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## Extreme homogeneity and low genetic diversity in *Kryptolebias ocellatus* from south-eastern Brazil suggest a recent foundation for this androdioecious fish population

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This study documents unexpectedly low levels of intra and interpopulation genetic diversity in *Kryptolebias ocellatus*, an androdioecious and predominantly self-fertilizing killifish from south-eastern Brazil. This finding generally is inconsistent with the established opinion that the *K. ocellatus* and *K. marmoratus* clade originated in this geographic region and later dispersed northward into the Caribbean.

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Key words: founder effect; hermaphroditism; mangrove killifish; mixed-mating system; reproductive modes; selfing.

Molecular markers have unearthed many otherwise cryptic features of organismal natural history, clarified phylogenies and taxonomies and shed light on the evolutionary histories of numerous phenotypic features including reproductive modes and mating behaviours (Avise, 2004, 2006). One common and successful set of applications has been in characterizing the geographical centres of origin and subsequent colonization routes for particular species and larger taxonomic groups (Avise, 2000, 2004).

With a range that stretches from central Florida and the Bahamas through the Antilles and along the Central and South American coastlines to south-eastern Brazil (Taylor *et al.*, 1995; Fig. 1), the mangrove rivulus *Kryptolebias marmoratus* (Poey 1880) was thought to have the widest geographic distribution of any species in the family Rivulidae. The apparent colonizing success of this hermaphroditic species can be attributed to a suite of biological characteristics including (1) a strong propensity to self-fertilize such that even a single individual can establish a new colony, (2) the physiological capacity to withstand diverse temperatures, salinities, and hydrogen sulphide levels, (3) cutaneous respiration (if need be) when temporarily emerged from water, (4) a habit of occupying hollowed mangrove logs, which might facilitate

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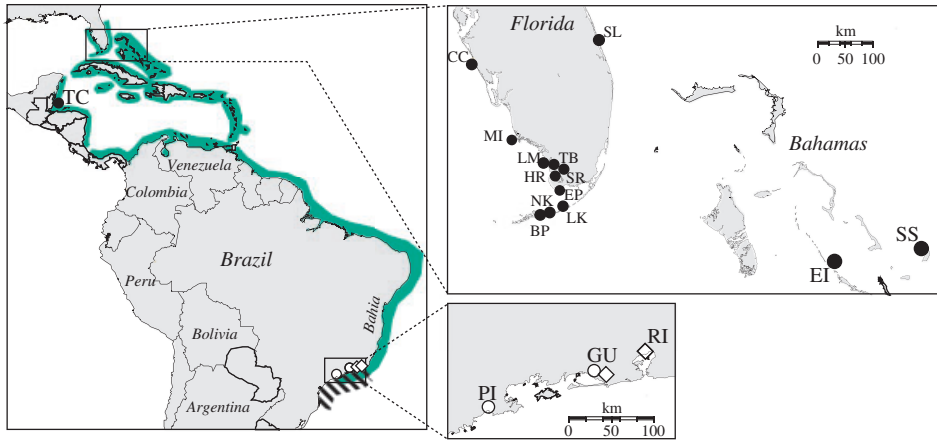


FIG. 1. Geographical distributions and sampling sites of three *Kryptolebias* species: *K. marmoratus*, *K. ocellatus* and *K. caudomarginatus*. The combined geographical range is shown for *K. marmoratus* and *K. ocellatus* because earlier reports did not discriminate between these taxa and also because of remaining uncertainties with regard to their species status. Sampling sites are labelled as in Table II. Distributions: *K. ocellatus* and *K. marmoratus* (■); *K. caudomarginatus* (▨). Sampling sites: *K. marmoratus* (●), *K. ocellatus* (○) and *K. caudomarginatus* (◇).

across-ocean dispersal of adults via drifting debris and (5) adhesive eggs that also might promote dispersal by sticking to flotsam (Davis *et al.*, 1990; Taylor, 2000; Tatarenkov *et al.*, 2007; Taylor *et al.*, 2008).

The wide geographic distribution of the Mangrove Rivulus, however, could be deemed suspect because *Kryptolebias ocellatus* (Hensel 1868), an earlier synonym of *K. marmoratus*, recently was promoted to full-species status (Costa, 2006), thereby implying that the clade's full range must be split somehow between these two sister taxa. To complicate matters further, other authors have described several additional sibling species or subspecies from the Caribbean: *Rivulus heyeyi* Nichols 1914, *Rivulus myersi* Hubbs 1936, *Rivulus garciai* de la Cruz & Dubitsky 1976 and *Rivulus marmoratus bonairensis* Hoedelman 1958. Because no diagnostic characters were provided in these studies, both the biological and the geographical status of these taxa remain to be verified. Pending further investigation, such populations therefore might represent *K. marmoratus*, *K. ocellatus* or some other entity.

Recently, molecular markers (both microsatellites and mtDNA) revealed rather large genetic differences between *K. ocellatus* and *K. marmoratus* (Tatarenkov *et al.*, 2009). Based on these genetic analyses, undoubted populations of *K. marmoratus* inhabit Florida, Bahamas, Belize and Honduras, whereas a *bona fide* population of *K. ocellatus* resides in south-eastern Brazil, the site from which this taxon was originally described. Furthermore, single genotyped specimens from Turks and Caicos, Panama, southern Cuba and Puerto Rico (Weibel *et al.*, 1999; Tatarenkov *et al.*, 2010; unpubl. data) are now documented to be genetically closer to *K. ocellatus* than to *K. marmoratus*.

The closest phylogenetic outlier to the *K. marmoratus* and *K. ocellatus* clade is *Kryptolebias caudomarginatus* (Seegers 1984) (Murphy *et al.*, 1999), a species thought to be confined to south-eastern Brazil where it occurs sympatrically with

*K. ocellatus* (Fig. 1). These three species are the only rivulids that inhabit brackish estuarine areas, unlike all of the other 330+ species of the family that live in freshwater swamps and streams (Costa *et al.*, 2010). Furthermore, populations of all three species consist either of males plus hermaphrodites (androdioecy) or of hermaphrodites exclusively, but unlike in the other species, selfing was not detected in *K. caudomarginatus* (Tatarenkov *et al.*, 2009; Costa *et al.*, 2010).

Principle of parsimony suggests that the origin of this three-species clade lies in south-eastern Brazil, with *K. caudomarginatus* and *K. ocellatus* probably occupying the ancestral range and the common ancestor of *K. ocellatus* and *K. marmoratus* (or perhaps *K. marmoratus* alone) forming a phylogenetic offshoot that later penetrated and populated huge areas of Florida, the Caribbean and South America. Generally accepted views are that selfing hermaphroditism arose in south-eastern Brazil, that *K. marmoratus* expanded northward throughout the Caribbean (Murphy *et al.*, 1999), and that ancestral populations of *K. marmoratus* thus are likely to be found somewhere in the southern range of modern-day *K. marmoratus* (Turner *et al.*, 2006). Here, however, evidence is presented that populations of *K. ocellatus* in south-eastern Brazil are genetically depauperate and homogeneous, a finding with repercussions for inferences about the geographical origin of the *ocellatus* and *marmoratus* clade. A new possibility proposed in this work is that both species have a Caribbean origin and that *K. ocellatus* secondarily invaded south-eastern Brazil. An alternative hypothesis is that *K. ocellatus* arose *in situ* in Brazil. Although the current findings are not definitive on this matter, they should be known to the broader community of ichthyologists in the hope that they will stimulate further research and sampling efforts in South America.

Genetic analyses of *K. ocellatus* have lagged well behind those of *K. marmoratus*, with only one population of the former (from the Guaratiba area of Rio de Janeiro, close to the type locality of the species, Fig. 1) having been examined previously (Tatarenkov *et al.*, 2009). One peculiar finding about that population was its low genetic variation:  $H_E$  (expected heterozygosity under random mating) = 0.060, considerably lower than values recorded in various populations of *K. marmoratus* (range, 0.135–0.688; Tatarenkov *et al.*, 2007, 2009). The initial explanation for such low variation was that Guaratiba population was rather small or passed through a severe bottleneck. The surprise grew, however, when fish from the Nagoya Higashiyama Zoo (and known as *Hy* line) were found to be genetically nearly identical to fish from Guaratiba at 31 microsatellite loci (Table I) and also had the same mtDNA sequence (at nearly 3000 bp) as the most common haplotype in Guaratiba (Tatarenkov *et al.*, 2010). The *Hy* line originated from a single specimen taken by an unknown collector from an unknown locality prior to 2003 (Kanamori *et al.*, 2006). As the original collecting site could not be traced, it was assumed that the specimen from Nagoya Zoo had been collected near Guaratiba.

Recently (in 2009), 25 specimens of *K. ocellatus* were collected from mangroves of central Picinguaba (Fazenda Beach, Parque Estadual da Serra do Mar, São Paulo State, Brazil, 23° 21' 56" S 44° 50' 10" W; shown in Fig. 1). Picinguaba is at least 130 km south of Guaratiba (as measured by a straight line overwater), but the actual coastline distance (by which *K. ocellatus* probably disperses) is much longer. Using techniques described previously (Tatarenkov *et al.*, 2009), these fish were genotyped at 31 microsatellite loci, with the results shown in Table I. For comparison, in Table II genetic data on *Kryptolebias* as taken from

TABLE I. Allele frequencies at 31 microsatellite loci in *Kryptolebias ocellatus*

Locus	Allele	Guaratiba	Picinguaba	Hy line	Locus	Allele	Guaratiba	Picinguaba	Hy line
		<i>n</i> = 10	<i>n</i> = 25	<i>n</i> = 1			<i>n</i> = 10	<i>n</i> = 25	<i>n</i> = 1
<i>R1</i>	270	1.00	1.00	1.00	<i>R28</i>	152	1.00	1.00	1.00
<i>R3</i>	135	1.00	1.00	1.00	<i>R30</i>	185		0.04	
<i>R4</i>	175	1.00	1.00	1.00		201	1.00	0.96	1.00
<i>R5</i>	263	1.00	1.00	1.00	<i>R33</i>	194	1.00	0.96	1.00
<i>R6</i>	196	1.00	1.00	1.00		198		0.04	
<i>R7</i>	232	1.00	1.00	1.00	<i>R35</i>	140	1.00	1.00	1.00
<i>R9</i>	194	1.00	1.00	1.00	<i>R37</i>	348	0.10		
<i>R10</i>	165	1.00	1.00	1.00		356	0.10		
<i>R11</i>	107	1.00	1.00	1.00		372	0.15		
<i>R17</i>	275		0.02			376	0.15		
	283	1.00	0.94	1.00		384		0.12	
	287		0.04			388	0.20	0.20	
<i>R18</i>	172	0.90	1.00	1.00		392	0.10	0.36	
	176	0.10				396		0.12	
<i>R19</i>	139		1.00	1.00		400	0.20	0.16	1.00
	143	1.00				404		0.04	
<i>R22</i>	109		0.04		<i>R38</i>	214	1.00	0.92	
	133	1.00	0.96	1.00		218		0.04	1.00
<i>R23</i>	272		0.04			222		0.04	
	276	1.00	0.92	1.00	<i>B10</i>	117	1.00	1.00	1.00
	304		0.04						
<i>R25</i>	98	1.00	1.00	1.00	<i>B86</i>	183	1.00	1.00	1.00
<i>R26</i>	162	1.00	1.00	1.00	<i>R86</i>	183	1.00	1.00	1.00
<i>R27</i>	186	0.20	1.00	1.00	<i>R90</i>	250	1.00	1.00	1.00
	194	0.80							
					<i>R92</i>	211	1.00	1.00	1.00
					<i>R93</i>	161	1.00	1.00	1.00
					<i>R103</i>	127	1.00	1.00	1.00
					<i>R22D</i>	237		0.04	
						245	0.30	0.92	1.00
						249	0.70	0.04	

*n*, sample size; *Hy* line, fish from Nagoya Higashiyama Zoo.

Tatarenkov *et al.* (2007, 2009) are also displayed. Descriptive genetic statistics and distances ( $D_{PS}$ ) based on the proportion of shared alleles (Bowcock *et al.*, 1994) were calculated using the software microsatellite analyser (MSA) (Dieringer & Schlötterer, 2003).

TABLE II. Summary of genetic variation in *Kryptolebias*. Estimates are based on 31, 32–33 and 28 microsatellite loci in populations of *K. ocellatus*, *K. marmoratus* and *K. caudomarginatus*, respectively. Data for *K. ocellatus* from Picinguaba, Brazil are new; those for the other populations are from Tatarenkov *et al.* (2007, 2009)

Species & locality	Locality abbreviation	<i>n</i>	Per cent loci polymorphic (95% criterion)	Mean number of alleles per locus	<i>H<sub>E</sub></i> (random mating)	<i>H<sub>O</sub></i>	Inbreeding coefficient ( <i>F<sub>IS</sub></i> )
<i>K. ocellatus</i> :							
Picinguaba, São Paulo, Brazil	PI	25	16.1	1.5	0.052	0.001	0.98
Rio Piracão, Guaratiba, Brazil	GU	10	12.9	1.3	0.060	0.003	0.95
<i>K. marmoratus</i> :							
Long Key, FL 2007	LK	6	78.1	2.7	0.463	0.020	0.96
No Name Key, FL 2007	NK	10	84.4	3.5	0.495	0.024	0.95
Big Pine Key, FL	BP	40	84.4	4.2	0.474	0.015	0.97
Exuma Island, Bahamas	EI	12	33.3	1.4	0.135	0	1.00
San Salvador Is., Bahamas	SS	5	60.6	2	0.326	0.012	0.97
Charlotte County, FL	CC	17	57.6	2.1	0.258	0.022	0.92
Marco Island, FL	MI	8	81.8	2.7	0.381	0.068	0.83
Everglades National Park, FL	EP	4	72.7	2.1	0.432	0	1.00
Lostman's River, FL	LM	6	54.5	2.3	0.354	0.005	0.99
Tarpon Bay, FL	TB	12	69.7	2.7	0.396	0.003	0.99
Shark River, FL	SR	8	63.6	2.6	0.361	0	1.00
Harney River, FL	HR	10	72.7	2.8	0.404	0.003	0.99
St Lucie County, FL	SL	12	84.8	3.6	0.495	0.081	0.84
Long Key, FL	LK	7	60.6	1.6	0.217	0.082	0.64
No Name Key, FL	NK	5	84.8	2.4	0.466	0.170	0.66
Twin Cays, Belize 1991	TC	21	93.9	6.8	0.660	0.472	0.29
Twin Cays, Belize 2005	TC	101	97.0	10.4	0.688	0.502	0.27
<i>K. caudomarginatus</i> :							
Rio Piracão, Guaratiba, Brazil	GU	24	75.0	7.8	0.522	0.486	0.07
Rio Iriri, Magé, Brazil	RI	51	64.3	10.7	0.501	0.480	0.04

*n*, sample size; *H<sub>E</sub>*, expected heterozygosity; *H<sub>O</sub>*, observed heterozygosity.

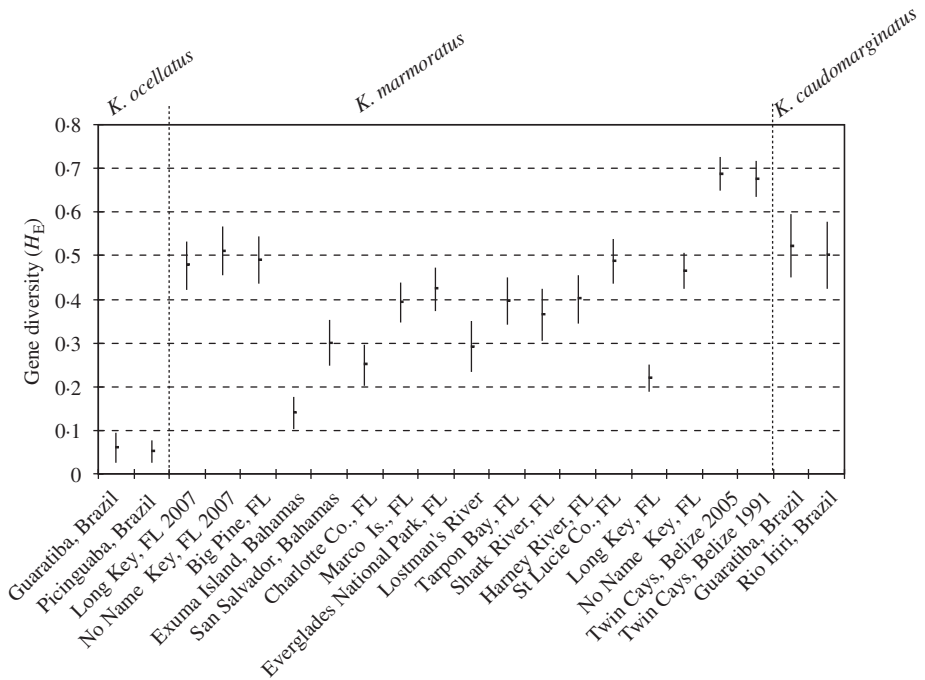


FIG. 2. Gene diversities (expected heterozygosity,  $H_E$ ; ■)  $\pm$  s.d. in populations of *Kryptolebias ocellatus*, *K. marmoratus* and *K. caudomarginatus*.

The Picinguaba population proved to have the same signatures of low genetic diversity as did the Guaratiba population (Table I). Only seven of 31 loci (23%) were polymorphic and six of those loci had the main allele present at frequency  $>90\%$ . Only one locus (*R37*) was highly polymorphic, and this locus was previously shown to have unusually high rates ( $3 \times 10^{-2}$ ) of *de novo* mutation (Tatarenkov *et al.*, 2010). In the Picinguaba population,  $H_E = 0.052$ , a value even lower than for Guaratiba and also considerably lower than in *K. marmoratus* where the least variable populations (from Charlotte County, Florida and Exuma Island in the Bahamas) had gene diversities of 0.26 and 0.13, respectively (Fig. 2 and Table II). Furthermore, at Picinguaba the proportion of polymorphic loci at the 95% cutoff level ( $P_{95}$ ) is 16%, noticeably lower than in *K. marmoratus* (range 33–97%) or *K. caudomarginatus* (64–75%). Even more striking is the high genetic similarity between the Guaratiba and Picinguaba populations (and also with the  $H_y$  line from Nagoya Zoo). These two populations are fixed for the same allele at 20 loci and have the same most common allele at seven others. Finally, only one heterozygous individual was observed at one locus in the Picinguaba population ( $H_0 = 0.001$  overall) as well as a high inbreeding coefficient ( $F_{IS} = 0.976$ ). Assuming that this single heterozygote is evidence of past outcrossing rather than *de novo* mutation, the selfing rate estimated from  $F_{IS}$  is 0.988. High selfing rates ( $>0.95$ ) have previously been reported in the Guaratiba population of *K. ocellatus* and in several populations of *K. marmoratus* from Florida and Bahamas (Mackiewicz *et al.*, 2006; Tatarenkov *et al.*, 2007, 2009).

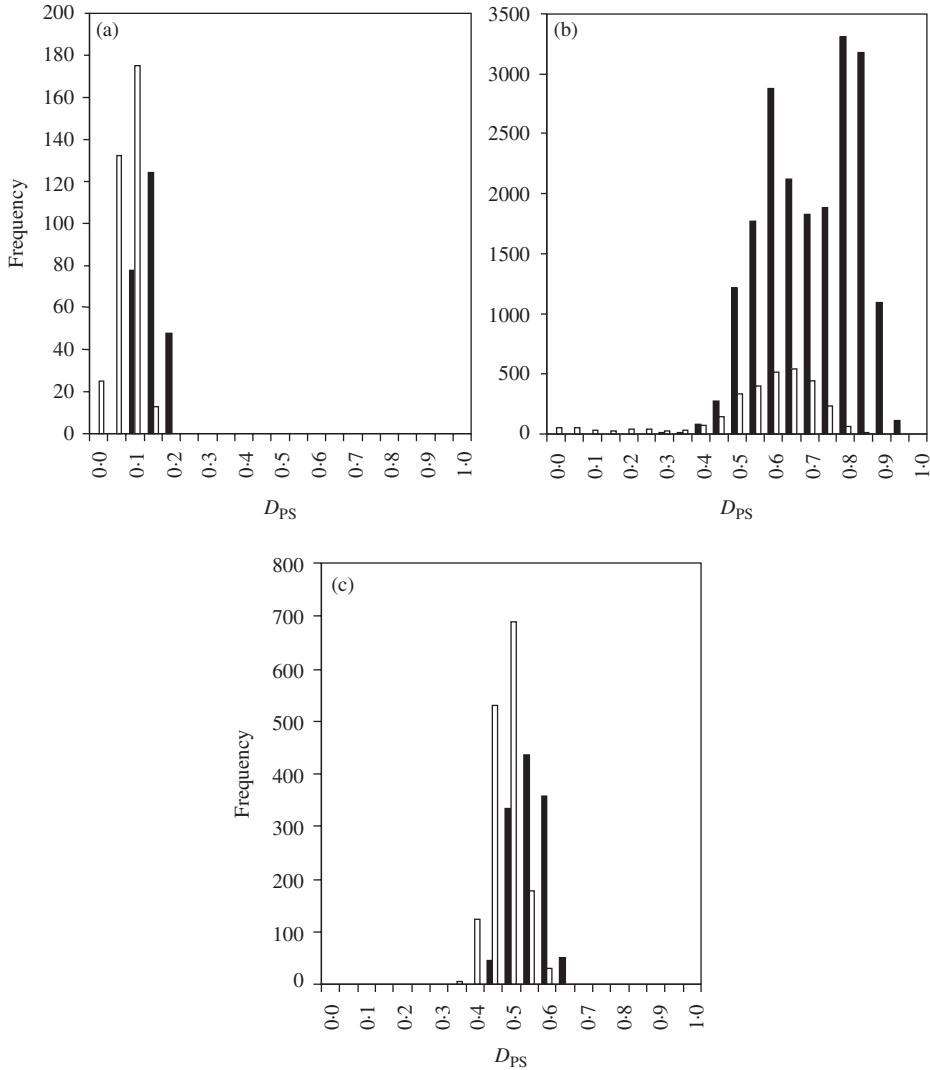


FIG. 3. Frequency distributions of genetic distance ( $D_{PS}$ ) between individuals from the same population (□) and from different populations (■) in three *Kryptolebias* species: (a) *K. ocellatus*, (b) *K. marmoratus* and (c) *K. caudomarginatus*.

The uniqueness of the population genetic profile of *K. ocellatus* from south-eastern Brazil is further exemplified by an analysis of genetic similarity at the level of single individuals (rather than populations). The distribution of genetic distances  $D_{PS}$  between individuals from the same and different populations of *K. ocellatus*, *K. marmoratus* and *K. caudomarginatus* is shown in Fig. 3. In the intrapopulation comparisons,  $D_{PS}$  among individuals of *K. marmoratus* has a median of 0.58 and reaches values as high as 0.80, although the distribution is skewed such that some genetically similar or identical individuals do exist. In *K. marmoratus*, this kind of skew is the result of self-fertilization, which when continued across generations



leads to the production of effectively isogenic individuals. Intrapopulation  $D_{PS}$  values are also high (median 0.46) in two populations of *K. caudomarginatus*, an androdioecious species that lacks self-fertilization. In comparison, individuals of *K. ocellatus* show much smaller intrapopulation genetic differences (median  $D_{PS}$  = 0.06; maximal  $D_{PS}$  = 0.13). This is yet another demonstration that gene diversity is drastically reduced in populations of *K. ocellatus* from south-eastern Brazil.

The  $D_{PS}$  values (which range from 0.06 to 0.19 with a median of 0.13) between individuals from different populations demonstrate that genetic diversity is decreased not only within but also between populations of *K. ocellatus*. The specimen from Nagoya Zoo (not included in Fig. 3) has a maximum  $D_{PS}$  of 0.17, with values as low as 0.03 in comparisons with three individuals from Pinguaba. By contrast, much higher values of  $D_{PS}$  characterize both *K. marmoratus* and *K. caudomarginatus*. Indeed, even the *minimal* values in both species (0.25 and 0.41, respectively) exceed the maximal values in *K. ocellatus*. Taken together, the results indicate that the two sampled populations of *K. ocellatus* are impressively similar in their genetic profiles.

One potential explanation for such low variation in *K. ocellatus* could be purely methodological. It is well known that microsatellite loci developed for a focal species are generally less variable when applied to other species and that the effect becomes stronger with increased divergence between the focal and target species (Primmer *et al.*, 2005). Because microsatellite loci developed for *K. marmoratus* were applied to four species of *Kryptolebias*, the bias can be ascertained. For these four species, the mt DNA sequence divergence between *K. marmoratus* and *K. ocellatus* is *c.* 4% (Tatarenkov *et al.*, 2009), a value not unusual in intraspecific comparisons in many other species (Avice, 2000). By contrast, mtDNA sequence divergence between *K. marmoratus* and *K. caudomarginatus* is higher [9.6% at locus *NADH* (Vermeulen & Hrbek, 2005); 10–11% at loci *CR1* and *ATP6* (unpubl. data)]. Yet, *K. caudomarginatus* displays high levels of microsatellite variation ( $P_{95}$ ,  $H_E$ ) that are comparable to those of *K. marmoratus*, and even the very divergent *K. brasiliensis* (Valenciennes 1821) [mtDNA *NADH* sequence divergence is *c.* 21% (Vermeulen & Hrbek, 2005) has higher variation than *K. ocellatus* at the microsatellites loci developed for *K. marmoratus*. Thus it was concluded that methodological bias is unlikely to be the cause of reduced variation observed in *K. ocellatus*.

Exceptionally low intrapopulation genetic variation can be caused by small effective population size, *e.g.* if populations recently were founded by only a few individuals or if they passed through a population bottleneck. Of these two explanations, a recent founding of two *K. ocellatus* populations from the same source would appear to be more likely. Severe bottlenecks lasting for several generations in established populations of *K. ocellatus* could also result in decreased intrapopulation variation, but they cannot so readily explain the extremely low interpopulation differentiation. Indeed, strong genetic drift caused by simultaneous bottlenecks in two populations would probably increase genetic differentiation between them due to random fixation of different alleles in each location. The distributions of genetic distance between individuals from different population of *K. marmoratus* and *K. caudomarginatus* suggest that the low  $D_{PS}$  values observed in *K. ocellatus* are unlikely to be produced by draws of genotypes from two independently bottlenecked populations. Nevertheless, another type of scenario involving bottlenecks could in principle explain low interpopulation differentiation: an environmental cataclysm that affects a large area might extinguish some populations while severely reducing others that later would

serve as a source for re-seeding the devastated locations. No such loss of variation can be seen, however, in two populations of *K. caudomarginatus* that are also from south-eastern Brazil and one of which (at Guaratiba) is sympatric with one of the *K. ocellatus* populations (Fig. 1). Because populations of *K. caudomarginatus* and *K. ocellatus* in south-eastern Brazil are syntopic as well as broadly sympatric (both occur in brackish mangrove forests), if there was an environmental disaster it probably would have affected both species. Thus, it seems unlikely that low intra and inter-population gene diversity in south-eastern Brazilian *K. ocellatus* resulted from an *in situ* decrease of population size. It follows, therefore, that founders of the Guaratiba and Picinguaba populations of *K. ocellatus* probably came from other sources. Furthermore, the colonization of both locations must have been quite recent because insufficient time has elapsed to accumulate substantial genetic variation and divergence.

The limited available data prevent plausible geographical sources of the colonists to be proposed. Circumstantial evidence, however, suggests that the source populations may be remote. As mentioned earlier, the geographic distribution of *K. marmoratus* and *K. ocellatus* is assumed to be continuous from south-eastern Brazil to central Florida. In fact, however, collections of these species from south-eastern Brazil, north to French Guiana are non-existent (Taylor, 2000). Limited surveys north of Rio de Janeiro in Brazil's Bahia state failed to find either species (unpubl. data). Such a gap in the distribution of *K. marmoratus*–*K. ocellatus* may simply reflect the paucity of research in the area, but it may also indicate scarcity and patchiness of the distribution. If *K. ocellatus* in south-eastern Brazil is indeed represented by genetically depauperate populations disconnected from the main area, this gives some credence to an exciting hypothesis that both *K. marmoratus* and *K. ocellatus* might have a Caribbean origin, with *K. ocellatus* only secondarily invading south-eastern Brazil. The exact area that was the source of colonists for south-eastern Brazil, however, remains to be established, with vast stretches of mangroves north of Rio de Janeiro being among the other candidates. More generally, examples are known in other maritime species in which source populations are quite remote. For example, whereas the main distribution of the ovoviviparous snail *Littorina saxatilis* is in the North Atlantic Ocean, isolated pockets of this species are known from Venice in the Mediterranean Sea and from South Africa (Janson, 1985; Knight *et al.*, 1987). The South African population also has very low genetic diversity in what otherwise is a highly polymorphic species. Another such example may be even more relevant to the current discussion: a mangrove land crab *Ucides cordatus* whose range overlaps that of *K. marmoratus*–*K. ocellatus* and likewise is associated with the mangrove habitat is thought to have undergone a dramatic southward expansion with concomitant losses of variation along the way (Oliveira-Neto *et al.*, 2007).

Following the discovery of self-fertilization in *K. marmoratus* (Harrington, 1961), considerable progress has been made in the study of this species' population genetics and mating system. Most of these studies, however, involved populations from Florida and Belize, with vast areas of the Caribbean including the Greater and Lesser Antilles as well as the coasts of Central and South America having remained virtually unexplored. The unusual population genetic structure that was found in south-eastern Brazilian populations of *K. ocellatus* (a sibling species or probable conspecific of *K. marmoratus*) is yet another reminder of the importance of broad

geographic coverage in genetic analyses. Further genetic sampling from these areas should help to clarify not only the taxonomy but also the evolutionary origins and historical dispersal routes of *Kryptolebias* lineages, and this in turn should help to single out biological and ecological features that have played a role in securing the broad distribution of *K. ocellatus*–*marmoratus* clade. Such knowledge could also be relevant for understanding phylogeographic patterns in other mangrove-restricted taxa.

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