

# Gene flow between an endangered endemic iguana, and its wide spread relative, on the island of Utila, Honduras: when is hybridization a threat?

## Does hybridization threaten an endangered iguana?

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**Abstract** The island endemic *Ctenosaura bakeri* was listed as critically endangered by the IUCN Redlist Assessment in 2004, 7 years after it was recognized as a distinct species. *C. bakeri* occupies a portion of Utila, a small continental island located off the northern coast of Honduras. Habitat destruction and over-harvesting are among the top threats facing this species. In addition, morphological evidence of hybridization was recently documented, raising the concern that gene flow from the common and widely distributed *C. similis* could threaten the genetic distinctiveness of *C. bakeri*. We show that hybridization occurs only at low levels and is not a current threat to *C. bakeri*. All ctenosaurs captured for this study were identified to species level without difficulty; none had intermediate or mosaic phenotypes. Sequence analysis of mitochondrial and nuclear markers revealed only two individuals with introgressed genotypes. Molecular analysis of the previously described hybrid showed it to be heterozygous for *C. bakeri* and *C. similis* alleles. Hybridization between these two species is possible and occurs occasionally in the wild, and the rate of hybridization could increase if habitat destruction or changes in relative abundance increase the probability of interbreeding. However, the level of gene flow indicated by current data is too low to

threaten *C. bakeri* with genetic swamping or deleterious fitness effects.

**Keywords** *Ctenosaura bakeri* · *Ctenosaura similis* · Iguanas · Hybridization · Gene flow

### Introduction

Natural hybridization is not intrinsically undesirable and may even enhance mean population fitness and responsiveness to natural selection (Arnold 2006; Fitzpatrick and Shaffer 2007a). However, interspecific hybridization in nature presents several challenges for conservation management when it occurs because of anthropogenic factors or when one of the species involved is protected (Rhymer and Simberloff 1996; Allendorf et al. 2001). Hybridization can be a direct biological threat if it reduces population viability owing to the introduction of alleles that are maladaptive in the local environment or genetic background (Reisenbichler and Rubin 1999; Allendorf and Luikart 2007). Gene flow between protected and non-protected taxa also creates difficulty in defining and distinguishing protected from unprotected individuals or populations thus complicating management decisions (O'Brien and Mayr 1991; Allendorf et al. 2001; Haig et al. 2004; Fitzpatrick and Shaffer 2007b). Thorough genetic analysis of wild populations is therefore necessary to guide conservation decisions. In this paper, we investigate the potential for hybridization and gene flow between the critically endangered island endemic, *Ctenosaura bakeri*, and its widespread relative, *Ctenosaura similis*.

The Utila Spiny-Tailed Iguana, *C. bakeri* (Stejneger 1901), along with three other species (*C. palearis*, *C. oedirhina*, and *C. melanosterna*), makes up a recent

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radiation of narrow range insular and mainland endemics, occurring in Honduras and Guatemala. *Ctenosaura palearis* was the first species of this group to be described (Stejneger 1899), and *C. bakeri* was later distinguished from this species based on scutellation of the tail, dewlap size, and dorsal crest coloration (Bailey 1928; de Queiroz 1987, 1990). In addition, more recent morphological and molecular work based on RAPDs (Köhler et al. 2000) and sequence data (SAP, unpublished data) supports this delimitation.

*Ctenosaura bakeri* is endemic to the small island (41 km<sup>2</sup>) of Utila, the most westerly of the Bay Islands (86°56' N; 16°06' W), which are located 15–50 km off the Caribbean versant of Honduras (McCranie et al. 2005). *C. similis* occupies lowland mainland habitats from the Isthmus of Tehuantepec in Mexico to Panama; and specifically within the Bay Islands is found on both Utila and Guanaja, the most easterly of the Bay Islands (McCranie et al. 2005). Preliminary data concerning the molecular phylogeography of *C. similis* suggest that the population occurring on Utila is not distinct from that on the adjacent mainland (SAP, unpublished data). In recent years the Bay Islands and Utila in particular have experienced extensive anthropogenic development, which threatens the native flora and fauna through habitat destruction, introduction of non-native species, and pollution. Additionally, over-harvesting of iguanas persists owing to the lack of environmental protection and enforcement.

In 1994 the Honduran government designated *C. bakeri* as ‘in need of protection’ and in 2004 *C. bakeri* was listed as critically endangered by the IUCN Red List Assessment (Zoerner and Köhler 2004). The latter listing was due primarily to its limited geographic range, increased habitat modification and destruction, and over-harvesting of adults and eggs. *Ctenosaura bakeri* is a charismatic and unique species that has been the subject of substantial conservation effort, including a captive breeding program, education, and community outreach. A recent report of hybridization between *C. bakeri* and its wide ranging congener, *C. similis* (Köhler and Blinn 2000), raises the concern that these conservation efforts will be in vain if *C. bakeri* is displaced via genetic swamping or suffers reduced fitness due to hybrid dysfunction or dilution of important adaptations.

Hybridization between *C. bakeri* and *C. similis* was first reported in 2000 when a freshly killed gravid female (Natur-Museum und Forschung-Institut Senckenberg [SMF] 78870) was brought to the Iguana Research and Breeding Station (IRBS) on Utila and identified as a hybrid on the basis of morphology (Köhler and Blinn 2000). Eggs rescued from this female were artificially incubated, resulting in two viable hatchlings. Coloration and scale characteristics of these hatchlings were intermediate between *C. bakeri* and *C. similis*. One of these hatchlings

was crossed with *C. bakeri* and fertile offspring were produced (pers. comm. Lutz Dirksen). In addition, Aurel Heidelberg (pers. comm.) informs us that ctenosaurs that were thought to be hybrids on the basis of morphological characteristics have been observed since the original documentation by Köhler and Blinn (2000).

Historically, *C. bakeri* has been said to thrive primarily in mangrove habitats where they seek refuge in hollows in the larger trees (Gutsche 2005). Zoerner and Köhler (2004) reported that these forests encompass only 8 km<sup>2</sup> of the island. Because of the vast destruction of the mangrove habitat, *C. bakeri* has been displaced and is now often found in dry open areas (Pasachnik 2006). The wide ranging species, *C. similis*, prefers dry open areas (Lee 1996; Savage 2002; Campbell 1998; Köhler et al. 2006), though there are some records of *C. similis* in other habitats (Stafford and Meyer 2000) throughout its range. It is possible that the destruction of *C. bakeri* habitat and expansion of *C. similis* habitat have created new opportunities for contact and gene flow. In addition, contact between the two species appears most extensive in two areas where there have been extensive anthropogenic changes. The first is an abandoned residential foundation where both species can be found in holes in the cinder block walls. The second is a bed-and-breakfast situated at the edge of a mangrove stand. The proprietor feeds wild ctenosaurs daily and both species can be observed in large numbers. These situations may provide new opportunities for the production and survival of hybrids (e.g., Blair 1941; Anderson 1948; Anderson and Stebbins 1954; Lewontin and Birch 1966; Seehausen et al. 1997; Arnold 2006).

With the destruction of mangrove habitats and declining numbers of *C. bakeri* on Utila, extensive gene flow from *C. similis* could lead to “genetic swamping” where *C. bakeri* alleles at many genes are replaced by *C. similis* alleles (Rhymer and Simberloff 1996; Allendorf et al. 2001). Introgression of *C. bakeri* alleles could also affect the genetic makeup of *C. similis*, however, the conservation concern is not as great in this case as this species has a wide distribution throughout Mexico and Central America. The elimination of unique characters of *C. bakeri*, potentially including adaptations to mangrove habitats, could be detrimental to the preservation of this endangered species. However, the effects of a low level of gene flow [e.g., fewer than one hybridization event per generation (Wright 1931)] would be negligible, except for universally advantageous alleles (Barton 1979).

## Methods

Several strategies for evaluating the possibility of hybridization have been used in different contexts (Arnold 2006).

We used the joint distribution of field identification characters to assess whether the *Ctenosaura* on Utila exist as two distinct phenotypic clusters, as a mixed array of individuals with diverse character combinations, or as clusters with a minority of intermediate or mosaic morphologies. We then compared mitochondrial and nuclear haplotype distributions between morphologically identified *C. bakeri* and *C. similis* to determine whether or not those two groups could be interpreted as distinct, isolated gene pools. Finally, DNA sequences from the putative natural hybrid housed at IRBF were examined to test whether she had a mixture of *C. bakeri* and *C. similis* alleles.

#### Field identification

A total of 599 ctenosaurs were opportunistically captured on Utila, Islas de la Bahia, Honduras during the summer of 2005, the spring and summer of 2006, and the summer of 2007. Individuals were collected from the entirety of the island with the exception of the interior on the western side of the canal, as it consists of unsuitable swamp habitat. Upon capture a digital photograph was taken and snout-vent length, tail length and weight were recorded. All individuals were morphologically evaluated for three characters that are typically used to distinguish between *C. bakeri* and *C. similis* (pers. obs. A.C.E. and S.A.P., McCranie et al. 2005, Köhler 2003): (1) Color: *C. bakeri* is blue to light gray to black, *C. similis* is a light brownish gray (green on juveniles); (2) Pattern: *C. bakeri* may have indistinct crossbands present on the dorsum, *C. similis* has distinct crossbands with a pale center along the dorsomedial line; and (3) Scalation: *C. bakeri* has one row of intercalary scales between the third and fifth tail whorls, *C. similis* has two intercalary scale rows. A unique mark was given to each individual for future mark-recapture population estimates and to avoid re-sampling from year to year. A one cm section of the tail tip was removed and stored in 100% ETOH for molecular analysis. Pressure was applied to the wound until bleeding ceased and the area was sealed with a topical skin adhesive to prevent infection.

#### DNA sequencing

Sequence data were collected from a subset of specimens ranging throughout the entire island of Utila (Fig. 1) and from the previously documented hybrid (progeny of SMF 78870). We sequenced 64 *C. bakeri* and 25 *C. similis* for the mitochondrial marker ND4, 61 *C. bakeri* and 25 *C. similis* for the nuclear marker LDHA, and 24 *C. bakeri* and 11 *C. similis* for the nuclear gene PACs. Both of the nuclear markers are in non-coding 3' UTRs. All sequences have been registered on Genbank (EU268007-EU268017, EU271874-EU271881).

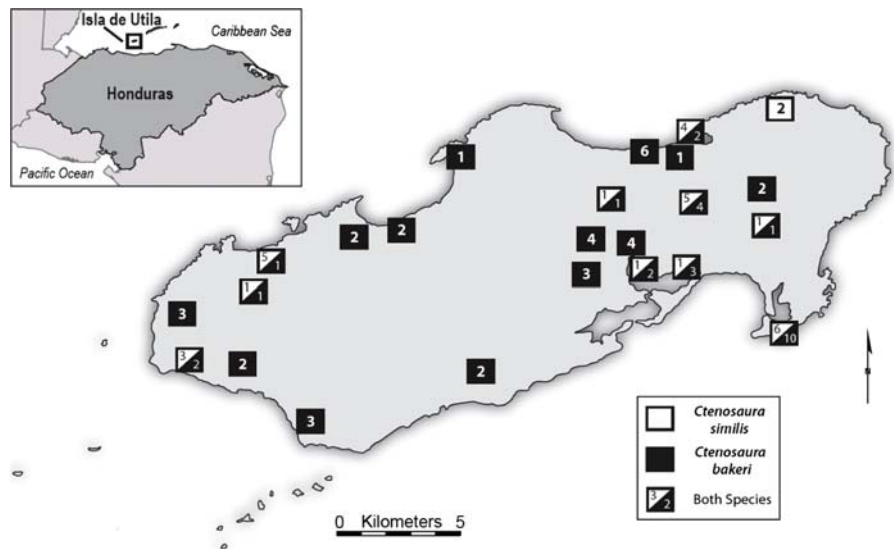
Sample processing, sequencing and sequence analysis was performed at the University of Tennessee with the exception of some samples which were sent to Yale University for sequencing. Genomic DNA was extracted using the Qiagen DNeasy extraction kit (Qiagen, Valencia, CA). The manufacturer's protocol was modified only by extending the cell lysis step (using Proteinase K) to at least 15 h. Blood samples from *Amblyrhynchus cristatus* were obtained from Scott Glaberman and Adalgisa Caccone at Yale University for use as an additional outgroup. Outgroup choice was based on phylogenetic proximity (Norell and Queiroz 1991; Sites et al. 1996; Rassmann 1997; Wiens and Hollingsworth 2000; Hollingsworth 2004). DNA extraction procedures for blood follow that of the Qiagen DNeasy manufacturer's protocol.

The polymerase chain reaction (PCR) was used to amplify one portion of the mitochondrial genome (674 bp of NADH dehydrogenase subunit 4) and two nuclear loci: 418 bp of Lactate dehydrogenase A (LDHA) and 569 bp of polymerase alpha catalytic subunit (PACs). The ND4 fragment was amplified using primers ND4 (Sites et al. 1996) and ND4R1 (5'-CGAAACACCTCTCGGTTTGC-3') developed specifically for ctenosaurs from data in Sites et al. (1996). The LDHA fragment was amplified using primers LDHA1 (5'-AGCCTGGTCTCCTGTCAT-3') and LDHA2 (5'-GCAGCAGTGTTGGGAAAAAT-3') developed from *I. iguana* sequence data (Hsu and Li unpublished data; AY130249). The PACs fragment was amplified using primers PACs1 (5'-AGACTTTGCTCCGGGGTATT-3') and PACs2 (5'-CTTTCCCCTCCCAAACAAAC-3'), developed from sequence data for *I. iguana* (Iwabe et al. 2005; AB178527). Amplifications for all fragments were conducted in a total volume of 25  $\mu$ l using: 2.5  $\mu$ l MgCl<sub>2</sub>, 2.5  $\mu$ l 10 $\times$  buffer, 2.0  $\mu$ l dNTPs, 1.25  $\mu$ l forward primer, 1.25  $\mu$ l reverse primer, 0.25  $\mu$ l *Taq* polymerase, 12.25  $\mu$ l ddH<sub>2</sub>O, and 3.0  $\mu$ l DNA template.

PCR cycling for the mitochondrial fragment was performed using the following conditions: denaturation at 94°C for 3 min., followed by 30 cycles at 94°C for 30 s, 50°C for 30 s, 72°C for 90 s, and final extension at 72°C for 5 min. PCR cycling for the two nuclear fragments was performed using the following conditions: denaturation at 94°C for 3 min, followed by 30 cycles at 94°C for 30 s, 60°C for 30 s, 72°C for 90 s, and final extension at 72°C for 5 min. PCR products were verified by gel electrophoresis and successful amplicons were purified using either exonuclease I/shrimp alkaline phosphatase (ExoSap) or a QIAquick PCR purification kit (Qiagen, Valencia, CA).

Sequencing reactions were performed using original PCR primers. Forward and reverse sequences for each template were aligned using Sequencher 4.6 (Gene Code Corporation). Ambiguous base calls were verified manually by examining the electropherograms for the forward

**Fig. 1** Markers indicate sampling sites for *C. bakeri* (black) and *C. similis* (white) on Utila, Honduras. Numbers indicate how many individuals of each species were used in the molecular analysis from each area



and reverse reads. Sequence alignment was verified using MacClade 4.07 (Maddison and Maddison 2005) with the aid of published sequences from GenBank for Iguaninae. A few LDHA and PACs sequences had heterozygous sites. Levels of heterozygosity, however, were relatively low and haplotypes could be determined unambiguously. LDHA had one species-specific indels. *Ctenosaura bakeri* consistently had five fewer bp than *C. similis*.

#### Sequence analysis

The program DNAsp 4.10.9 (Rozas et al. 2003) was used to collapse redundant haplotypes. Haplotype trees were constructed using maximum likelihood (ML) and Bayesian analyses with each locus analyzed separately. ML analysis was conducted using Paup\* 4.0 (Swofford 2002) using a heuristic search with 1,000 random-taxon-addition replicates. Confidence at each node was assessed using nonparametric bootstrapping (Felsenstein 1985) based on 100 pseudo-replicates with 10 random-taxon-addition replicates per pseudo-replicate. The optimal model of sequence evolution for each locus was determined in Modeltest 3.7 (Posada and Crandall 1998). Bayesian posterior probabilities were estimated using MrBayes 3.1 (Ronquist and Huelsenback 2003) with four Markov chains, the temperature profile at the default setting of 0.2, run for 2 million generations and sampling every 100th generation with a final burnin of 10,000 generations.

We compared haplotype distributions between morphologically described groups and used the estimated haplotype trees to examine the genealogical concordance of the three markers. To provide a numerical evaluation of the potential for gene flow, we used allele frequencies to estimate  $F_{ST}$  between *C. bakeri* and *C. similis* following Weir (1996). While other analyses could be implemented

in cases of extensive hybridization, the simplicity of our results (see below) makes additional analyses unnecessary to address the question of whether hybridization with *C. similis* is likely to have significant effects on the *C. bakeri* gene pool.

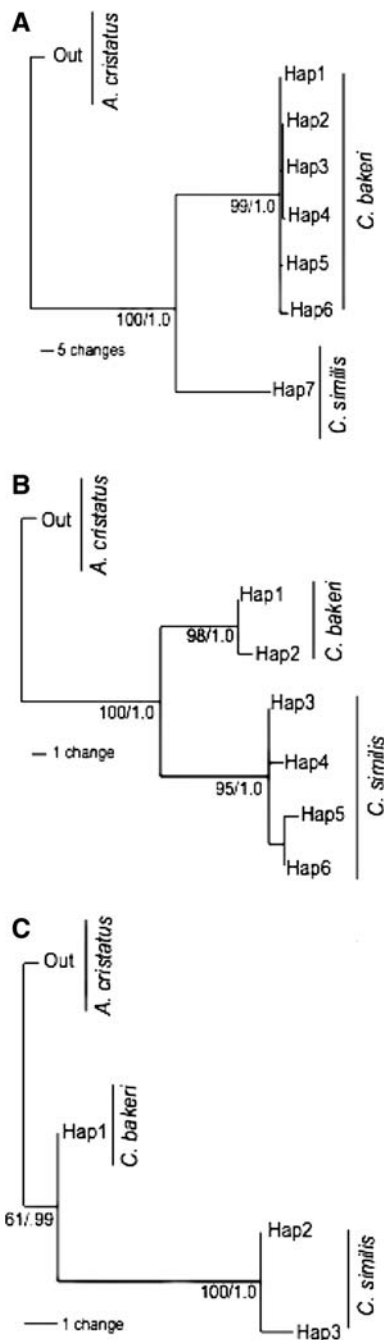
## Results

#### Field identification

Visual identification of morphological characters did not reveal intermediate states between *C. bakeri* and *C. similis* for any of the 599 ctenosaurs captured within the time frame of this study. A total of 496 *C. bakeri* and 103 *C. similis* from the island of Utila were visually consistent in coloration, pattern, and intercalary tail whorls with the character states described and seen in photographs in standard field guides (Köhler 2003; McCranie et al. 2005). Intermediate or mosaic character combinations were not observed. Additionally, coloration, pattern, and intercalary tail whorls observed in *C. similis* from Utila were consistent with those observed in *C. similis* on the adjacent mainland of Honduras (SAP, pers. obs.).

#### Sequence analysis

ND4 sequence data obtained for 89 opportunistically collected ctenosaurs revealed seven unique haplotypes. Haplotypes 1–6 were found primarily within *C. bakeri*; these haplotypes are very similar to one another and form a strongly supported group in the haplotype tree (Fig. 2a). Haplotype 7 was found primarily within *C. similis* and is very distinct from all other haplotypes (Fig. 2a). Only one *C. similis* (of 25) did not have haplotype 7; this individual



**Fig. 2** Consensus phylograms of *C. bakeri* and *C. similis* haplotypes, from Utila, Honduras. (a) ND4, (b) LDHA, (c) PACs. Numbers before nodes indicate bootstrap support from Maximum Likelihood analysis and posterior probabilities from Bayesian analyses. *Amblyrhynchus cristatus* was used as the outgroup

had ND4 haplotype 1, one of the two most common *C. bakeri* haplotypes (Table 1). Likewise, only one *C. bakeri* (of 64) did have haplotype 7 (Table 1). Thus, the morphologically identified *C. bakeri* and *C. similis* corresponded to two genealogically distinct groups of ND4 haplotypes with the exception of two out of 89 individuals.

**Table 1** Distribution of ND4, LDHA and PACs *C. bakeri* and *C. similis* haplotypes from Utila, Honduras

	ND4 haplotypes							LDHA haplotypes						PACs haplotypes		
	1	2	3	4	5	6	7	1	2	3	4	5	6	1	2	3
<i>C. bakeri</i>	27	28	1	2	1	4	1	120	2	0	0	0	0	48	0	0
<i>C. similis</i>	1	0	0	0	0	0	24	0	0	17	17	12	4	0	18	4

Haplotype numbers correspond to Fig. 2

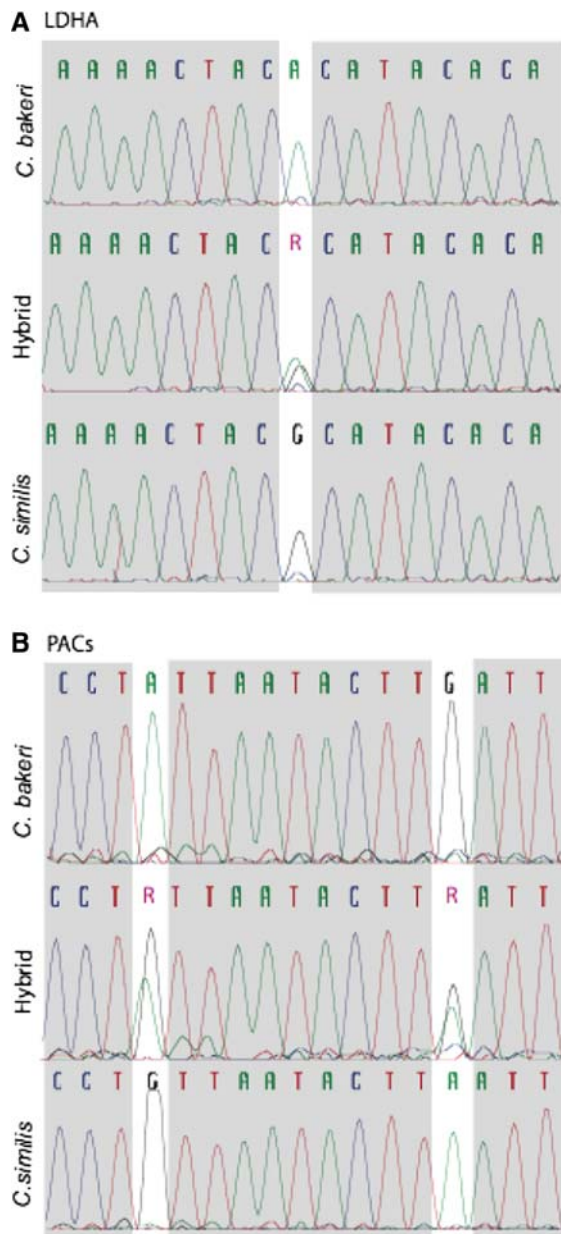
This may indicate initial misidentification or past hybridization events.

The LDHA sequence data obtained from 86 opportunistically collected ctenosaurs revealed two haplotypes in *C. bakeri* and four in *C. similis* (Fig. 2b). Twenty-five morphologically identified *C. similis* and 61 *C. bakeri* were sequenced. Molecular data were consistent with morphological data for all individuals including those that showed discordance in the ND4 analysis (Table 1). This supports the hypothesis of past introgression and not initial misidentification.

A subset of individuals (11 *C. similis* and 24 *C. bakeri*) was sequenced for an additional nuclear marker PACs. Molecular and morphological data were consistent for all individuals (Table 1). Phylogenetic analysis shows one haplotype in *C. bakeri* and two in *C. similis* for this marker (Fig. 2c). The smaller PACs data set reinforces the agreement between morphological and molecular data (Table 1).

Reciprocal monophyly of the gene trees (with the two exceptions noted) indicates that most of the DNA variation within species is caused by mutation rather than gene flow between species. Because  $F_{ST}$  is decreased by both mutation and gene flow (Weir 1996), the best way to estimate gene flow ( $Nm$  the average number of “immigrants” or number of individuals contributing to the other species’ gene pool per generation) is to first factor out the variation caused by mutation. In this case, it was simple to categorize haplotypes as “*similis* alleles” or “*bakeri* alleles” and estimate  $F_{ST}$  using a two-allele model for each marker. This yields estimates of  $F_{ST} = 0.9522$  for mtDNA, and  $F_{ST} = 1.0$  for each nuclear marker for an overall average of 0.984. The corresponding estimate of  $Nm = 0.004$ , thus, an average of approximately 4 hybrid matings every thousand generations is estimated.

Sequence data for two nuclear markers, LDHA and PACs, and one mitochondrial marker, ND4, were collected for the offspring of the putative hybrid, SMF 78870. The LDHA and PACs nuclear sequence data show that all sites distinguishing *C. bakeri* and *C. similis* were heterozygous (Fig. 3), indicating that this individual is indeed a hybrid between *C. bakeri* and *C. similis*. The mitochondrial sequence data shows this individual’s maternal lineage to be *C. bakeri*.



**Fig. 3** Examples of heterozygous sites in (a) LDHA and (b) PACs nuclear genes for *C. bakeri*, *C. similis*, and the putative hybrid described by Köhler and Blinn (2000) from Isla Utila, Honduras

## Discussion

Our analysis of distinguishing morphological characters and DNA sequence data show that *C. bakeri* and *C. similis* are morphologically and genealogically distinct and nearly completely reproductively isolated. Rare instances of genealogical discordance and marker heterozygosity of a putative natural hybrid indicate that fertile hybrids are occasionally produced in the wild. The level of gene flow consistent with these observations is far too low to present a threat to the distinctiveness of *C. bakeri*. However, the

possibility that hybridization may increase owing to future destruction of habitat justifies continued genetic monitoring of ctenosaurs on Utila.

Hybridization has been regarded as a complicating factor and even a threat in conservation contexts (O'Brien and Mayr 1991; Rhymer and Simberloff 1996; Allendorf et al. 2001; Allendorf and Luikart 2007); however, it also may play an important role in evolutionary processes in both plants and animals (Arnold 2006). To present a credible threat, hybridization must in some way reduce the viability of a population or result in the replacement of a protected taxon by a less desirable admixture. Both effects are dependent upon on a high degree of gene flow. Genetic swamping by foreign alleles that have no effect on fitness is unlikely when the number of immigrants per generation ( $Nm$ ) is less than one (Wright 1931). Alleles causing local maladaptation or hybrid dysfunction would affect *C. bakeri* only if gene flow is also large relative to the strength of selection (e.g., Lenormand 2002). Thus, even if our estimate of gene flow ( $Nm = 0.004$ ) is off by a factor of 100, hybridization with *C. similis* is unlikely to have a substantial effect on *C. bakeri*. This conclusion is supported by the absence (in a sample of 599) of individuals with intermediate or mosaic morphological characteristics.

From the molecular evaluation of the putative hybrid described by Köhler and Blinn (2000) and the fact that she produced viable offspring in captivity, we know that fertile hybrids can be produced when *C. bakeri* and *C. similis* mate. One may thus speculate that the frequency of hybrids would increase if the frequency of interspecies matings were to increase or if ecological conditions favoring hybrid genotypes were to become widespread. Habitat disturbance can have the effect of breaking down barriers to interbreeding and/or generating an array of potential “hybrid niches” (Anderson 1948). While the two wild-caught individuals with introgressed genotypes were sampled from disturbed habitats (described in the introduction), we have no statistical power to support a proposition that hybrids are more likely to be encountered in disturbed areas than in pristine areas.

*Ctenosaura bakeri* is listed as critically endangered by the IUCN and as in need of protection by the Honduran government because this species requires two rapidly disappearing habitats on Utila. Adults depend on mature red, black and white mangrove forest habitats for food and retreat sites, and relatively undisturbed sandy beaches for nesting (Zoerner and Köhler 2004). As this optimal habitat is destroyed, ecological interactions with *C. similis* may contribute to the decline of *C. bakeri* if competitive interactions prevent the latter from using a broader range of habitats. Although concerns over a genetic threat from hybridization are not supported by our research, increased habitat destruction could increase the potential for

hybridization. Thus, it seems clear that conservation efforts should focus on preservation and restoration of mangrove stands and nesting beaches in order to combat both direct and indirect effects of this destruction and to improve the chances of persistence for *C. bakeri*.

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