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GENETIC RELATIONSHIPS IN A COMPLEX OF *Cynolebias* SPECIES (CYPRINODONTIFORMES, RIVULIDAE) FROM EASTERN URUGUAY AND SOUTHERN BRAZIL

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ABSTRACT

The genus *Cynolebias* (Cyprinodontiformes: Rivulidae) is an endemic group of Neotropical killifishes. One well-supported intrageneric clade from previous mitochondrial phylogenetic analysis includes *Cynolebias cf. adloffi,* which has always been considered a complex of populations and its systematic affinities with two other taxa (*C. cf. adloffi* and *C. viarius*) have been historically discussed. We present allozyme and cytogenetic data to clarify the relationships among them. Most of the 15 loci analyzed by allozyme electrophoresis are monomorphic. Nei´s genetic similarity coefficient between *C. cf. adloffi* and *C. viarius* (0.812) populations is similar to that found between subspecies. However, two diagnostic loci (GPI-11 and G6PD1), and two other exclusive alleles (GPI-21d and PGM1b) found at high frequency in *C. viarius* but absent in *C. cf. adloffi* populations suggest little or no gene flow between both populations. We report partial sterility and viability in laboratory F1 hybrids between *C. adloffi* and *C. cf. adloffi*. This fact shows the existence of postzygotic reproductive barriers among both taxa. These results suggest that *C. cf. adloffi* is a separate species belonging to "Bañados del Este" a region from Uruguay.

Keywords: killifishes, *Cynolebias*, Rivulidae, species complex

RESUMEN

Relaciones genéticas un complejo de la especie *Cynolebias* (Cyprinodontiformes, Rivulidae) del este de Uruguay y sur de Brasil

El género *Cynolebias* (Cyprinodontiformes: Rivulidae) es un grupo endémico de peces anuales Neotropicales. Uno de los clados resultantes en análisis filogenéticos previos basados en ADN mitocondrial incluye a *Cynolebias cf. adloffi*, integrado por un complejo de poblaciones cuya afinidad sistemática con *C. adloffi* y *C. viarius* ha sido históricamente discutida. El presente trabajo intenta aclarar las relaciones entre dichos taxa en base a electroforesis de enzimas y datos citogenéticos. De los 15 loci analizados la mayor parte resultaron ser monomórficos. El coeficiente de similitud de Nei presentó un valor de 0.812 entre poblaciones de *C. cf. adloffi* y *C. viarius.* Sin embargo, se encontraron dos loci diagnósticos (GPI-11 and G6PD1) y otros dos alelos exclusivos para cada uno de estos taxa (GPI-21 y PGM1b), lo cual sugiere la interrupción del flujo génico. Se describe además, la esterilidad parcial y viabilidad en híbridos F1 obtenidos en el laboratorio, entre *C. adloffi*

and *C. cf. adloffi* sugiriendo la existencia de barreras reproductivas poscigóticas entre dichos taxa. Estos resultados sugieren la divergencia de *C. cf. adloffi* en los "Bañados del Este" de Uruguay.

Palabras clave: killifishes, *Cynolebias*, Rivulidae, complejo de especies

INTRODUCTION

The genus *Cynolebias* is in the Neotropical family Rivulidae (Cyprinodontiformes) which has an annual life cycle. *Cynolebias* is highly variable in morphology (Vaz-Ferreira & Sierra 1972, Costa 1990, 1995, Loureiro & de Sá 1998), behavior (Vaz-Ferreira & Sierra 1973) and chromosomes (García *et al.* 1993, 1995). Based on morphology, Costa (1998) proposed that species of *Cynolebias* from Argentina, Uruguay and southern Brazil belong to two newly erected genera: *Austrolebias* and *Megalebias*. However, recent phylogenetic analyses based on partial sequences of cytochrome b sequences (*cyt b*) do not suppot such arrangement (García *et al.* 2000, 2002). These phylogenetic analyses are concordant with the existence of different intrageneric clades grouping species from Uruguay and southern Brazil. One of these well-supported clades includes *Cynolebias adloffi* from Porto Alegre (POA), southern Brazil, *Cynolebias* sp. (named *C. cf. adloffi* in our analyses) and *Cynolebias viarius,* both from the Atlantic coast basin (eastern Uruguay). *Cynolebias viarius* and *C. cf. adloffi* are distributed parapatrically, whereas *C. adloffi* is allopatric (Fig. 1).

Cynolebias cf. adloffi has been considered a complex of populations and its systematic affinities have been controversial (Katz 1982, Vaz-Ferreira & Melgarejo 1984). High levels of morphological variability and chromatic gradation between *C. viarius* and *C. adloffi* were reported in *C. cf. adloffi* populations, which inhabit the Uruguayan-Brazilian border (Vaz-Ferreira & Melgarejo 1984). Chromosomal studies of *C. adloffi* showed unique diploid (2n=48) and fundamental (FN=50) numbers. Nevertheless, two cytotypes in *C. viarius* (2n=48, FN=50 and 2n=46, FN=50) and three in *C. cf. adloffi* (2n=48, FN=52; 2n=46, FN=52 and 2n=48, FN=56) have been described (García *et al.* 1993, 1995).

Mitochondrial *cyt b* sequence analyses showed that the lowest sequence divergence in this species complex, was found between *C.adloffi* and *C. cf. adloffi* (12.1%), whereas *C. viarius-C. cf. adloffi* and *C. viarius-C.adloffi* differed by 18.2% and 16.6%, respectively (García *et al.* 2000, 2002). However, total protein electrophoresis studies by isoelectrofocusing had shown that *C. cf. adloffi* appeared more closely related to *C. viarius* than *C. adloffi* (Bellini *et al.* 1992). The same results emerged from the Principal Components Analysis based on morphological data (Loureiro 1999). Therefore relationships among these taxa suggested by mitochondrial *cyt b*, total protein electrophoresis and morphological analyses are in conflict. Reciprocal monophyly of *C.adloffi* y *C. cf. adloffi* is well supported only by mitochondrial sequence data. These contrasting genealogical perspectives were explained previously by the possible occurrence of episodes of introgressive hybridization between *C. viarius* and *C. adloffi*, which differentially affected mitochondrial and nuclear genes (García *et al*. 2000). Because mitochondrial phylogenetic relationships could be evidence for the retention of ancestral polymorphisms and/or differential lineage sorting between *C. adloffi* and *C. cf. adloffi* populations, nuclear genes could show the occurrence of introgressive hybridization events between *C. cf. adloffi* and *C. viarius*.

Herein we investigate the systematic relationships among this species complex using genetic markers. The goal of our study is to contribute to the understanding the dynamics of population differentiation in the annual killifishes of "Bañados del Este" a region from Uruguay, which was declared a Biosphere Reserve site by MAB UNESCO in 1976.

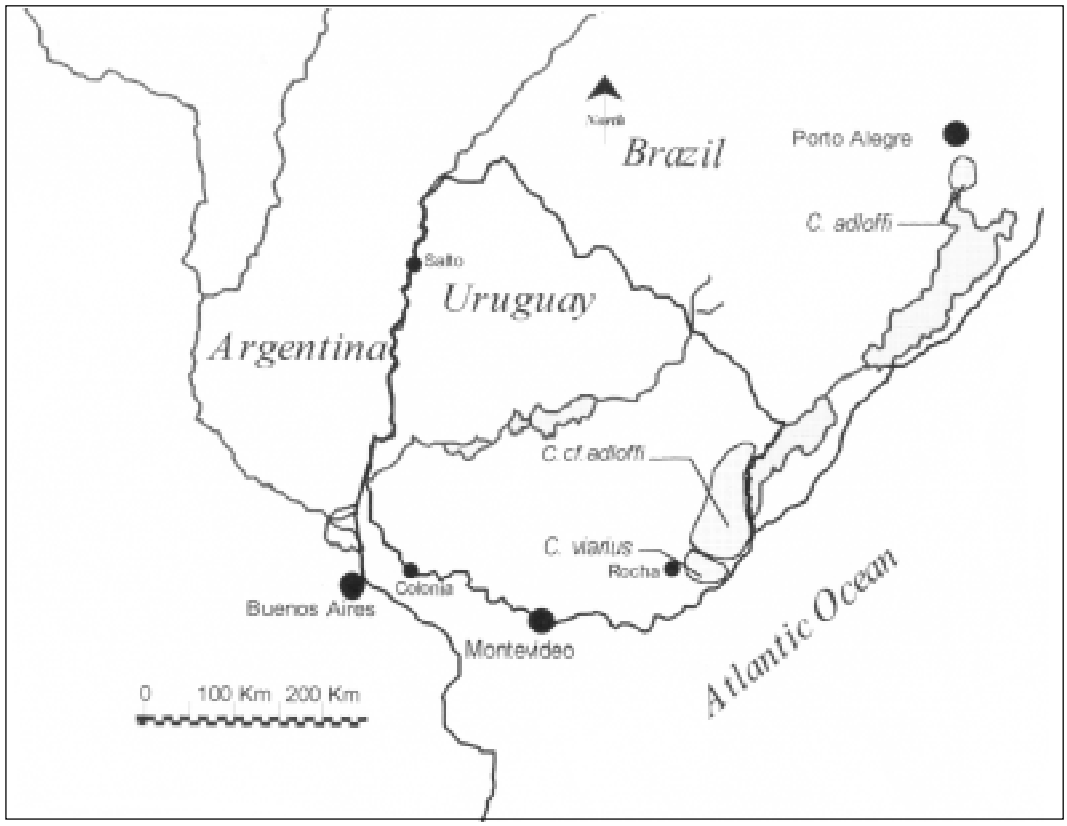

Fig. 1. Geographic distribution of *C. adloffi*-1 (southern Brasil), *C. adloffi*-2 and *C. viarius* (eastern Uruguay). *C. adloffi*-2 and *C. viarius* are parapatric. *C. adloffi*-1 is allopatric.

MATERIALS AND METHODS

Fishes

We sampled natural populations of *C. viarius*, *C. cf. adloffi* and *C. luteoflammulatus* from "Bañados del Este" in Uruguay. F1 offsprings were obtained in the laboratory from occasional crosses between *C. adloffi* from Rio Grande Do Sul (southern Brazil) and *C. cf. adloffi*. Tissues and voucher specimens are deposited in Sección Genética Evolutiva (GP= *Cynolebias*),

Facultad de Ciencias, Universidad de la República, Montevideo, Uruguay. No specimens of *C. adloffi* were available for allozyme electrophoresis analyses.

Allozyme electrophoresis

Extract of water-soluble proteins of skeletal muscle from each individual was obtained. Horizontal starch gel electrophoresis and interpretation of banding patterns were performed according to Shaklee & Keenan (1986). Sixteen individuals of *C. luteoflammulatus* (from R10 K. 225, Dpto. Rocha), twenty six specimens of *C. viarius* (from R.10 and R.16, Dpto. Rocha) and sixteen of the *C. cf. adloffi* (from R. 14 K. 504, Dpto. Rocha) were analyzed for the following enzymes: aspartate aminotransferase (E.C. 2.6.1.1, AAT), malic enzyme (E.C. 1.1.1.40, ME), adenilate kinase (E.C. 2.7.4.3, AK), L-lactate dehydrogenase (E.C. 1.1.1.27, LDH), malate dehydrogenase (E.C. 1.1.1.37, MDH), isocitrate dehydrogenase (E.C. 1.1.1.42, IDH), glycerol-3-phosphate dehydrogenase (E.C. 1.1.1.8, G3PD), 6-phosphogluconate dehydrogenase (E.C. 1.1.1.44, 6PGD), glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49, G6PD), phosphoglucoisomerase (E.C. 5.3.1.9, PGI), phosphoglucomutase (E.C. 5.4.2.2, PGM), esterases (E.C. 3.1.1.1, EST). Stainings were performed according to Shaklee & Keenan (1986) (LDH, MDH, IDH, G3PD, ME, AAT, and EST) or according to Selander *et al.* (1986) (GPI, G6PD, AK, PGM and 6PGD). Polymorphism index (P) and mean heterozygosity (H) were calculated for each of the three taxa studied. A significance test using exact probabilities was performed to evaluate deviation of observed genotypic frequencies from those expected under Hardy-Weinberg equilibrium. Expected genotypic frequencies were calculated using Levene´s (1949) correction for small samples and the Fixation Index (F) was calculated to estimate heterozygote deficiency or excess. Nei's (1972) coefficient of genetic identity was obtained for each species. Computations were done with BYOSIS-1 (Swofford & Selander 1981).

Chromosome Studies

Two individuals (F1 hybrid offsprings) were available for cytogenetic studies from the cross between *C. adloffi* and *C. cf. adloffi*. Female *C. adloffi* and male *C. cf. adloffi* crossings resulted in a male offspring (specimen #394) which exhibited normal testis appearance. The reciprocal cross, resulted in two morphological males offspring: specimen #414, which showed a hypertrophied white tissue within the abdominal cavity (most probably non-functional testis) and specimen #415 with low viability, which died.

Metaphase mitotic chromosomes were obtained using conventional techniques (Kligerman & Bloom 1977, Bertollo 1978). At least twenty metaphases per individual were examined. Chromosomes were classified according to Levan et al. (1964) following Denton's (1973) modifications for fish cytogenetics. Meiotic studies in male individuals of *C. adloffi*, *C. cf. adloffi* and F1 hybrid were performed following standard protocols (Kligerman & Bloom 1977). Thirty-five F1 hybrid cells and twenty of each parental population were examined. Mann-Whitney U tests were performed using STATISTICA (Statsoft, Inc. 1999) to detect significant differences in the mean of chiasmata number per cell and its position.

RESULTS

Allozyme analysis

Table 1 shows allelic frequencies, polymorphism index and heterozygosities found in the three taxa studied. Most of the 15 loci analyzed were monomorphic. In *C. viarius* genetic variation was detected in GPI_2 and PGM, each with two alleles. *Cynolebias luteoflammulatus* showed variation in GPI_2 and *C. cf. adloffi* in GPI_1, both with two alleles. Locus G6PD has a different allele for each taxon. Mean heterozygosity for each species was low and more similar between *C. cf. adloffi* and *C. luteoflammulatus*. Nei´s coefficient of genetic identity based on 15 loci was 0.812 between *C. viarius* and *C. cf. adloffi*, and 0.408 between any of them and *C. luteoflammulatus*. The observed genotypic frequencies agreed with those expected under Hardy-Weinberg equilibrium. However, we found that PGM locus in *C. viarius* showed significative excess of homozygotes ($p=0.028$) as shown by a high fixation index ($F=0.614$).

Cytogenetics analyses of F1 offspring

Specimen #394 had 2n=48, NF=50 and a chromosomal constitution of two Meta-Submetacentric (M-SM) type chromosomes, and forty-six Subtelo-Acrocentric (ST-A) type chromosomes (Fig. 2a). On the other hand, specimen #414 had 2n=48, NF=58 and a chromosomal constitution of ten (M-ST) type chromosomes and nineteen pairs of ST-A type chromosomes (Fig. 2b). Meiotic analysis of specimen #394 with conventional techniques showed similar stages of meiotic process to those found in parental populations. However, a comparative analysis of meiotic stages (diakinesis and metaphase I) between parental populations and F1 hybrid reveals some interesting differences in chiasmata number and localization. F1 hybrid cells showed a significant reduction (Mann-Whitney U test, $p<0.01$) in the mean of chiasmata number per cell (mean=19.7, S.D.= 2.28) compared to the parental populations (*C. adloffi*: mean=23.3, S.D.=1.15; *C. cf. adloffi*: mean =22.6, S.D.=1.57) considered as one. Mann-Whitney U test showed that there are not significant differences between parental populations considering total number of chiasmata, and chiasmata localized in proximal, distal and interstitial positions. In the parental populations chiasmata were found mainly in interstitial positions. On the other hand, hybrid #394 showed a significant increase in distal and proximal chiasmata positions ($p<0.01$), and a significant reduction in the interstitial ones ($p<0.01$) with respect to parental populations.

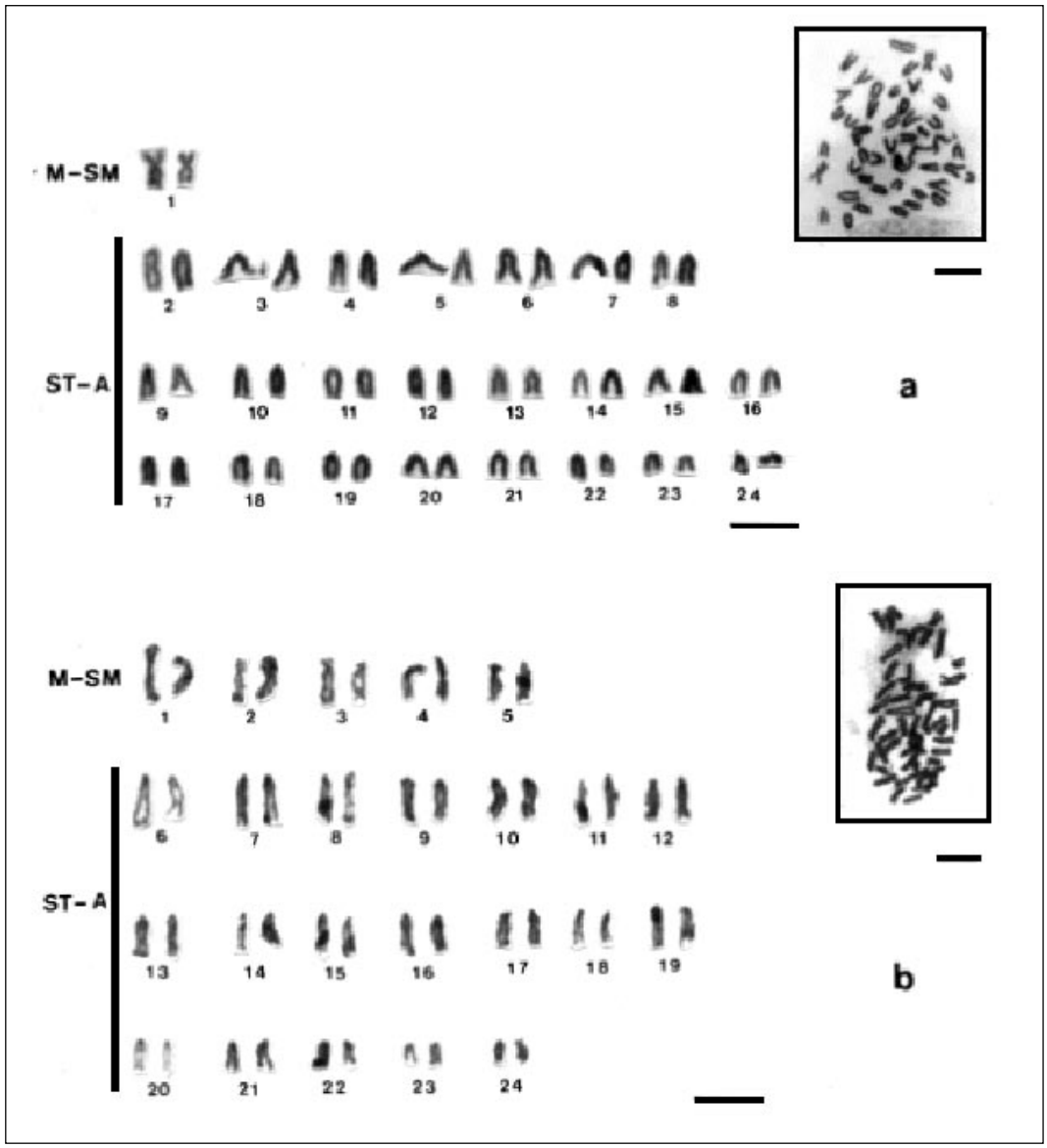

Fig. 2. Karyogram with Giemsa staining of somatic cells of two F1 laboratory hybrids. a) individual 394 (male): 2n=48, (M=2, ST-A=46), b) individual 414 (male) 2n=48, (M-SM=10, ST-A=38). Bar=10 mm.

Table 1 shows allelic frequencies for 15 loci of three species of genus *Cynolebias*. Polymorphism index (P) and mean heterozygosity (H) found in the three taxa studied. Letters represent individual alleles in order of decreasing mobility starting with "a". (N, number of individuals; p, probability of Chi^2 Test for deviation from Hardy-Weinberg equilibrium corrected for small samples with Levene´s (1949) correction.

	LOCUS	*C. luteoflammulatus*	*C. adloffi-2*	*C. viarius*
GPI$_1$	N	4	4	9
	a	1.000	0.000	0.000
	b	0.000	0.000	1.000
	c	0.000	0.875	0.000
	d	0.000	0.125	0.000
	p	>0.99	>0.99	>0.99
GPI$_2$	N	4	4	11
	a	0.125	0.000	0.000
	b	0.875	0.000	0.000
	c	0.000	1.000	0.864
	d	0.000	0.000	0.136
	p	>0.99	>0.99	*0.675*
G6PD	N	2	2	4
	a	1.000	0.000	0.000
	b	0.000	1.000	0.000
	c	0.000	0.000	1.000
	p	>0.99	>0.99	>0.99
G3PD	N	2	3	5
	a	1.000	1.000	1.000
	p	>0.99	>0.99	>0.99
6PGD	N	4	5	11
	a	1.000	1.000	1.000
	p	>0.99	>0.99	>0.99
AATm	N	2	3	7
	a	1.000	1.000	1.000
	p	>0.99	>0.99	>0.99
AATc	N	2	3	7
	a	1.000	0.000	0.000
	b	0.000	1.000	1.000
	p	>0.99	>0.99	>0.99
LDH	N	4	5	9
	a	1.000	1.000	1.000
	p	>0.99	>0.99	>0.99
MDHm	N	4	5	9
	a	1.000	0.000	0.000
	b	0.000	1.000	1.000
	p	>0.99	>0.99	>0.99
MDHc	N	12	11	19
	a	0.000	1.000	1.000
	b	1.000	0.000	0.000
	p	>0.99	>0.99	>0.99
IDH	N	4	5	9
	a	1.000	0.000	0.000
	b	.000	1.000	1.000
	p	>0.99	>0.99	>0.99
ME	N	12	11	19
	a	1.000	1.000	1.000
	p	>0.99	>0.99	>0.99

AK	N	4	5	9
	a	1.000	0.000	0.000
	b	0.000	1.000	1.000
	p	>0.99	>0.99	>0.99
PGM	N	4	3	11
	a	1.000	0.000	0.000
	b	0.000	0.000	0.864
	c	0.000	1.000	0.136
	p	>0.99	>0.99	*0.143*
EST	N	12	11	19
	a	1.000	1.000	1.000
	p	>0.99	>0.99	>0.99
	H	0.017	0.017	0.033
	P	6.7	6.7	13.3

DISCUSSION

The genetic variation found herein, expressed by mean heterozygosities (H=0.017-0.033) and polymorphism index (P=0.067-0.133) (Table 1) are among the lowest values found in fishes (H=0.080; P=0.30; Selander, 1976). *Aphanius iberus* showed mean values of genetic variability of H=0.075, P=0.29 (Doadrio et al. 1996), in the genus *Cyprinodon* H=0.012-0.123, P=0.04-0.29 (Echelle et al. 1987) and in other eurythermic and euryhaline species of Cyprinodontiformes H=0.049-0.180, P=0.21-0.56 (Nevo 1978). It seems that these values are not uniform within Cyprinodontiformes.

Homozygote excess detected in PGM locus from *C. viarius* and the low heterozygosity found in three taxa could be explained by either genetic drift or/and inbreeding in these populations. Discontinuity of aquatic habitats may affect the genetic structure of freshwater fish populations perhaps lowering heterozygosity (Avise & Smith 1974). In fact, members of the genus *Cynolebias* are restricted to annual ponds and have been associated with organisms living in small isolated populations or with limited vagility. Moreover, the effective number (Ne) in *Cynolebias*' populations could be smaller considering mate preferences in the species of this genus proposed by Vaz-Ferreira & Sierra (1973). In this sense, sexual selection could contribute to enhance the inbreeding and genetic drift effect in natural populations of *Cynolebias*.

Nei's genetic similarity coefficient between *C. cf. adloffi* and *C. viarius* populations (0.812) is similar to that found between subspecies (Avise 1976). Moreover, a similar value was found between subspecies of the cyprinodontid *Aphanius iberus* (Doadrio et al. 1996), between two conspecific characins *Astyanax mexicanus* (Avise & Selander 1972) and between two bluegill sunfish *Lepomis macrochirus* subspecies (Avise & Smith 1974).

In contrast, the relatively low value of genetic similarity coefficient (0.408) and nearly 50% differences at the allelic composition loci level between *C. luteoflammulatus* and *C. viarius* or *C. cf. adloffi,* provide references for comparison at species level (Avise 1976). This agrees with mitochondrial *cyt b* analysis where *C. luteoflammulatus* is grouped in another intrageneric clade (García et al. 2000).

Although Nei´s genetic similarity coefficient between *C. cf. adloffi* and *C. viarius* was high, the allozymic data showed that there are two diagnostic fixed loci (GPI-11 and G6PD1) that

separate these taxa. Moreover, two other exclusive alleles (GPI-21d and PGM1b) found in high frequency in *C. viarius* were absent in *C. cf. adloffi*. This suggest an interruption of gene flow between both populations.

We found partial sterility and viability in the occasional laboratory F1 hybrids between *C. adloffi* and *C. cf. adloffi*. This points to postzygotic reproductive barriers among natural populations of *C. adloffi* and *C. cf. adloffi* taxa, with an allopatric distribution.

Moreover, in both crosses hybrids inherited the maternal karyotype structure (Fig. 2) and karyotypic patterns were similar to those previously described from *C.adloffi* and *C. viarius* populations (García et al. 1993, 1995). Although our sample is small, we found no F_1 viable hybrid females. A different F_1 hybrid sex bias was also observed by Katz (1982) who obtained low F1 hybrid success from crosses between a male *C. viarius* and a female *C. adloffi*. In these experiments, individuals had a female appearance, caudal black spots similar to *C. adloffi*, dark flank spots similar to *C. viarius* and low viability. Further hybridization experiments among these taxa are needed to clarify this preliminary bias in favor of one or the other F1 hybrid sex.

Interspecific hybrids and their offspring have reduced chiasmata frequency and show unrestricted chiasmata distribution (Sybenga 1975). We show here that chiasmata frequency is significantly reduced. In the hybrid #394, they evidence changes in the distribution compared with parental cells. However, chiasma frequency does not show significant differences between parental species *C. cf. adloffi* and *C. adloffi* and this fact does not support the previous hypothesis that *C. cf. adloffi* could be an introgressed population (García et. al. 2000).

In this sense, an alternative hypothesis of differentiation among this complex of species is proposed, considering a likely scenario of allopatric differentiation. The hypothetical cladogenetic events could be concordant with geological scenarios of Pleistocene and Post-Pleistocene marine transgressions (Sprechmann 1980) in this region, probably producing habitat modification, fragmentation and isolation of populations from a more extensive ancestral population. The isolation during this period could have produced differentiation between populations as evidenced by the lack of genetic exchange between *C. viarius* and *C. cf. adloffi* and the differential allele fixation in both taxa. Subsequent reinforcement of isolation by secondary contact may be responsible for the observed reproductive postzygotic barriers between *C. adloffi* and *C. cf. adloffi*. In this scenario, the greater affinity of mitochondrial *cyt b* sequences of *C. adloffi* and *C. cf. adloffi* populations could be explained by random retention of mitochondrial lineages, whereas morphological similarities between *C. viarius* and *C. cf. adloffi* can reflect the real origin from neighbor populations, with actual parapatric distribution.

Further analyses are needed to reassess the taxonomic status of *C. cf. adloffi* populations as a distinct taxon, and as well as the establishment of post-zygotic isolation barriers among these taxa.

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