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Evolutionary biology

Natural hybridization between divergent lineages in a selfing hermaphroditic fish

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By definition, mating between individuals is infrequent in highly selfing organisms, and so too, therefore, hybridization should be rare between genetically divergent lineages in predominantly self-fertilizing species. Notwithstanding these expectations, here we report a remarkable case of natural hybridization between highly diverged phylogeographic lineages of the mangrove rivulus, a small killifish that reproduces predominantly by self-fertilization and typically is found as highly homozygous lines in most parts of its extensive geographical range. Two distinctive genetic lineages (*Kryptolebias marmoratus* and a 'Central clade' closely related to *K. hermaphroditus*) previously were not known in sympatry, but were found by us to co-occur on San Salvador, Bahamas. Genetic analyses of a mitochondrial and multiple nuclear markers determined the direction of a cross producing a hybrid fish. Furthermore, we show that this hybrid individual was viable, as it successfully reproduced by self-fertilization for two generations. Additional sampling of this population will be necessary to determine if backcrossing of hybrids to the parental lineages occurs in nature and to analyse whether such backcross progeny are viable. Application of the biological species concept (BSC) is traditionally difficult in clonally reproducing organisms. Our results show that although mangrove rivulus fish are mostly highly selfing in nature (resulting in isogenic, effectively clonal and homozygous progeny), classification within this taxonomic complex need not be incompatible with the BSC.

1. Introduction

The mangrove rivulus (a killifish) is the only known vertebrate that routinely reproduces by self-fertilization [1,2]. Currently, two selfing species of mangrove rivulus are recognized: *Kryptolebias marmoratus* from Florida and the Caribbean, and *K. hermaphroditus* from Brazil [3,4]. A recent phylogeographic study found that populations of mangrove rivulus formed three main clades [5]: a 'Northern' clade that includes populations from Florida, northern Cuba, Bahamas, Belize, and Honduras and that corresponds to *K. marmoratus*; a 'Southern' clade that encompasses populations from Brazil and corresponds to *K. hermaphroditus* and a 'Central' clade that includes populations from Panama, the Dutch Caribbean, Puerto Rico, Turks and Caicos, and southern Cuba (electronic supplementary material, figure S1). The Central clade is phylogenetically closer to *K. hermaphroditus* despite its geographical proximity to *K. marmoratus*. Sequence divergence at the mitochondrial *cox1* gene is 1% between the Central clade and *K. hermaphroditus*, whereas *K. marmoratus* is 3% divergent from them. The taxonomic status of the Central clade has not yet been designated formally [5], but its level of genetic divergence from

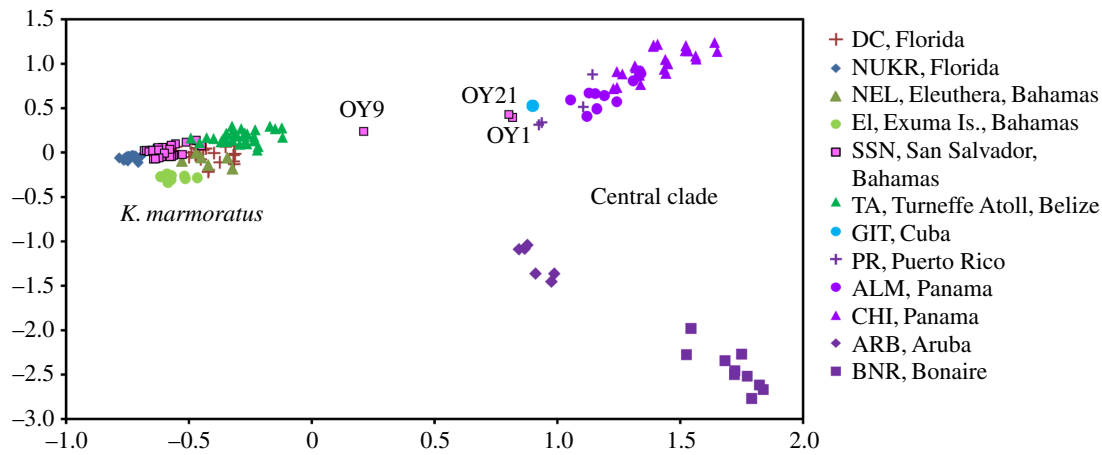


Figure 1. Clustering of *Kryptolebias* in principal coordinate analysis (PCA) using 33 microsatellite loci. For additional details on samples, including geographical coordinates, see [5].

K. marmoratus is on par with values often observed between many other 'good' vertebrate species [6,7].

Among the questions motivating this research was a possibility of natural hybridization between *K. marmoratus* and the Central clade. A laboratory experiment produced a viable hybrid by *in vitro* fertilization of an egg of a fish from the Central clade by sperm of a Belizean *K. marmoratus* [8], indicating that despite relatively high divergence, genomes of the two clades are functionally compatible.

Nevertheless, hybridization in nature seemed unlikely for geographical and biological reasons: first, the known geographical ranges of the two clades in the Caribbean do not overlap (the nearest known locations are hundreds of kilometres apart); second, predominant selfing would be an impediment to hybridization even if two clades were sympatric and syntopic (populations of *K. marmoratus* in Florida and Bahamas are 91–100% selfing, those of the Central clade are effectively 100% selfing and selfing rates in Belize range from 39–77% [5]); and third, occasional outcrossing in mangrove rivulus is mediated by males which typically are rare (approx. 1% in most areas, except on some Belize islands where their frequency can reach 25% [9]).

Notwithstanding these considerations, here we report the discovery that a site in San Salvador, Bahamas harbours fish belonging to both the Central clade and to *K. marmoratus*. Furthermore, we unequivocally document that fish from these distinct clades successfully hybridize at this site in nature. These results raise broader evolutionary issues about taxonomy and species concepts in taxa that reproduce by mechanisms that entail joint elements of sexuality and clonality.

2. Material and methods

Twenty specimens of mangrove rivulus were collected from two sites on San Salvador, Bahamas: Reckley Hill Lake (RHL, $N = 10$; $N 24^{\circ}06.915'$, $W 074^{\circ}27.428'$) and Oyster Lake (OY, $N = 10$; $N 24^{\circ}06.650'$, $W 074^{\circ}27.796'$). All these fish were genotyped at 33 microsatellite loci (electronic supplementary material, file S1). The cytochrome oxidase I (*cox1*) gene was sequenced in seven of them (electronic supplementary material, file S2). Molecular genetic analyses followed protocols described in [5]. Other data used in this study, including 41 additional specimens from San Salvador Island, were retrieved from Tatarenkov *et al.* [5].

3. Results and discussion

Forty-one mangrove rivulus from San Salvador were included in our previous studies, all genetically identified as *K. marmoratus*. While genotyping the 20 new fish from San Salvador, we noted that three individuals from OY possessed microsatellite alleles that previously were exclusively or predominantly found in the Central clade. Furthermore, one of these fish (OY9) was heterozygous for alleles that otherwise were characteristic of either *K. marmoratus* or the Central clade, suggesting that it might be a hybrid individual. Clustering of samples in a principal coordinate analysis based on 33 microsatellite loci confirmed the initial suspicion (figure 1). Among the total of 61 mangrove rivulus collected from San Salvador, 58 fish were firmly embedded in a cluster consisting of *K. marmoratus* from Florida, Belize, and Bahamas; another two fish (OY1 and OY21) were closely affiliated with the Central clade, and one fish (the hybrid OY9) occupied an intermediate position between these two groups.

Kryptolebias marmoratus and the Central clade are strongly divergent at mitochondrial loci: 3% sequence divergence at the cytochrome oxidase 1 (*cox1*) gene and 4.2% at cytochrome B (*cytB*) [5]. This enabled us to further verify the taxonomic affiliation of the fish and to use maternal inheritance of mtDNA to determine the direction of the cross. For this analysis, we included OY1, OY21, OY9 and another four fish from OY (figure 2). For six of these fish, the deduced position on the mitochondrial *cox1* gene tree was as expected based on microsatellite loci. The position of OY9, on the other hand, could not be predicted because it would depend on whether *K. marmoratus* or the Central clade provided the mother for the hybrid. OY1 and OY21 clustered with the Central clade, whereas four other OY fish were in the *K. marmoratus* cluster, as expected. The *cox1* gene of hybrid OY9 was identical to those of OY1 and OY21, indicating that a hermaphroditic fish of the Central clade was the mother of the hybrid, whereas a *K. marmoratus* fish was the sire.

The heterozygosity of individual fish can be used to estimate the number of generations that passed since the last outcrossing event [10]. In the case of hybridization between two species, a hybrid resulting from outcrossing will be heterozygous at all diagnostic loci distinguishing those species. If such a hybrid resumes reproduction by self-fertilization, it would quickly lose heterozygosity, with 50% of loci becoming homozygous at each successive generation of selfing. If

Table 1. Observed and expected distributions of the number of homozygous and heterozygous loci in a hybrid fish under assumption of up to three generations of selfing.

	observed	expected		
		1 selfing generation	2 selfing generations	3 selfing generations
homozygotes Central clade	9	5.5	8.25	9.625
homozygotes <i>K. marmoratus</i>	6	5.5	8.25	9.625
heterozygotes Central/ <i>K. marmoratus</i>	7	11	5.5	2.75
χ^2 -sum		3.73	1.09	7.97
probability (p)		0.16	0.58	0.02

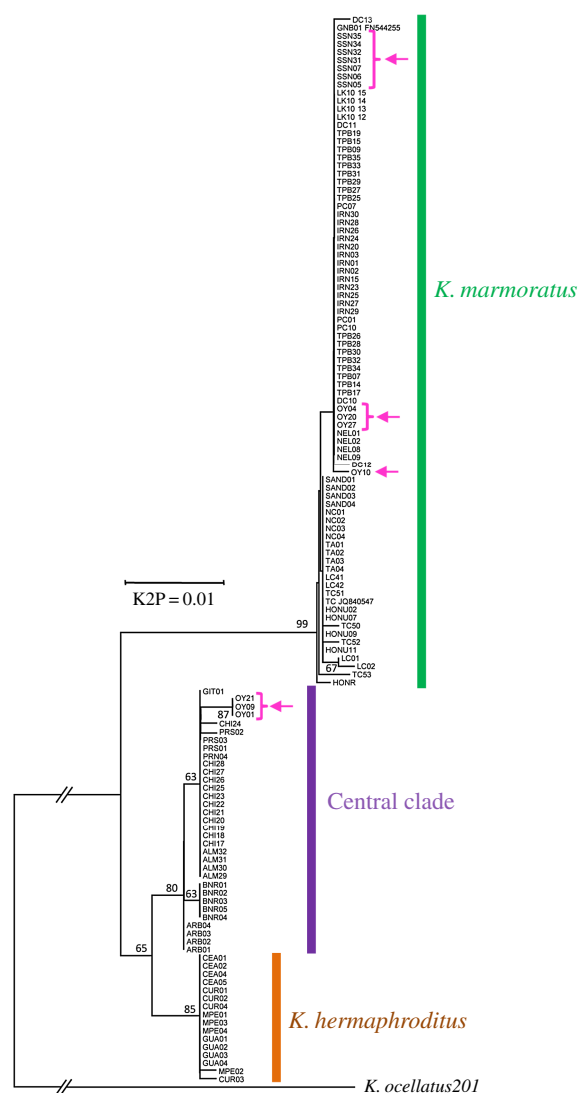


Figure 2. mtDNA *cox1* neighbour-joining tree. Pink arrows show specimens from San Salvador Island, Bahamas. K2P, Kimura-2-parameter distance. Bootstrap support values below 50% are not shown. Branch leading to the outgroup species *Kryptolebias ocellatus* is not up to scale.

the loci are unlinked and the assortment of alleles is random, half of the homozygous loci would carry alleles of one parental species and the other 50% of such loci would be homozygous for alleles of the second parental species. In the OY9 hybrid, we identified 22 loci with a distribution of alleles distinct enough for their reliable assignment to either *K. marmoratus* or the Central clade. Table 1 shows the observed distribution of homozygous and heterozygous loci

in this hybrid, the expected distributions for up to three generations of selfing, and the results of χ^2 -tests. The observed distribution best fit the expected distribution under the assumption of two generations of selfing; the hypothesis of three generations of selfing was not supported ($p = 0.02$); and the hypothesis of one generation of self-fertilization could not be rejected but yielded a fit between observed and expected values that was worse than that for two selfing generations (table 1). Thus, we conclude that hybrids between *K. marmoratus* and the Central clade are not only produced and survive in nature, but that they successfully reproduce by self-fertilization, which is the reproductive mode normally characteristic of both parental species. Specifically, the OY9 hybrid resulted from a cross between a male of *K. marmoratus* and a hermaphrodite of the Central clade, followed by two generations of selfing. Furthermore, the OY9 fish had a body length of 25 mm, which is a size of a sexually mature mangrove rivulus.

Our discovery of interclade hybridization adds significantly to knowledge about the breeding biology of mangrove rivulus, but also raises several broader evolutionary and taxonomic issues. For the first time, we show that two highly divergent clades of selfing *Kryptolebias* occur in sympatry, opening prospects to study their biological interactions in nature. Second, we show that these two clades can and do mate in nature and produce viable progeny. This is remarkable, considering the high rates of self-fertilization (often approaching 100%) normally characteristic of *Kryptolebias* fishes in these two clades. Perhaps this indicates some degree of negative assortative mating in this taxonomic complex. Third, we show that hybrids resulting from crosses between these clades can successfully propagate in nature by self-fertilization. Future genetic work should include a search for backcrossing of a hybrid with members of one or another parental clade (which should inform on the extent, if any, of gene exchange between these distinct genetic pools).

Our finding of the hybridization between two sibling species of mangrove rivulus has consequences for the taxonomy of these fishes. On the one hand, hybridization shows that *K. marmoratus* and the Central clade are not completely isolated reproductively and can successfully cross even in the face of predominant reproduction by self-fertilization, which might be an argument that these taxonomic groups are not distinct species. On the other hand, evidence of hybridization does not automatically negate distinct species status. Many valid species are known to hybridize in nature [11], with fishes particularly well known for this phenomenon [12,13], and yet distinct genetic pools and separate

evolutionary trajectories seem to be retained. We do not yet know how common hybridization is between *Kryptolebias* lineages in nature, whether it leads to gene introgression, and whether introgression is uni- or bidirectional. The fact that two OY fish had a genetic composition typical of the Central clade might indicate an absence of introgression, but it also might reflect a recent arrival of these fish on San Salvador (so that there was not sufficient time for erosion of genetic pools). The latter scenario could not be ruled out, because San Salvador is washed by the Antilles current which originates near Puerto Rico and passes Turks and Caicos, with both areas known to harbour the Central clade. Furthermore, San Salvador lies in a region often hit by hurricanes, which create abundant flotsam that could carry *Kryptolebias* eggs. However, if future investigations find that two *Kryptolebias* lineages live side-by-side for a long time, their sympatric occurrence might help taxonomists to decide on their species status, because introgression will be easily detectable through the presence of fish of intermediate genetic composition.

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Ethics. Experimental research was approved by University of Alabama Institutional Animal Care and Use Committee (protocols nos. 13-10-0048 and 14-05-0071).

Data accessibility. GenBank accessions for nucleotide sequences are MH319827–MH319837. Electronic supplementary materials are available from the Dryad Digital Repository (http://dx.doi.org/10.5061/dryad.t5p71ch) [14].

Authors' contributions. A.T., J.C.A. and D.S.T. conceived and designed the study. R.L.E. and J.C.A. obtained funding. R.L.E., D.S.T. and W.P.D. collected fish. A.T. conducted molecular work, analysed data and wrote original draft. A.T., R.L.E., D.S.T., W.P.D. and J.C.A. reviewed and critically revised the manuscript. All authors approved the final version and agreed to be accountable for the content of the manuscript.

Competing interests. We declare we have no competing interests.

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