Ann. Zool. Fennici 51: 00–00 ISSN 0003-455X (print), ISSN 1797-2450 (online)
Helsinki 2014

Natural hybridization in lizards of the genus *Tupinambis* (Teiidae) in the southernmost contact zone of their distribution range

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Received 20 May 2013, final version received 15 July 2013, accepted 19 Aug. 2013

Cabaña, I., Gardenal, C. N., Chiaraviglio, M. & Rivera, P. C. 2014: Natural hybridization in lizards of the genus *Tupinambis* (Teiidae) in the southernmost contact zone of their distribution range. — *Ann. Zool. Fennici* 51: 00–00.

Studies on the mechanisms of speciation and maintenance of lineages have paid great attention to hybridization between species because this process is considered an important source of variability and evolution. In recent years, the use of molecular markers has provided more detailed information on the distribution and magnitude of hybridization in natural populations. Here we present a phylogenetic analysis using one mitochondrial and one nuclear DNA segment as molecular markers in two closely related lizard species, *Tupinambis merianae* and *T. rufescens*, which are present in a continuous area including allopatric and sympatric populations. Consensus trees obtained with the mitochondrial gene showed two well-supported clades. Some individuals clustered with one of the species in the tree obtained with mitochondrial DNA, and with the other species in the tree recovered using the nuclear gene, demonstrating the occurrence of hybridization between these species. Hybrid individuals were captured in the area of sympatry, suggesting the existence of a hybrid zone in the contact area of the distribution ranges of these two lizards, which corresponds to the ecotone between Dry Chaco and Espinal. This work presents the first evidence of natural hybridization within the genus *Tupinambis*.

Introduction

Hybridization between species is an interesting process in speciation and lineage maintenance. By backcrossing, hybridization may result in the transfer of alleles between species; the introgression of new genetic information can be an important source of variability and subsequent evolution, even at low levels (Anderson 1949, Barton 2001, Seehausen 2004, Mallet 2007, 2008).

Hybrid individuals may originate from mating between females of one species and males of the other species and vice versa (reciprocal hybridization), or from mating of females of one species with males of the other species only (unidirectional hybridization) (Wirtz 1999). Detection of hybrid individuals has traditionally been performed by morphological, cytogenetic and/or histocompatibility studies (Dowling & Secor 1997). Today, the use of molecular markers enables the study of directionality, distribution and/or extent of hybridization in natural populations (Mallet 2008).

Natural hybridization has been studied in teiids of the genera *Aspidoscelis, Cnemidophorus* and *Kentropix* (Dessauer *et al*. 2000, Taylor *et al*. 2001, 2003, Reeder *et al*. 2002, Cole *et al*. 2007, Manríquez-Morán 2007) with the aim to identify the parental species that gave rise to parthenogenetic forms. None of these studies included hybrids able to sexually reproduce among them or with their parental species.

To identify the origin of hybrids and obtain a comprehensive picture of lineage evolution, it is convenient to combine analysis with mitochondrial (mtDNA) and nuclear (nDNA) DNA markers, since the nDNA evolves at lower rates than the mtDNA, recombines and represents the history of both parents, rather than that of the maternal lineage (Leache & McGuire 2006, Zarza *et al*. 2008). If hybrids share mtDNA haplotypes with only one of the parental species, the process behind it is unidirectional hybridization. However, if some hybrids share haplotypes with one of the parental species and other hybrids do so with the other parental species, reciprocal hybridization can be assumed.

The genus *Tupinambis* belongs to the family Teiidae, with their two southernmost distributed species being present in Argentina. *Tupinambis merianae* is widespread, spanning several biogeographic regions, whereas *T. rufescens* has a range restricted mainly to Arid Chaco (Cei 1986, 1993). Although the habitats of these species differ, in central and north-central Argentina their distributions overlap in the ecotones of Arid Chaco and Espinal, and Arid Chaco and Humid Chaco. Hitherto, there is no evidence of hybridization in natural populations of the genus *Tupinambis*. Cei (1993) proposed the existence of a possible hybrid specimen between *T. merianae* and *T. rufescens* on the basis of its pattern scalation. Fitzgerald *et al*. (1999) found a paraphyletic relationship between *T. rufescens* and *T. duseni* and argued incomplete lineage sorting as the most probable explanation for their results, introgression of mtDNA being a less likely possibility since the species are not in sympatry. In the present study, we evaluate the occurrence of hybridization between *T. merianae* and *T. rufescens* in the southernmost contact zone of their distribution, by analyzing nDNA and mtDNA data using a phylogenetic approach. We also explore the directionality and distribution of this phenomenon.

Material and methods

Study area and data collection

The sample area was located in central Argentina (29°–32°S and 61°–65°W; Fig. 1), and covered two biogeographic regions, Arid Chaco and Espinal, and their ecotone zone. Individuals belonging to *T. merianae* and *T. rufescens* were identified phenotypically on the basis of their coloration according to Cei (1993), who established coloration as a valid character to differentiate these species: *T. merianae* is dark olive green, sometimes almost black, and *T. rufescens* is reddish. These individuals were from localities belonging to areas of sympatry and allopatry of those species, as determined in Cardozo *et al*. (2012). We obtained samples (muscle tissue or scales) from individuals hunted by rural people, since commercial exploitation is permitted (Porini 2006), and from road-killed individuals. In both cases, we recorded the coordinates using GPS. All tissue samples were stored in 70% ethanol at –20 °C and were deposited in the tissue collection of the Behavioural Biology Laboratory (IDEA, CONICET-UNC). Scientific capture was authorized by the government environmental agency.

Genomic DNA extraction and sequencing

Genomic DNA was obtained from muscle tissue

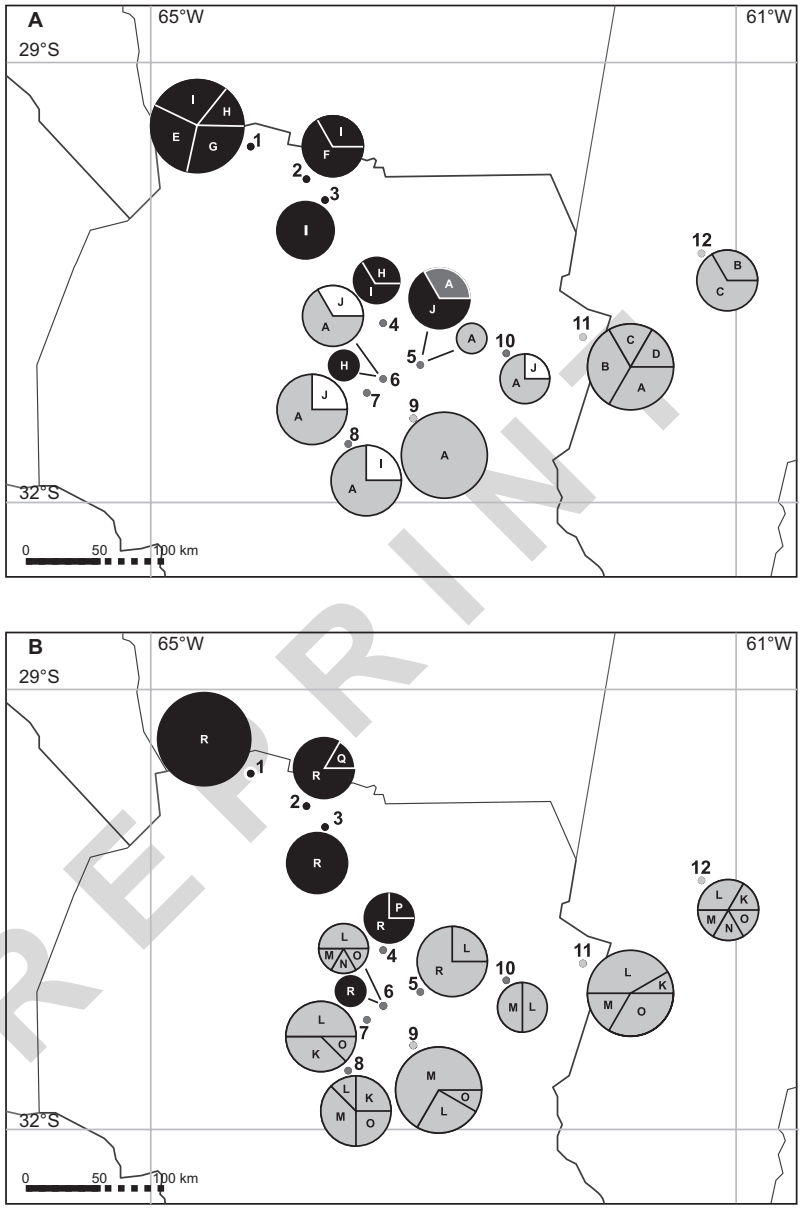

Fig. 1. Spatial distribution of localities, sample size and frequency of (**A**) *ND4* haplotypes and (**B**) *ACA4* alleles. The dots show the localities (black dots: populations of *T. rufescens* in allopatry; light-gray dots: populations of *T. merianae* in allopatry; dark-gray dots: populations in the sympatric area). The circles represent the sample size for each species at each locality (black slices: individuals of *T. rufescens*; light-gray slices: individuals of *T. merianae*; white slices: hybrid individuals classified as *T. merianae* with mtDNA from *T. rufescens*; dark gray slice: hybrid individual classified as *T. rufescens* with mtDNA from *T. merianae*). Letters within the circles indicated haplotypes for *ND4* gene in **A**, and alleles for *ACA4* gene in **B**. For explanations *see* Table 1.

or scales, using a saline extraction method (Bruford *et al*. 1992). We used a fragment of the Nicotinamide Adenine Dinucleotide Dehydrogenase subunit 4 gene (*ND4*) as the mtDNA marker and a fragment of the α-Cardiac-Actin Intron 4 gene (*ACA4*) as the nDNA marker. Both markers showed high polymorphism and have been used in previous studies on Squamata (Pinho *et al*. 2007, Giffor & Larson 2008, respectively). We performed amplification of the *ND4* and *ACA4* genes via the polymerase chain reaction (PCR) using specific primers described by Forstner *et al*. (1995) and Waltari and Edwards (2002), respectively, following the protocol of Martínez *et al*. (2009) with an annealing temperature of 50 °C. All purifications and sequencing reactions were performed by Macrogen Inc. (Seoul, South Korea) in 3´ to 5´ direction. The sequences obtained in the present study were deposited in GenBank (Table 1).

Phylogenetic analysis

Chromatograms were examined using Chromas lite ver. 2.01 (Technelysium Pty Ltd., USA). Sequences were aligned using the Muscle software (Edgar 2004) with the default parameters. In the case of *ND4*, sequences were translated into amino acids to confirm alignment. For the nuclear gene, alleles were determined using the PHASE software (Stephens & Donnelly 2003), which allowed us to estimate the allele that was more likely to occur when a sequence had more than one heterozygous site, by using the probabilistic approach. This method assumes Hardy-Weinberg equilibrium and uses a coalescent-based Bayesian method to infer haplotypes. The file input used in this software was obtained from SeqPHASE (available at http://jfflot.mnhn.fr/seqphase/). The number of iterations, thinning intervals and burn-in values were the default parameters.

We estimated phylogenetic relationships using two approaches, Maximum Parsimony (MP) and Bayesian inference (BI), for mtDNA (*ND4*) and nDNA (*ACA4*), separately. For MP analysis, we used PAUP 4.0b10 (Swofford 1998) software considering equal weighting for all characters. The node support was evaluated with 1000 bootstrap replicates. For BI, we estimated HKY + G for the mitochondrial gene and F81 for the nuclear gene as the most appropriate models of sequence evolution using JModeltest (Posada 2008), under the Akaike information criterion. Bayesian analyses were performed using MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003). For each data set, the analyses were made for two million generations. In both analyses two independent runs were simultaneously performed on the data, each using one cold and three heated chains, with sampling intervals of 1000 generations. We discarded the first 25% of the samples as "burn-in". We determined support for tree nodes according to the values of Bayesian posterior probability obtained from a majority-rule consensus tree. We included sequences of the *ND4* gene for *T. merianae* and *T. rufescens* available from GenBank. Based on previous phylogenies (Fitzgerald *et al*. 1999, Giugliano *et al*. 2007) and sequence availability, we used *T. quadrilineatus* and *T. longilineus* as outgroups for phylogenetic reconstructions with the *ND4* gene and *Ameiva chrysolaema* with the *ACA4* gene.

To get a clear picture of the haplotype and allele frequencies, and the relationships among the co-existing lineages, we constructed networks for each gene. They were obtained with a median-joining approach using the program Network 4.6.1 (Bandelt *et al*. 1999) with default parameters. For *ACA4*, we considered both alleles for each individual.

Results

We analyzed 29 individuals phenotypically classified as *T. merianae* from eight localities and 19 individuals classified as *T. rufescens* from six localities (Table 1 and Fig. 1). An 807-bp fragment of the mtDNA gene and a 413-bp fragment of the nDNA gene were sequenced from each specimen. All the different sequences obtained were deposited in GenBank (accession numbers KF034084–KF034101).

The MP and BI phylogenetic trees using the *ND4* data matrix showed great congruency and similar topology in estimating phylogenetic relationships among the taxa (Fig. 2A). Two well supported clades were obtained, one with most of the *T. merianae* sequences and the other one with most of the *T. rufescens* sequences. Four individuals identified phenotypically as *T. merianae* presented sequences grouped within the clade corresponding to *T. rufescens* and one individual classified as *T. rufescens* presented an *ND4* haplotype grouped within the *T. merianae* clade.

The MP and BI phylogenetic trees using the *ACA4* data matrix also showed similar topology in estimating phylogenetic relationships among the taxa (Fig. 2B). Sequences of *T. merianae* form a polytomy that also includes all specimens classified as *T. merianae* but presenting *T. rufescens* mtDNA. Only one well supported clade grouped sequences of *T. rufescens*. The sequence of the individual identified phenotypically as *T. rufescens* and presenting *T. merianae* mtDNA was grouped within this clade, with high posterior probability in the Bayesian analysis.

In summary, five individuals had nuclear alleles of one species, according to their pheno-

Table 1. Localities, coordinates, specimens captured and GenBank accession numbers for each haplotype for *ND4* gene or allele for *ACA4* gene sequence for the two species of *Tupinambis* (the letters in parentheses next to the numbers are codes used in Figs. 1 and 3). Individuals were classified according to skin coloration (Cei 1993). Individual identification that starts with R stands for *T. rufescens* and with M, for *T. merianae*. Hybrid individuals are denoted with an asterisk (*).

Locality	Latitude	Longitude	Individual	GenBank accession numbers		
				ND4	*ACA4*	*ACA4*
1	–29.573.764	–64.312.264	R490	KF034091 (H)	KF034101 (R)	KF034101 (R)
			R491	KF034092 (I)	KF034101 (R)	KF034101 (R)
			R590	KF034088 (E)	KF034101 (R)	KF034101 (R)
			R601	KF034092 (I)	KF034101 (R)	KF034101 (R)
			R606	KF034090 (G)	KF034101 (R)	KF034101 (R)
			R607	KF034088 (E)	KF034101 (R)	KF034101 (R)
			R610	KF034090 (G)	KF034101 (R)	KF034101 (R)
2	–29.789.739	–63.932.783	R586	KF034092 (I)	KF034101 (R)	KF034101 (R)
			R598	KF034089 (F)	KF034100 (Q)	KF034101 (R)
			R608	KF034089 (F)	KF034101 (R)	KF034101 (R)
3	–29.932.872	–63.798.817	R587	KF034092 (I)	KF034101 (R)	KF034101 (R)
			R734	KF034092 (I)	KF034101 (R)	KF034101 (R)
			R735	KF034092 (I)	KF034101 (R)	KF034101 (R)
4	–30.781.515	–63.412.460	R722	KF034092 (I)	KF034099 (P)	KF034101 (R)
			R723	KF034091 (H)	KF034101 (R)	KF034101 (R)
5	–31.063.261	–63.151.619	R741	KF034093 (J)	KF034101 (R)	KF034101 (R)
			RIC23	KF034093 (J)	KF034101 (R)	KF034101 (R)
			MIC28	KF034084 (A)	KF034095 (L)	KF034095 (L)
			R681*	KF034084 (A)	KF034101 (R)	KF034101 (R)
6	–31.151.253	–63.403.454	M691*	KF034093 (J)	KF034096 (M)	KF034098 (O)
			M694	KF034084 (A)	KF034095 (L)	KF034095 (L)
			M697	KF034084 (A)	KF034095 (L)	KF034097 (N)
			R744	KF034091 (H)	KF034101 (R)	KF034101 (R)
7	–31.250.008	–63.522.806	MIC26	KF034084 (A)	KF034095 (L)	KF034095 (L)
			MIC27	KF034084 (A)	KF034094 (K)	KF034094 (K)
			MIC31*	KF034093 (J)	KF034095 (L)	KF034095 (L)
			MIC38	KF034084 (A)	KF034094 (K)	KF034098 (O)
8	–31.606.478	–63.640.197	MIC6*	KF034092 (I)	KF034096 (M)	KF034096 (M)
			MIC8	KF034084 (A)	KF034098 (O)	KF034098 (O)
			MIC17	KF034084 (A)	KF034094 (K)	KF034096 (M)
			M507	KF034084 (A)	KF034095 (L)	KF034094 (K)
9	–31.426.060	–63.193.195	M54	KF034084 (A)	KF034096 (M)	KF034096 (M)
			M55	KF034084 (A)	KF034096 (M)	KF034096 (M)
			M56	KF034084 (A)	KF034095 (L)	KF034096 (M)
			M57	KF034084 (A)	KF034095 (L)	KF034096 (M)
			M58	KF034084 (A)	KF034095 (L)	KF034098 (O)
			M427	KF034084 (A)	KF034096 (M)	KF034096 (M)
10	–30.991.400	–62.571.333	M341	KF034084 (A)	KF034095 (L)	KF034096 (M)
			M342*	KF034093 (J)	KF034095 (L)	KF034096 (M)
11	–30.866.506	–62.043.315	M303	KF034085 (B)	KF034095 (L)	KF034096 (M)
			M304	KF034087 (D)	KF034095 (L)	KF034098 (O)
			M305	KF034085 (B)	KF034094 (K)	KF034098 (O)
			M306	KF034084 (A)	KF034095 (L)	KF034096 (M)
			M391	KF034086 (C)	KF034095 (L)	KF034098 (O)
			M392	KF034084 (A)	KF034095 (L)	KF034098 (O)
12	–30.306.904	–61.237.816	MIC47	KF034086 (C)	KF034095 (L)	KF034094 (K)
			MIC48	KF034085 (B)	KF034095 (L)	KF034097 (N)
			MIC49	KF034086 (C)	KF034096 (M)	KF034098 (O)

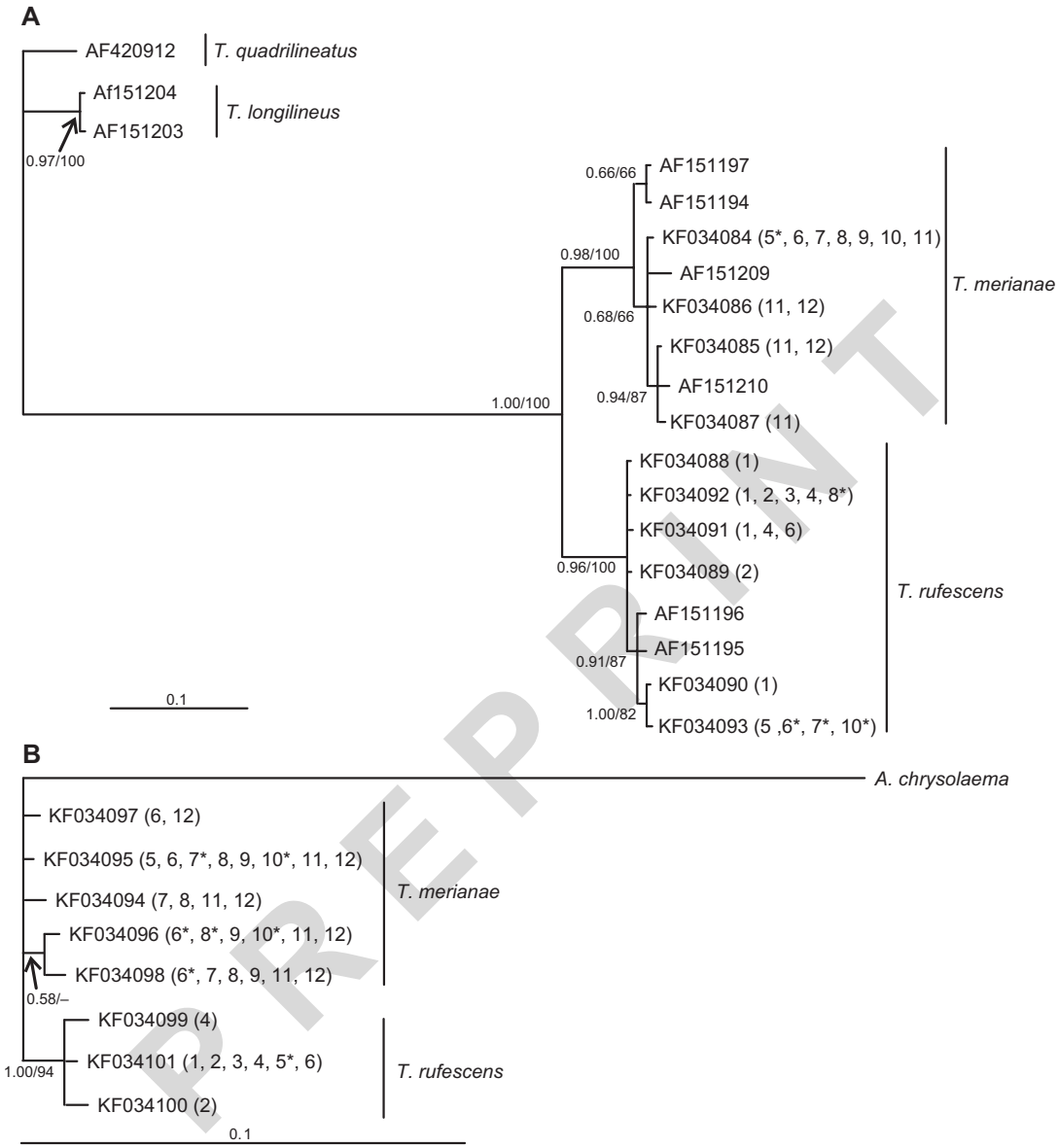

Fig. 2. Consensus tree obtained from Bayesian inference with the matrix of (**A**) mtDNA (*ND4*) and (**B**) nDNA (*ACA4*). Node support has the following order: Bayesian posterior probability/MP after 1000 bootstrap replicates. The localities (numbers as in Table 1) where each haplotype or allele was found are given in parentheses; the localities where hybrid individuals were found are indicated with an asterisk (*).

typic classification, but showed mitochondrial sequences corresponding to the other species, i.e., they presented introgressed haplotypes. All these specimens were located in the areas of sympatry of both species (localities 5, 6, 7, 8 and 10) (Table 1 and Fig. 1A).

Our results showed four haplotypes for the *ND4* gene and five alleles for the *ACA4* gene in *T. merianae*, whereas in *T. rufescens*, six haplotypes were identified for the mtDNA gene and three alleles for the nDNA gene (Fig. 2). Introgressed haplotypes were not considered when counting the haplotypes of each species.

The haplotype networks obtained showed two well-separated groups (Fig. 3), one belonging to *T. merianae* and the other to *T. rufescens*.

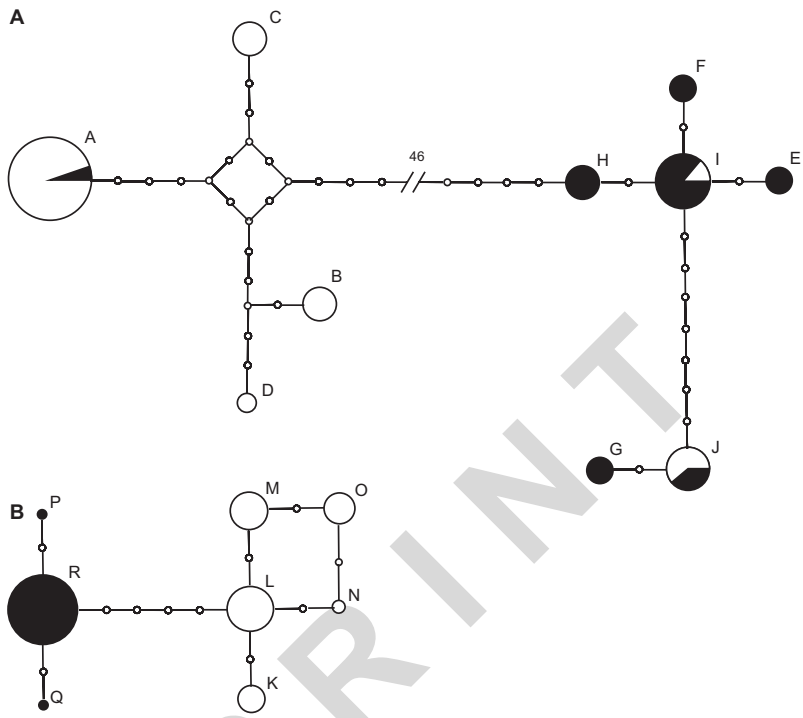

Fig. 3. Networks based on the matrix of (**A**) mtDNA (*ND4*) and (**B**) nDNA (*ACA4*). White circles and slices represent specimens identified as *T. merianae*; black circles and slices represent specimens identified as *T. rufescens*. Each haplotype or allele is indicated by a circle and coded with the same letter as in Table 1. The size of each circle represents the frequency of the denoted haplotype or allele. The dots in the branches that connect the circles represent numbers of mutations separating the different haplotypes and alleles.

The five individuals with introgressed haplotypes presented in both networks the most frequent variants (Table 1 and Fig. 3). None of the specimens presented nDNA alleles belonging to both parental species, indicating that F_1 hybrids were not found in this study.

Discussion

In this study, we present the first evidence of natural hybridization between species of *Tupinambis*: some individuals were grouped in different clades, depending on whether the phylogenetic trees and/or haplotype network were built based on the mitochondrial or nuclear dataset. The inconsistency in phylogenetic estimates from different sources of evidence (mtDNA and nDNA) has been interpreted in many taxa as the result of hybridization (Seehausen 2004). Another possible explanation could be an incomplete lineage sorting, which means that some individuals of these two species present identical or very similar haplotypes due to common ancestry and the short time elapsed since the separation between them. However, the identified hybrids occur only in the sympatry zone and are not randomly distributed across the entire study area, as expected if they were the result of common ancestry. Therefore, current gene flow between *T. merianae* and *T. rufescens* in the sympatric zone is the most likely explanation.

As mentioned above, Cei (1993) suggested the existence of a hybrid individual between the two studied species based on its scalation pattern. Fitzgerald *et al*. (1999) also proposed the possibility of introgression of mitochondrial DNA between *T. rufescens* and *T duseni* to explain the incongruence between molecular and morphological data. However, in their study the specimens of *T. duseni* that presented mtDNA of *T. rufescens* did not occur in sympatry with the latter species; for this reason, the authors considered introgression as a less likely explanation for their results. In our study, the use of both mtDNA and nDNA markers analyzing specimens of *T. merianae* and *T. rufecens* in areas of sympatry and allopatry provides the first evidence of the existence of hybridization in the genus.

The presence of the mitochondrial gene sequences of *T. rufescens* in four *T. merianae* individuals suggests that females of the former

species mate with males of the latter. On the other hand, the fact that a specimen of *T. rufescens* presented a *T. merianae* mitochondrial haplotype indicates that hybridization can also occur in the opposite direction; thus, the process would be reciprocal.

Hybrid individuals detected originated from backcrossing (no F_1 hybrids were found), demonstrating the occurrence of introgression between *T. merianae* and *T. rufecens*. Furthermore, mitochondrial haplotypes found in the hybrid specimens were different, suggesting multiple hybridization events. Bolnick and Near (2005), in a study of fishes of the Centrachidae family established that natural hybridization is common even among taxa that have been separated for up to 14 mya. Péres (2003) estimated that the divergence between *T. merianae* and *T. rufescens* occurred during the late Miocene (10 mya). According to our results, this amount of time would not have been enough to reach a degree of reproductive isolation that prevents backcrossing between these species.

The hybrid zone corresponding to the area of sympatry between *T. merianae* and *T. rufescens* is the ecotone between Arid Chaco and Espinal (Cardozo *et al*. 2012). However, due to the low number of hybrids detected we cannot establish other characteristics of this zone, such as size, or if it corresponds to a continuous region across the ecotone or to a mosaic of patches.

The finding of natural hybridization in populations of *Tupinambis* spp. raises many questions about how reproductive strategies, patterns of dispersal and the ecological characteristics of hybrids shape the hybrid zone influencing the evolution of the lineages. New studies using highly variable molecular markers, microsatellites, might answer these questions.

Acknowledgments

We are grateful to the local people for their invaluable assistance in data collection. We also thank the members of Laboratorio de Biología del Comportamiento, Sergio Naretto, Cecilia Blengini, Gabriela Cardozo and Valeria Di Cola, for field assistance and comments that helped to improve this manuscript. We also thank two anonymous reviewers for their careful reading of the first draft of the manuscript and their constructive comments and suggestions. Our work was funded by Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Fondo para la Investigación Científica y Tecnológica (FONCyT), MinCyT Córdoba-Préstamo BID-PID no. 013/2009, Secretaría de Ciencia y Tecnología (SeCyT) and Universidad Nacional de Córdoba, Argentina.

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