

## REVIEW PAPER

# Population genetics and evolution of the mangrove rivulus *Kryptolebias marmoratus*, the world's only self-fertilizing hermaphroditic vertebrate

J. C. AVISE\* AND A. TATARENKOV

*Department of Ecology and Evolutionary Biology, University of California, Irvine, CA 92697, U.S.A.*

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The mangrove rivulus, *Kryptolebias marmoratus* (Rivulidae, Cyprinodontiformes), is phylogenetically embedded within a large clade of oviparous (egg laying) and otherwise mostly gonochoristic (separate sex) killifish species in the circumtropical suborder Aplocheiloidei. It is unique in its reproductive mode: *K. marmoratus* is essentially the world's only vertebrate species known to engage routinely in self-fertilization as part of a mixed-mating strategy of selfing plus occasional outcrossing with gonochoristic males. This unique form of procreation has profound population-genetic and evolutionary-genetic consequences that are the subject of this review.

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**Key words:** androdioecy; mixed mating; outcrossing; phylogeny; population structure; selfing.

## INTRODUCTION

Mangrove rivulus *Kryptolebias* (formerly *Rivulus*) *marmoratus* (Poey 1880) is a small New World rivulid that inhabits mangrove forests along the tropical and subtropical western Atlantic basin. Although diminutive, it has had an enormous effect on population-genetic and evolutionary-genetic thought. The reasons for special scientific interest in *K. marmoratus* stem from this species' peculiar reproductive lifestyle, which is unique among vertebrate animals. *Kryptolebias marmoratus* routinely reproduces by self-fertilization, when the eggs and sperm (produced by an internal ovotestis) unite to yield zygotes that are then laid outside each hermaphroditic fish's body. The occurrence of self-fertilization in a vertebrate offers a unique opportunity to examine the population-genetic ramifications of selfing in a higher animal. This begs for comparison with analogous reproductive syndromes in many plants and invertebrates that are likewise simultaneous hermaphrodites with mixed-mating systems and predominant selfing.

\*Author to whom correspondence should be addressed. Tel.: +1 949 8244747; email: [javise@uci.edu](mailto:javise@uci.edu)

## BACKGROUND ON TAXONOMY AND SYSTEMATICS

*Kryptolebias marmoratus* is part of a small clade of cyprinodontiform killifishes in the New World family Rivulidae of the suborder Aplocheiloidei (Murphy *et al.*, 1999; Costa, 2006) (Fig. 1). Two members of this *Kryptolebias* species group are unique among vertebrates by virtue of containing hermaphroditic individuals with self-fertilization (Costa *et al.*, 2010). In addition, the group is unique among rivulids in that the species live in brackish waters (Costa, 2006), in contrast to all other New World aplocheiloids that are confined to freshwater habitats.

The history of species-level taxonomy in the *K. marmoratus* group is highly confusing because of probable specimen misidentifications as well as shifting synonymies within the complex (Seegers, 1991). For simplicity, this review will focus on three nominal taxonomic species in the *K. marmoratus* complex: *K.* (= *Rivulus*) *marmoratus*, *Kryptolebias ocellatus* (Hensel 1868) and *Kryptolebias caudomarginatus* (Seegers 1984) (which some authors have subsumed under *K. marmoratus*). This paper follows the taxonomical classification employed in the most recent release of the Catalogue of Fishes (Eschmeyer, 2015), where all these species are considered valid. Readers should appreciate that these species' designations and their relevance to actual biological species boundaries in this taxonomic complex are provisional. In fact, in a recent revision, Costa (2011) compellingly showed that *K. caudomarginatus* is a junior synonym of *K. ocellatus*. As a part of the same revision, the latter species was re-described as *Kryptolebias hermaphroditus* Costa 2011. Such radical re-arrangement of taxonomy in a group of fishes' popular among scientists and aquarists shows that more genetic research is needed to achieve a more complete understanding of evolutionary relationships within and outside the *K. marmoratus* complex. Among the many topics such research should address are: (1) the distributional gap in collections of *Kryptolebias* between south-eastern Brazil and the Caribbean countries, (2) additional genetic analyses of populations throughout the Caribbean and (3) taxonomic problems involving several other nominal species (such as *Rivulus heyei* Nicols 1914, *Rivulus bonairensis*

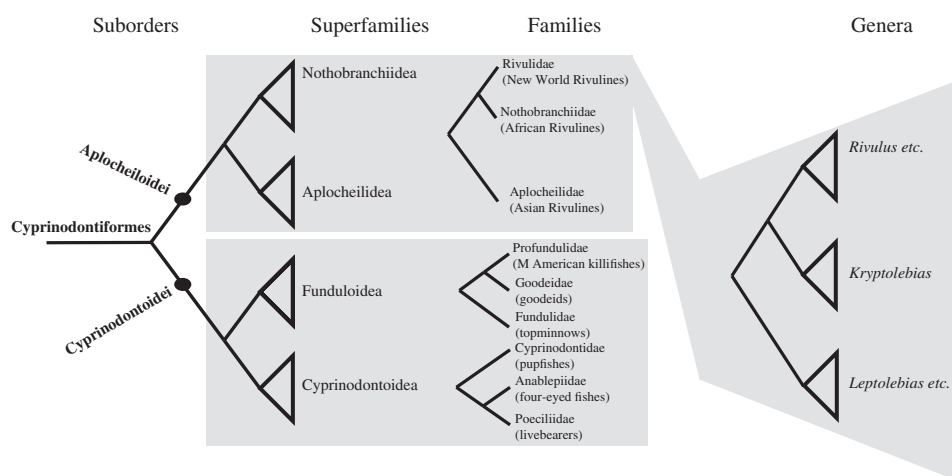


FIG. 1. Macro-phylogeny for cyprinodontiformes showing the general placement of the genus *Kryptolebias* within the broader framework of related piscine taxa.

Hoedeman 1958 and *Rivulus garciai* de la Cruz & Dubitsky 1976). All these nominal species have been synonymized with *K. marmoratus* but they might prove to belong to *K. ocellatus* or deserve a distinct species status.

## A BRIEF HISTORY OF GENETIC RESEARCH ON *K. MARMORATUS*

### THE DISCOVERY OF SELFING

The remarkable selfing reproductive mode of *K. marmoratus* was discovered a half-century ago by Harrington (1961) and has since been the subject of many detailed genetic and evolutionary analyses. Early on, Kallman & Harrington (1964) and Harrington & Kallman (1968) found that they could successfully graft fins and organs between a hermaphroditic individual and its offspring, or between progeny within a sibship, thus indicating that the fish were genetically identical (or nearly so) and probably were homozygous at histocompatibility loci. Furthermore, artificial tissue grafts between some of the self-fertilizing lines were acutely rejected by the recipient fish, thus implying that particular selfing strains must differ genetically from one another. Harrington & Kallman (1968) used the word clone to refer to each such presumably highly inbred line of *K. marmoratus*, a terminological practice that continues today (Avisé, 2008). This usage is not without pitfalls, however, because the rivulus clones that emerge *via* continued selfing could be misconstrued by naïve observers to have arisen *via* some non-meiotic process (as in ameiotic parthenogenesis), and also because the genetic delimitation of a rivulus clone can be ambiguous or susceptible to change when refined molecular assays uncover cryptic genetic variation within a previously identified clonal lineage (Avisé, 2008).

Since the 1960s, many new laboratory methods to assay DNA and proteins in ever-closer detail at the molecular level have been devised (Avisé, 2004), and many of these approaches have been applied to population genetic issues in *K. marmoratus* (Avisé, 2011). These methods have included protein electrophoresis (Massaro *et al.*, 1975; Vrijenhoek, 1985), multi-locus DNA fingerprinting (Turner *et al.*, 1990, 1992a; Laughlin *et al.*, 1995), mitochondrial (mt) DNA restriction site analyses (Weibel *et al.*, 1999), assays of mtDNA gene sequences (Murphy *et al.*, 1999; Lee *et al.*, 2001; Tatarenkov & Avisé, 2007; Tatarenkov *et al.*, 2009), molecular surveys of loci in the major histocompatibility complex (MHC) (Sato *et al.*, 2002; Ellison *et al.*, 2012a) and, recently, appraisals of large suites of unlinked nuclear microsatellite loci (Mackiewicz *et al.*, 2006a, b, c; Tatarenkov *et al.*, 2007, 2009, 2010, 2011, 2012; Ellison *et al.*, 2011, 2012b; Avisé & Tatarenkov, 2012). The last approach has been particularly informative because microsatellite loci tend to be highly polymorphic and because microsatellite alleles usually display straightforward Mendelian inheritance without dominance and thereby lend themselves to standard population-genetic models and interpretations.

### OUTCROSSING WITH MALES UNVEILED

The various molecular approaches mentioned above confirmed that natural populations of *K. marmoratus* often consist of strains that are so inbred as to represent, in effect, clonal lineages. Some of the molecular assays, however, also revealed much more genetic variation in *K. marmoratus* than formerly had been appreciated. Furthermore, by revealing the mechanistic sources of this genetic variation, some of the

latest laboratory techniques identified previously unsuspected population-genetic and reproductive phenomena within this species. For example, Floridian populations of *K. marmoratus* often show near-zero heterozygosity but extensive between-individual genetic variation (clonal diversity). The low heterozygosity within strains is apparently due to intense inbreeding that accompanies multi-generation selfing, whereas the clonal diversity traditionally had been attributed mostly to *de novo* mutations and interlocality gene flow (Turner *et al.*, 1990; Laughlin *et al.*, 1995). The newer microsatellite data, however, have made it abundantly clear that occasional outcrossing between different inbred lineages is another major contributor to the observed population-genetic patterns (Mackiewicz *et al.*, 2006a, b, c; Tatarenkov *et al.*, 2007).

Harrington & Kallman (1968) were aware that pure (gonochoristic or dioecious) males occur at low frequency in *K. marmoratus*, and also that these males can mediate outcross events with hermaphrodites in contrived laboratory settings. This can happen when a hermaphrodite occasionally releases a few unfertilized eggs, onto which a male (who has no intromittent organ) sheds sperm externally (Harrington, 1963; Nakamura *et al.*, 2008). The males in *K. marmoratus* are of two types: secondary males who are hermaphroditic when young but later in life lose ovarian function (Harrington, 1971), and primary males who have functional testicular but not ovarian tissue throughout life. Harrington (1967, 1968) discovered that he could readily generate primary males in the laboratory, for example, by incubating self-fertilized eggs from a hermaphrodite at low temperature. Male *K. marmoratus*, however, appear to be very uncommon in eastern Florida where Kallman & Harrington (1964) obtained their strains, and this fact, in conjunction with the mostly clonal makeup of natural populations, which led them to conclude that outcrossing probably is rare or absent in nature.

Males, however, subsequently were uncovered in much higher frequencies (>20%) in some other populations of *K. marmoratus* (Davis *et al.*, 1990), notably at Twin Cays in Belize (Turner *et al.*, 1992b, 2006). Initial molecular assays (by DNA fingerprinting) also documented the occurrence of natural outcrossing at the Belize sites (Lubinski *et al.*, 1995; Taylor *et al.*, 2001) and recent population-genetic reappraisals based on highly polymorphic microsatellite loci suggest that up to 60% of the matings in Belize may be outcross events and 40% involve selfing (Mackiewicz *et al.*, 2006c; Tatarenkov *et al.*, 2015). Outcrossing has also been genetically confirmed in Floridian populations (Mackiewicz *et al.*, 2006b, c), albeit at much lower inferred frequencies (ranging from zero to 20% across 10 surveyed populations). Outcross events often are presumed to be male mediated (Sakakura & Noakes, 2000) because hermaphrodites of *K. marmoratus* in the presence of males show courtship and spawning behaviours typical of females in other killifish species (Kristensen, 1970) and because male participation in spawning events has been documented in the laboratory (Mackiewicz *et al.*, 2006a). It remains at least theoretically possible that pairs of hermaphrodites sometimes cross as well.

Another possibility is that some of the outcross events involve crosses between males and young individuals that function solely as females. Using gonadal dissections, Cole & Noakes (1997) found that some relatively young specimens of laboratory-reared *K. marmoratus* are pure females that only later, as adults, add sperm production to their overall reproductive repertoires. It remains unclear whether these young females actually reproduce or whether they are merely in a transient developmental stage on the path to functional hermaphroditism. Moreover, a thorough histological examination of wild-caught fish gonads remains to be done. Regardless, such specimens raise the possibility that simultaneous hermaphroditism in *K. marmoratus* might have

evolved from a transitional condition of protogynous (female-first-in-life) sequential hermaphroditism (Avisé, 2011).

If, for the moment, one puts aside the possibility of functionally pure females, then the presence of male individuals in addition to hermaphrodites means (by definition) that *K. marmoratus* is an androdioecious species (Mackiewicz *et al.*, 2006c) rather than a strictly hermaphroditic species. Furthermore, the presence of outcrossing means that *K. marmoratus* actually has a mixed-mating system (Mackiewicz *et al.*, 2006b) rather than one of constitutive self-fertilization. Both of these features, which appear to be unique traits among all vertebrate animals, add to the list of biological superlatives for *K. marmoratus*.

## EXPERIMENTAL LABORATORY STRAINS

Following Harrington's (1961) discovery of hermaphroditic selfing in *K. marmoratus*, plus Kallman & Harrington's (1964) report that highly selfed laboratory lines of this species could be highly homozygous, researchers began to use *K. marmoratus* as a model species for experimental studies in many areas of biology including: developmental biology (Vogelbein *et al.*, 1987; Grageda *et al.*, 2004), behaviour (Hsu & Wolf, 1999, 2001; Earley *et al.*, 2000; Martin, 2007; Earley & Hsu, 2008; Luke & Bechler, 2010), ecology (Abel *et al.*, 1987; Davis *et al.*, 2003), ecotoxicology (Koenig & McLean, 1980; Davis, 1984, 1988; Lin & Dunson, 1993; Lee *et al.*, 2008), oncology (Koenig & Chasar, 1984; Park & Kim, 1984; Thiyagarajah & Grizzle, 1986; Courtney & Fournie, 1988; Grizzle & Thiyagarajah, 1988; Park *et al.*, 1990, 1992, 1993, 1994; Park & Lee, 1992; Goodwin & Grizzle, 1994a, b, c; Lee *et al.*, 1994, 1995; Couch, 1995; Thiyagarajah *et al.*, 1995) and physiology (Grizzle & Thiyagarajah, 1987; Heath *et al.*, 1993; Frick & Wright, 2002; Litwiller *et al.*, 2006; Ong *et al.*, 2007). As described below, however, an important caveat sometimes applies to the findings that emerge from such experimental studies.

Typically, in many of the studies cited above, the scientists merely assumed that each laboratory stock was effectively clonal, having been established and maintained (Harrington & Kallman, 1968; Laughlin *et al.*, 1995) by isolating a hermaphrodite and its offspring. Many publications refer to the presumed clonality of laboratory lines, and conclusions sometimes were based on the assumption that genetic variation was eliminated by the use of such clones (or that relevant comparisons were being drawn among distinct clonal lineages). The presumed homozygosities and isogenicities of longstanding laboratory lines could in principle be compromised by several factors: residual heterozygosity (genetic variation retained and segregated from variation in the ancestral wild progenitor), post-formational mutations (*de novo* variants that postdate a strain's laboratory origin) or mislabelling of stocks. Furthermore, the fact that outcrossing and the ensuing segregation of recombinant genotypes are known to occur occasionally in the laboratory (Harrington & Kallman, 1968; Mackiewicz *et al.*, 2006a), plus the documentation of high outcrossing rates in some natural populations of *K. marmoratus* (Lubinski *et al.*, 1995; Mackiewicz *et al.*, 2006b, c), raised the possibility that occasional outcross events might have introduced unrecognized genetic variation into particular laboratory stocks (Mackiewicz *et al.*, 2006b). Another potential complication is that most laboratory lines of *K. marmoratus* were derived from field-caught specimens of unknown genotype and heterozygosity. Thus, for any of the reasons listed above, various laboratory stocks

that were presumed to be clonal might actually contain multiple genetically distinct sub-lines.

To test this possibility, Tatarenkov *et al.* (2010) genetically surveyed essentially all laboratory stocks of *K. marmoratus* from around the world using a battery of microsatellite loci. The goals were four-fold: (1) genetically identify established laboratory stocks, (2) evaluate heterozygosities within these stocks, (3) address the origins and genetic relationships of the lines and (4) provide an accessible database of microsatellite genotypes to standardize stocks and thereby facilitate future laboratory research involving *K. marmoratus*.

The results of that study proved quite surprising. At 32 microsatellite loci, the laboratory stocks showed extensive interline differentiation as well as some intraline variation, much of which could be attributed to post-origin *de novo* mutations and to the segregation of polymorphisms from wild progenitors. The genetic findings also documented that many of the laboratory strains were not what they had been labelled, apparently due to mishandling or unintended mixing of various laboratory stocks over the years. Overall, this genetic inventory clarified much of this confusion about the clonal identities and genetic relationships of laboratory lines of *K. marmoratus*, and thereby should help to rejuvenate interest in *K. marmoratus* as a reliable vertebrate model for experimental research (Earley *et al.*, 2012; Orlando, 2012) that requires or can capitalize upon genetically replicated specimens.

## A BRIEF HISTORY OF POPULATION-GENETIC RESEARCH ON MIXED-MATING SYSTEMS

Any hermaphroditic population that displays both self-fertilization and outcrossing is said to have a mixed-mating system (Clegg, 1980; Brown, 1989). Genetic markers have been used to estimate selfing and outcrossing rates ( $s$  and  $t$ , respectively, where  $s + t = 1.0$ ) in dozens of hermaphroditic plant and animal species (Schemske & Lande, 1985). A direct approach, multi-locus paternity analysis, is applicable when the female parent of each offspring is known (*e.g.* as is often true in seed-bearing plants). Any embryo (inside a seed) that displays alleles other than those carried by its mother must have resulted from an outcross event (barring rare *de novo* mutations). Any offspring that displays only the dam's alleles at every gene was probably also sired by that same hermaphroditic parent. By accumulating such genetic information on many progeny of known mothers, researchers can derive estimates of outcrossing rates. A less direct approach, population-genetic analysis, is applicable when the dams of progeny are unknown (Hedrick, 2000). If a mixed-mating population is at inbreeding equilibrium with respect to  $s$  and  $t$ , then the observed heterozygosity ( $H_{\text{obs}}$ ) falls below random-mating expectations ( $H_{\text{exp}}$ ) and the inbreeding coefficient for the population becomes  $F = (H_{\text{exp}} - H_{\text{obs}})(H_{\text{exp}})^{-1}$ . Given an observed level of inbreeding ( $F$ ) within a population, the selfing rate ( $s$ ) can then be estimated as  $s = 2F(1 + F)^{-1}$ .

Beginning in the early 1970s, a series of seminal papers was produced on the adaptive significance of mixed-mating systems in annual plants (Allard, 1975). Much of this empirical work focused on the slender wild oat *Avena barbata*, a Mediterranean native that was introduced to California by Spanish missionaries. Each individual oat plant synchronously produces pollen and ova that usually unite in self-fertilization but sometimes participate in outcross events. Two highly distinctive multi-locus genotypes



predominate in Californian populations of the wild oat, one adapted to xeric conditions and the other to wetter (Clegg & Allard, 1972; Hamrick & Allard, 1972). It was concluded that consistent selfing had been adaptively advantageous in the ecological short term because it yielded offspring with identical copies of a co-adapted multi-locus genotype that nature already had field-tested for genetic fitness in a given habitat. Clegg & Allard (1972) and Hamrick & Allard (1972) also demonstrated that occasional outcrossing was also important, because parents from different inbred lines who outcross produce genetically diverse progeny, at least some of which might happen to be well suited to any new ecological conditions. A resumption of routine selfing then tends to perpetuate, nearly intact, any such multi-locus genotype until the next round of outcrossing occurs. By dramatically reducing heterozygosity, selfing in effect inhibits standard genetic recombination in a population and thereby can in theory facilitate the origin and (in conjunction with natural selection) the maintenance of suites of co-adapted loci. Furthermore, unlike the case for genetic linkage *per se*, the suppression of genetic recombination *via* selfing extends in principle across all loci in the genome.

Also in the early 1970s, an analogous series of studies on the adaptive significance of mixed-mating systems in invertebrates was produced (Selander & Kaufman, 1975). What Allard (1975) had demonstrated for monoecious plants, Selander & Kaufman (1975) would likewise implicate for hermaphroditic animals. Some of this genetic research involved a hermaphroditic species of land snail *Rumina decollata* that, similar to *A. barbata*, engages in a mixture of facultative self-fertilization and outcrossing. In its native range in southern France, two highly distinct inbred strains of *R. decollata* predominate, one typically associated with xeric habitats and the other with mesic conditions (Selander & Hudson, 1976). Yet, these clones also outcross occasionally, thereby releasing vast stores of genetic variation available for sexual recombination and selective scrutiny. Together with Allard's (1975) population-genetic discoveries on monoecious plants, Selander & Kaufman's (1975) genetic findings on hermaphroditic snails provided a powerful example of how the mating systems of plants and invertebrate animals have adaptively converged to a mixed-mating evolutionary strategy that apparently combines some of the favourable elements of both sexuality and clonality.

Thus, both Allard (1975) and Selander & Kaufman's (1975) provided empirical evidence consistent with population-genetic theory predicting that co-adapted multi-locus genotypes can arise and be maintained under a mixed-mating system with predominant selfing. Another popular hypothesis envisions a very different adaptive role for facultative self-fertilization. In 1955, Baker (1955) noted that the capacities for self-fertilization and for long-distance dispersal are positively correlated across species in both plants (Baker, 1948, 1953) and marine invertebrates (Longhurst, 1955), and that a plausible explanation involves the reproductive assurance that automatically comes with being a selfing hermaphrodite. Baker (1955) thus suggested that natural selection favours the capacity for self-fertilization in highly dispersive species because even a single self-fertile individual can be a successful colonist. Plants and marine invertebrates that are effective colonizers usually have dispersive propagules (seeds and larvae) that may be carried for long distances by winds or ocean currents, and upon arrival an immigrant's ability to reproduce self-sufficiently (rather than requiring a partner) is often advantageous if not crucial. The empirical association between self-fertilization and colonizing potential became known as Baker's rule. In theory, Baker's rule would also apply to any species in which individuals experience special

difficulties in finding a mate. For example, in any hermaphroditic species in which population densities are low or individuals are sedentary or solitary, outcrossing opportunities might be severely limited and a selfing capacity might therefore be selectively favoured.

#### MIXED MATING IN *K. MARMORATUS*

In several ways, *K. marmoratus* can play an important role in illuminating the selective advantages and disadvantages of selfing as part of a mixed-mating system. First, this species demonstrates that the reproductive mode as well as the general population-genetic architecture of a vertebrate species has evolutionarily converged on those of many hermaphroditic plants and invertebrate animals that likewise have mixed-mating systems with predominant selfing.

Second, experiments using *K. marmoratus* can inform broader population-genetic theory by facilitating experimental studies of inbreeding depression. Consistent selfing is a most intense form of inbreeding, and is otherwise unknown among hermaphroditic vertebrates (Avisé, 2011). In many plant and animal species, inbreeding is often associated with diminished survival or fertility (Ralls & Ballou, 1983; Frankham *et al.*, 2002). The fitness costs of inbreeding can not only be high (sometimes 50% or more) but also are variable among species. The fact that consistent selfing characterizes many populations of *K. marmoratus* should yield special opportunities to address the survivorship and performance of inbred strains experimentally or even in nature. For example, Ellison *et al.* (2011) demonstrated that outcrossed progeny of *K. marmoratus* in the wild are less susceptible to multiple parasite infections than are their selfed counterparts. Furthermore, because each inbred strain of *K. marmoratus* can offer multiple specimens with the same multi-locus genotype, such strains should lend themselves well to common-garden research protocols with genetically controlled animals and replicated environmental conditions in the laboratory.

Third, *K. marmoratus* can help to test the co-adaptation *v.* reproductive assurance hypotheses regarding the selective advantages of routine selfing. In principle, microhabitat selection might operate in conjunction with a mixed-mating system to foster the survival and proliferation of particular multi-locus genotypes in *K. marmoratus* (as has been documented in *A. barbata* and *R. decollata*). Currently available population-genetic data for *K. marmoratus*, however, offer little empirical support for the possibility that particular multi-locus genotypes are either common or consistently associated with particular ecological conditions (Tatarenkov *et al.*, 2012). Instead, the genetic data currently available appear more consistent with Baker's (1955) contention for the adaptive significance of self-fertilization in *K. marmoratus* (Avisé & Tatarenkov, 2012).

On the other hand, few experimental studies to date have addressed the possibility that particular multi-locus genotypes in *K. marmoratus* are well matched to particular microhabitats [Lin & Dunson (1995) provide an exception], despite the fact that mangrove environments are highly variable in many features (such as salinity, water temperature and hydrogen sulphide concentration) that might affect fitness in genotype-specific fashion (Davis *et al.*, 1995; Taylor, 2012). Thus, overall, with the current paucity of such experimental data on genotype-specific selection, at present there is no compelling empirical evidence for or against the proposition that particular multi-locus genotypes are uniquely well suited to specific microhabitats. This leaves



reproductive assurance as the most compelling hypothesis for any selective advantage that selfing routinely affords *K. marmoratus*.

The behaviour and natural history of *K. marmoratus* can indeed be interpreted as highly consistent with Baker's rule in several regards: the species has a huge geographic range (from southern Brazil to Florida and including many isolated Caribbean islands), so it must be an effective colonizer; individuals occupy coastal mangrove leaf litter and insect cavities in rotting logs (Davis *et al.*, 1990) where they may be predisposed to long-distance dispersal *via* floating debris (*e.g.* following storms); the dispersive proclivity of adults is further enhanced by the fish's capacity to move overland by flipping and to survive out of water for up to 10 weeks (Abel *et al.*, 1987; Grizzle & Thiagarajah, 1987; Taylor, 1990; Frick & Wright, 2002; Litwiller *et al.*, 2006); the fertilized ova are also well suited for dispersal because they can survive out of water for prolonged periods and hatch quickly when rehydrated (Ritchie & Davis, 1986); embryos readily attach to leaves, twigs or other floating plant debris that often washes out to sea; many adult *K. marmoratus* probably are rather isolated and independent much of the time (*e.g.* living in a rotting log or a crab burrow); finally, individuals tend to be belligerent towards conspecifics (Earley *et al.*, 2000). All of these ecological and behavioural attributes would appear to favour self-fertilization as a routine alternative to outcrossing in *K. marmoratus*. Given the species' habits and natural history, a mixed-mating strategy presumably enables this self-fertilizer to ameliorate the disadvantage of having to find a mate (as would otherwise be necessitated under constitutive outcrossing). Indeed, given that many highly isogenic and homozygous strains of *K. marmoratus* apparently have escaped the perils of intense inbreeding (at least over the short term), this androdioecious species probably enjoys the best of the two worlds: the long-term as well as the short-term advantages of outcrossing (continued genetic health and adaptability), plus some immediate benefits from selfing (especially reproductive assurance).

## MIXED MATING AND MICRO-GEOGRAPHIC POPULATION STRUCTURE

Mating systems can profoundly affect genetic variation and population structure (Charlesworth & Wright, 2001). For example, self-fertilization is an extreme form of inbreeding with a cascade of possible population-genetic consequences including: (1) a reduction in effective population size ( $N_e$ ) such that  $N_e$  in a population of strict selfers is only 50% that of constitutive cross-fertilizers, all else being equal; (2) a corresponding diminution of genetic variation at neutral loci; (3) elevated homozygosity, which within several generations can reach nearly 100% in a population of strict self-fertilizers; (4) a drastic reduction in the number of realized recombination events, in effect tightening linkage and heightening gametic-phase disequilibrium throughout the genome; (5) associated enhanced opportunities for natural selection to detect both beneficial and deleterious mutations and thereby to drive selective sweeps and promote background selection, eventuating in further reductions of within-population genetic diversity. Furthermore, because the effect of genetic drift is higher with smaller  $N_e$ , population structure should be magnified by inbreeding, as has been demonstrated in several species of selfing plants and animals. Moreover, whereas genetic variation in local populations is expected to be low in selfing species, genetic diversity over a species' entire range might be exceptionally high due to pronounced population subdivision that conserves distinct genetic variants. Finally, the ability of a single

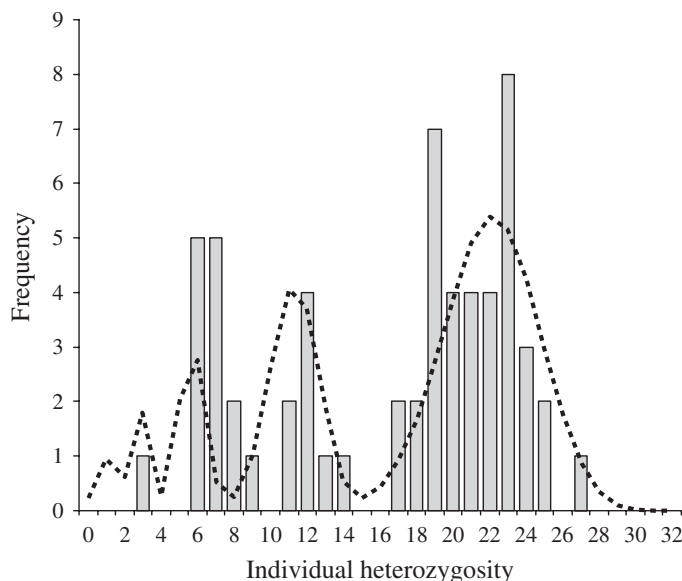


FIG. 2. Histogram showing the frequency distribution of individual heterozygosities in one Belizean population ( $n = 59$ ) of *Kryptolebias marmoratus* (after Tatarenkov *et al.*, 2015). The x-axis shows the observed number of heterozygous loci (among 32 surveyed) in each individual. ...., the distribution expected under an equilibrium mixed-mating model given the same level of heterozygosity ( $H_{exp}$ ) and selfing rate ( $s$ ) as was actually observed in this population.

selfing hermaphrodite to establish a new population may further contribute to low intra and high inter-population variation *via* genetic bottlenecking.

Two recent studies involving microsatellite loci have documented several of these effects for regional assemblages of *K. marmoratus* populations from the Florida Keys (Tatarenkov *et al.*, 2012) and for several sites in Central America (Tatarenkov *et al.*, 2015). In both cases, geographic population-genetic structure was highly statistically significant and arranged in patterns that were marginally consistent with models of isolation by distance. Also showing significant spatial variation in Central America were the deduced frequencies of outcrossing, which proved to be associated (albeit tenuously) with the frequencies of males at the various locales. Observed distributions of individual heterozygosities in Central America also showed very good agreement with the distributions expected under an equilibrium mixed-mating model with the same selfing rates (Fig. 2) (Tatarenkov *et al.*, 2015). This latter finding suggests that despite pronounced spatial heterogeneity in genotypic frequencies, selfing rates within populations may tend to be rather stable through time. A stability of selfing was also demonstrated more directly by the documentation of similar selfing rates at one site (Twin Cays, Belize) in samples spanning 14 years (Tatarenkov *et al.*, 2007).

## THE FUTURE OF MIXED-MATING RESEARCH

As researchers move further into the genomics era and gain access to expanded legions of molecular markers, refined genetic dissections of the population-genetic

consequences of mixed-mating systems can be expected. A helpful prelude to such analyses was provided by Bomblies *et al.* (2010) who assayed >1000 individuals at nearly 500 single nucleotide polymorphisms (SNP) scattered across the nuclear genome of the mustard *Arabidopsis thaliana*, another plant species with a mixed-mating reproductive system of predominant selfing plus occasional outcrossing. Results showed that the (mostly homozygous) genomes of *A. thaliana* are composed of blocks of linked genes arranged in mosaics of alternating marker identity and divergence, presumably registering histories of selfing punctuated by relatively rare outcross events of varied historical depths in different lineages. Until recently, such large suites of marker loci were not yet available for *K. marmoratus* [see Kelley *et al.* (2012) for an exception] nor has it been easy to obtain huge numbers of specimens from nature. If the logistical hurdles can be overcome, such population-genetic surveys across space and time, especially when combined with replicated experimental appraisals of selection on particular genomic regions, should reveal much about the functional adaptive significance of mixed-mating systems in any plant or animal species. For *K. marmoratus*, a start in this research direction was provided by Mesak *et al.* (2014).

### MESO-EVOLUTIONARY PHYLOGENY

The genus *Kryptolebias* (Costa, 2004) contains about four to eight named species (depending on a complicated nomenclature due to changing synonymies and different degrees of taxonomic splitting; Costa, 2011) constituting a distinct clade within the Rivulidae. Recent molecular genetic appraisals based on mtDNA sequences and microsatellite data (Tatarenkov *et al.*, 2009) have illuminated the phylogenetic relationships of several of these species and also helped to clarify the evolutionary histories of their reproductive modes (Costa *et al.*, 2010). In particular, microsatellite analyses documented a high selfing rate (97%) in a nominal species from southern Brazil (*K. ocellatus*) which, based on mtDNA sequences and other evidence, is the sister taxon (closest living relative) to *K. marmoratus*. In contrast, the microsatellite data uncovered no evidence of self-fertilization in *K. caudomarginatus* (an androdioecious species closely related to the *marmoratus*–*ocellatus* clade), and the data confirmed (as expected) that outcrossing is the norm in *Kryptolebias brasiliensis* (Valenciennes 1821), which is a phylogenetic outlier species with separate sexes.

When the various reproductive conditions of extant members of the *Kryptolebias* clade are phylogenetically mapped (Avice, 2006) onto the rivuline tree, what emerges are the following evolutionary inferences (Fig. 3): (1) the syndrome of synchronous hermaphroditism (as well as androdioecy and the associated loss of females) within this taxonomic assemblage probably arose on the stem lineage leading to the subclade composed of *K. caudomarginatus*, *K. ocellatus* and *K. marmoratus*; (2) the capacity for self-fertilization in the Rivulidae probably arose in the shared ancestor of the *marmoratus*–*ocellatus* clade. With regard to the second conclusion, the genetic data also thereby indicate that the self-fertilization capacity (as a component of a mixed-mating system) apparently has persisted in these rivuline fishes for at least several hundred thousand years (Tatarenkov *et al.*, 2009), based on considerations of a molecular clock for mtDNA.

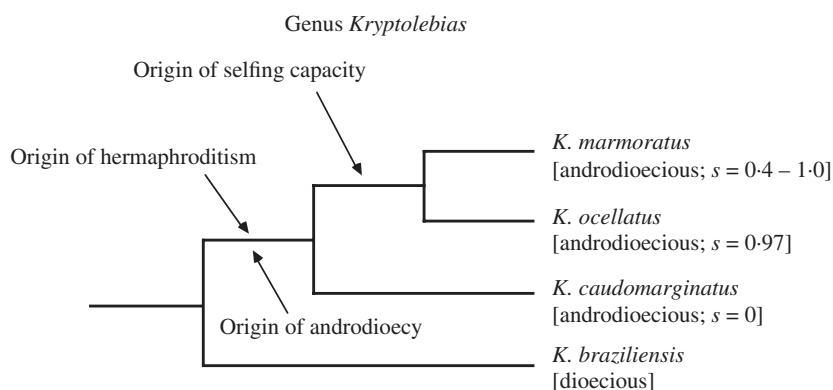


FIG. 3. Meso-phylogeny for several recognized species of *Kryptolebias* (after Costa *et al.*, 2010). Also plotted on this cladogram are the deduced evolutionary origins of several indicated reproductive conditions [note that for *Kryptolebias ocellatus* the presence of males has not been confirmed from direct observation, but rather is deduced from the phenomenon of outcrossing (which in *Kryptolebias marmoratus* is mediated by males)].  $s$ , Selfing rate.

## PHYLOGEOGRAPHY AND MACROGEOGRAPHIC POPULATION STRUCTURE

Due to a paucity of biological samples and a low variability in the available genetic markers, few of the early genetic studies compared multiple populations of *K. marmoratus*. Vrijenhoek (1985) genotyped several individuals from Florida, Yucatan and Curacao at 31 allozyme loci. All fish from Florida and Curacao were homozygous and identical to each other, and they differed from Yucatan fish (likewise homozygous and identical) at four loci. Other DNA markers, such as DNA fingerprinting loci (Turner *et al.*, 1990, 1992a), detected ample interindividual variation within populations, but the data were difficult to quantify in comparisons between populations. Of the current methods, microsatellites and mtDNA sequences appear to be most informative for studying population-level variation in *K. marmoratus* (Mackiewicz *et al.*, 2006a, b, c; Tatarenkov *et al.*, 2007, 2009, 2012). For example, Tatarenkov *et al.* (2007) compared microsatellites in several populations from Florida, the Bahamas and Belize, and they found significant population-genetic structure at all spatial scales, with values of  $F_{ST}$  ranging from nearly 40% over the entire studied area to 13% within a single river basin. Such high levels of differentiation indicate infrequent or limited genetic exchange among populations in the Caribbean region. Even more divergent, however, was a population of *K. ocellatus* from a locality in southern Brazil (Tatarenkov *et al.*, 2009), which showed pronounced differences from Caribbean samples of *K. marmoratus* at microsatellite loci as well as mtDNA sequences (Fig. 4), confirming the validity of the two species.

Non-recombining mtDNA is a popular marker in phylogeographic assessments, *i.e.* for unravelling the spatial distributions of gene lineages (Avise, 2000). Extensive mtDNA analyses are still lacking for *K. marmoratus*, mostly due to the difficulty of obtaining samples from many widely separated locales across the species' range. Weibel *et al.* (1999) observed that specimens from Turks and Caicos Islands and from Brazil had a *cytb* mtDNA haplotype that was highly divergent from a haplotype found

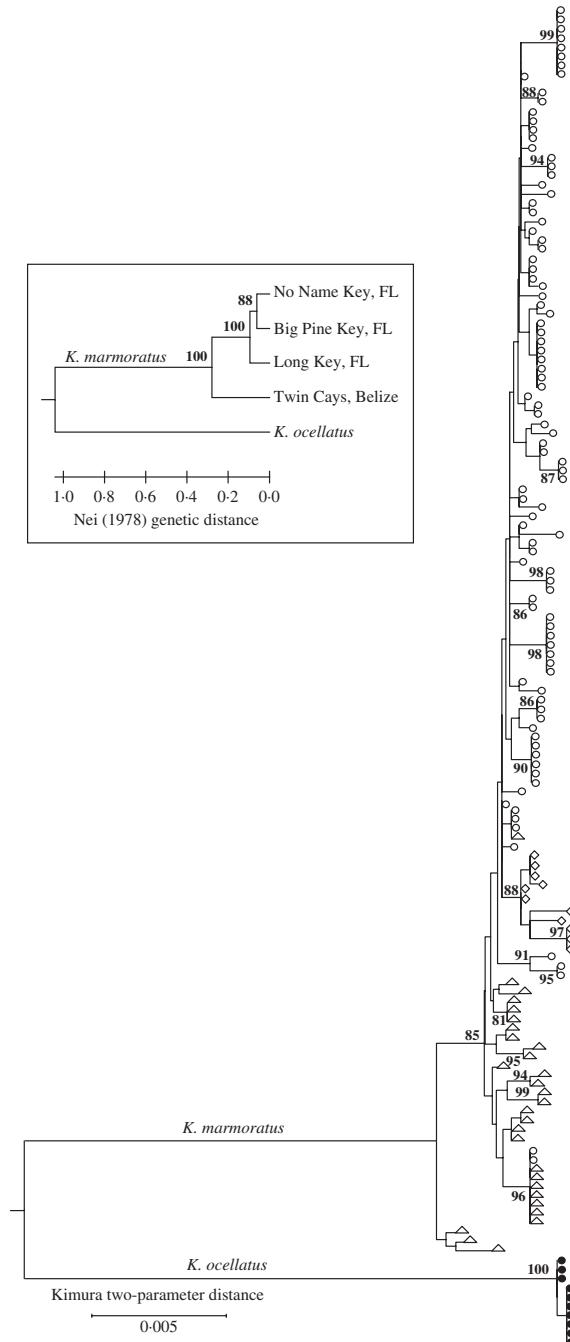


FIG. 4. Micro-phylogeny for *Kryptolebias* showing the phylogeographic orientation of genetically surveyed populations of *Kryptolebias marmoratus* and its close relative *Kryptolebias ocellatus* (●; after Tatarenkov *et al.*, 2009). Main figure: genealogy for 146 individuals based on nearly 3000 bp of mtDNA sequences per specimen. *Kryptolebias marmoratus* from Belize (△), various locations in Florida (○) and the Bahamas (◇). Inset: population phenogram for various populations based on a cluster analysis of Nei's genetic distances from 31 microsatellite loci.



in Belize and Florida. Sato *et al.* (2002) detected only limited variation in mitochondrial control region (CR) sequences in 10 specimens from Caribbean locales, despite the fact that the CR is usually highly variable in vertebrates. The shallow haplotype structure at CR in a larger sample was confirmed by Tatarenkov & Avise (2007). Pooling of several mtDNA regions for a total sequenced length of nearly 3000 bp yielded a somewhat finer resolution of geographical clustering and revealed some major lineages in *K. marmoratus* (Tatarenkov *et al.*, 2009; Fig. 4). Three groups were apparent (although bootstrap support was not particularly strong): (1) a basal position in the tree involving individuals from Belize, (2) Bahamian individuals that occupy a more derived position in the tree and (3) Floridian specimens that form another broadly derived position. Mean mtDNA sequence divergence within *K. marmoratus* is rather low (0.26%), although it reaches values as high as 0.69% in some pair-wise comparisons.

Recent findings suggest that the phylogeographic structure for *K. marmoratus* in the Caribbean may be more dramatic than described above in preliminary surveys. A genetic assessment of laboratory stocks revealed that strains from Panama and Cuba are genetically more similar to *K. ocellatus* from Brazil than to geographically proximate population of *K. marmoratus* from Florida and Belize (Tatarenkov *et al.*, 2010, 2011). Coupled with the findings by Weibel *et al.* (1999) mentioned above, this suggests that at least two highly diverged lineages may be present in the Caribbean, with their taxonomic relationships remaining uncertain. The Antilles and South American countries bordering the Caribbean are in special need of attention in further genetic assays of the *K. marmoratus* complex.

## CONCLUSIONS

*Kryptolebias marmoratus* and its close genetic relatives have informed population-genetic and evolutionary thought in ways that belie the diminutive size of these secretive little fish. In particular, the reproductive lifestyle of *K. marmoratus*, which includes androdioecy and hermaphroditic mixed mating with predominant selfing, offers a unique vertebrate paradigm for evolutionary comparisons with plants and invertebrate animals. Continued genetic study of these remarkable creatures appears likely to expand the understanding of the comparative ecology and evolution of alternative reproductive modes.

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