozygosity of experimental strains when evaluating plasticity or adaptive responses in *K. marmoratus* phenotypes (Lin & Dunson 1995).

Outcross matings in *K. marmoratus* probably involve hermaphrodites acting as females paired with primary (gonochoristic) males or secondary males (hermaphrodites that have lost ovarian function and developed male behaviours and colours). Harrington (1967, 1968, 1975) was able to generate primary males in aquaria by incubating self-fertilized eggs from hermaphrodites at low temperatures and secondary males by exposing immature hermaphrodites to high temperatures or

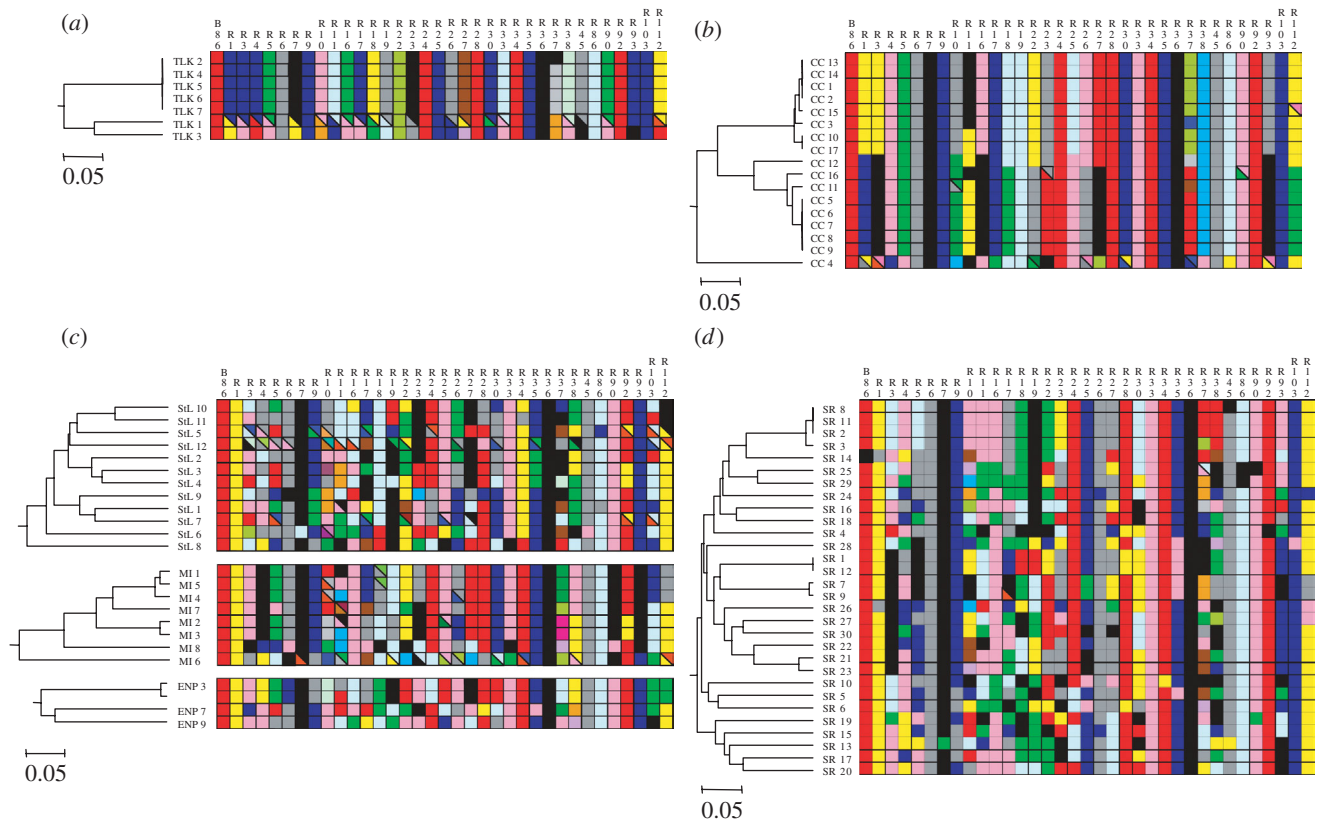


Figure 2. Genotypes at 35 microsatellite loci (columns) for each of 78 specimens (rows) of *K. marmoratus* representing six Florida locales. Different colours indicate different alleles. Homozygous and heterozygous genotypes are indicated by uni-coloured and bi-coloured cells, respectively, and black hatching indicates missing data. Phenograms summarizing pairwise genetic distances between individuals (based on proportions of shared alleles, using the program MSA (Dieringer & Schlötterer 2003)) were generated by MEGA3 software (Kumar *et al.* 2004) and are shown to the left of the panels. (a) Long Key (TLK) near the middle of the Florida Keys. (b) Charlotte Co. (CC) near Venice. (c) St Lucie Co. (StL); Marco Island (MI) in Collier Co.; Everglades National Park (ENP) near Flamingo in Monroe Co. (d) Shark River (SR) in Monroe Co.

shortened photoperiods. Most ova emitted by hermaphrodites are self-fertilized internally, but small numbers of unfertilized eggs are laid that could be available for external fertilization by males (who have no intromittent organ). Indeed, in contrived laboratory settings, we have recently documented outcrossed progeny resulting from crosses between gonochoristic males and hermaphrodites (Mackiewicz *et al.* submitted). However, males seem to be extremely rare if not absent in Floridian populations (Holsinger 1991; Lubinski *et al.* 1995), so another possibility not excluded by current data is that some of the outcrossing involves pairs of hermaphrodites.

The mixed-mating strategy of selfing and outcrossing evidenced by the genetic architecture in natural populations of *K. marmoratus* bears analogy to the reproductive modes of various hermaphroditic (including gynodioecious and androdioecious) plants and invertebrate animals (Allard 1975; Nollen 1983; Selander & Ochman 1983; Holsinger 1991; Pannell 2002). Although key questions persist about the maintenance of mixed-mating systems (Goodwillie *et al.* 2005), conventional wisdom and empirical evidence suggest that this reproductive tactic is adaptively significant especially in low-density colonizing species that occupy heterogeneous, ephemeral, or marginal habitats. In such circumstances, the 'best-of-both-worlds' advantages of selfing (fertilization assurance (Baker 1955, 1967) and the intact propagation of locally selected multi-locus genotypes) and outcrossing (which generates multitudinous genetic recombinants for selective scrutiny) may

be present concurrently or in alternating sequence within local populations.

Similar arguments can be made for *K. marmoratus*. The mangrove habitats occupied by this species are notoriously harsh and variable in factors such as water level, temperature, salinity and hydrogen sulphide concentrations (Davis *et al.* 1990, 1995). Furthermore, population densities are often thought to be low and reproductive uncertainties high, as these fish typically occupy small ephemeral pools of water, the hollows of logs or the burrows of land crabs. Finally, this species is a highly effective colonizer as judged by its broad geographic distribution (including isolated islands, Huber 1992) and natural history (which probably includes many opportunities for long-distance dispersal when storms or ocean currents move mangrove flotsam along the coast or across open water). Thus, both the reproductive system and the resulting population genetic pattern in *K. marmoratus* represent a remarkable instance of adaptive evolutionary convergence on the mixed-mating tactic heretofore known only in various hermaphroditic plants and invertebrates.

We thank William P. Davis, William Dunson, John Grizzle, Carole McIvor and D. Scott Taylor for specimen collections. Work was supported by funds from the University of Georgia and the University of California at Irvine.

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