

# Evolutionary history of the critically endangered Cozumel dwarf carnivores inferred from mitochondrial DNA analyses

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## Keywords

*Procyon*; *Nasua*; conservation genetics; Cozumel Island; management unit; procyonids; coati; raccoon.

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## Abstract

The pygmy raccoon *Procyon pygmaeus* and dwarf coati *Nasua nelsoni*, both endemic to Cozumel Island, Mexico, are two of the most endangered carnivores in the world, and their persistence requires active management. However, the taxonomic status of these populations remains unclear. Therefore we investigated mitochondrial DNA variation using the control region to examine the genetic uniqueness and evolutionary history of these taxa. Using strict phylogenetic criteria, species-level uniqueness of the Cozumel taxa was difficult to discern based solely on haplotype groupings and identification of unique alleles. However, population genetic approaches indicate significant population differentiation between Cozumel and mainland populations and we suggest that these taxa should be treated as distinct management units from their mainland conspecifics. Coalescent analysis indicated that the pygmy raccoon diverged from the mainland about 3050–200 111 years before present (ybp) and the Cozumel coati 1263–82 896 ybp; dates that can be further constrained by incorporating known-age subfossil specimens from Cozumel as well as the geological history of the island. Thus, although it is likely that the island taxa colonized Cozumel before the Mayan peoples populated the island, we are unable to definitively reject the hypothesis that colonization by these taxa was not human facilitated.

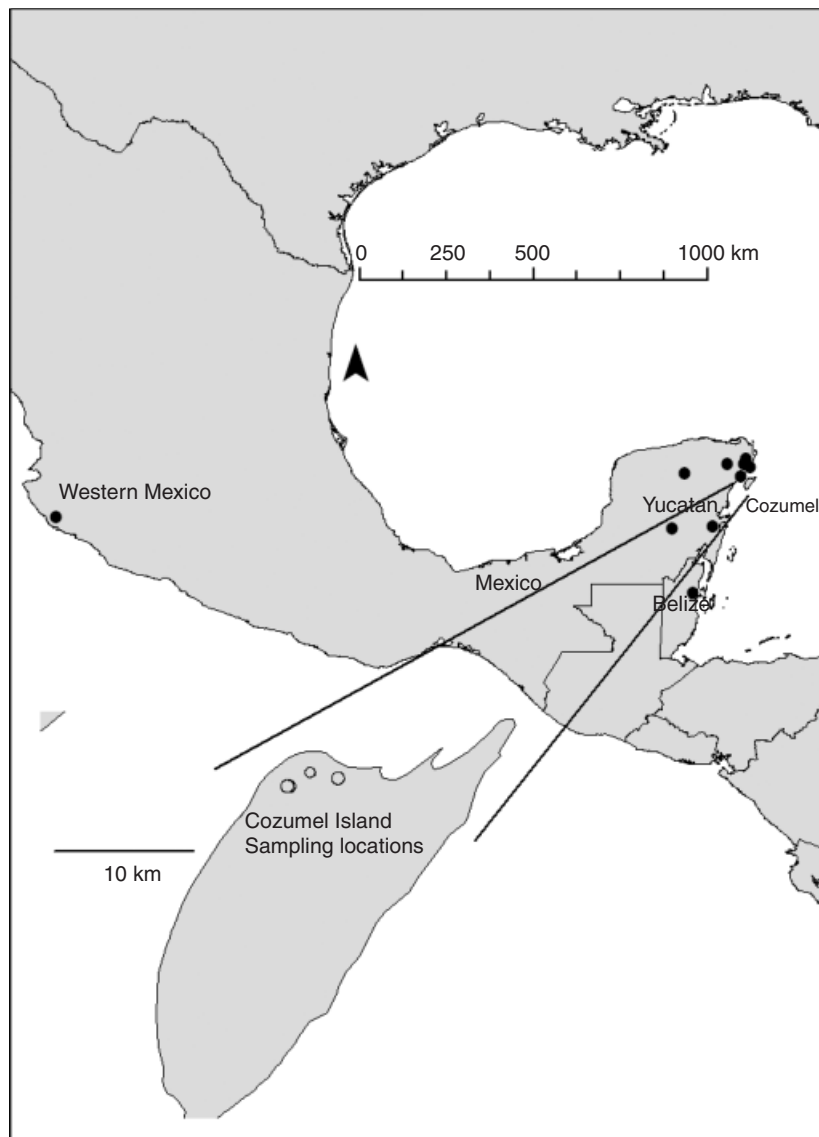
## Introduction

Island carnivores are known to have high risks of extinction which is often further compounded by additional threats such as increasing island habitat degradation and predation by introduced species (Burkey, 1995; Roemer *et al.*, 2001; Cardillo *et al.*, 2004). Here, we use a population genetic approach to understand the history and demography of two critically endangered island carnivores, the endemic dwarf coati *Nasua nelsoni* and the pygmy raccoon *Procyon pygmaeus* of Cozumel Island, Mexico. Based on the excavation of known-age bones on Cozumel Island, both taxa are known to have existed on the island for at least several thousand years (Hamblin, 1984). Currently, however, only several hundred *P. pygmaeus* and far fewer *N. nelsoni* are known to persist, making these taxa among the world's most threatened Carnivora; both are recognized as endangered by the World Conservation Union (IUCN) and Mexico (SEMARNAT, 2002; Cuarón *et al.*, 2004; McFadden, 2004).

Insular and mainland populations often differ morphologically (Lawlor, 1982). For the Cozumel procyonids, smaller body size and lighter coat color, have resulted in species-level

designations (Merriam, 1901; Thomas, 1901). More recent examinations of osteology and external morphology suggest a *c.* 20–40% stature reduction relative to mainland sibling taxa (*Nasua narica* and *Procyon lotor*) (Decker, 1991; Cuarón *et al.*, 2004; McFadden, 2004; Helgen & Wilson, 2005). Despite the morphologic differences, however, the taxonomic status of these populations remains unclear. Some have suggested that the taxa may not deserve species-level designation based solely on morphology and that Mayan peoples may have introduced these taxa when humans colonized the island *c.* 2500 years before present (ybp) (Decker, 1991; Glatston, 1994; Zeweloff, 2003). If true, a conservation emphasis that treats these taxa as distinct species must be reevaluated, with close attention to data on morphological, ecological and evolutionary distinctiveness. We therefore examined the genetic uniqueness and evolutionary history of these taxa.

Cozumel Island (20°16'20"26'N and 86°44'87"20'W) is a 486 km<sup>2</sup> oceanic island separated from the mainland by a 18 km wide and 914-m-deep channel (Fig. 1). The island was formed *c.* 200 000 years ago, but was completely submerged with increased sea levels until *c.* 121 000 ± 6000 ybp (Spaw, 1978; Ward, 1985). Earliest evidence of procyonids on



**Figure 1** Map of Mexico and Central America showing Belize, mainland Mexico, and Cozumel Island, Mexico sampling localities.

Cozumel are carnivore bones dated to *c.* 2500 ybp which were found from Mayan feeding middens (Hamblin, 1984). Thus, procyonids must have colonized Cozumel 2500–121 000 ybp.

Using sequence divergence data to definitively discern exact time of origin of the Cozumel taxa is problematic because of the high probability that these intraspecific phylogenies will not have had time to sort into distinct lineages (Avice, 1994); that is, mainland–island variance is unlikely to be greatly divergent from cross-population differences on the mainland. Traditional models of population subdivision assume that populations have reached equilibrium between migration and drift; however, a species undergoing lineage sorting is not in equilibrium and thus models that do not assume equilibrium are necessary (Nielsen & Wakely, 2001). Coalescent theory has experienced significant development in the last decade (Kingman, 1982; Griffiths & Tavaré, 1997; Fu & Li, 1999) and during this

period, Nielsen & Wakely's (2001) coalescent model emerged. Coalescent models estimate divergence times for populations not necessarily in genetic equilibrium using DNA sequence data. Therefore, to address island–mainland divergence of procyonids, we use Nielsen & Wakely's (2001) coalescent model to analyze mitochondrial control region DNA sequences. We use the resulting data to make inferences about the conservation genetic and population level divergence of the Cozumel procyonids to the mainland taxa and as a basis for recommending management strategies for the island taxa.

## Materials and methods

Genetic samples of *Nasua* and *Procyon* were obtained from Cozumel and throughout the mainland Yucatán Peninsula (Fig. 1, Table 1). On Cozumel, animals were sampled from

**Table 1** *Procyon* and *Nasua* samples used in this study

| Species                 | Haplotype               | <i>n</i> | Geographic origin     |
|-------------------------|-------------------------|----------|-----------------------|
| <i>Procyon pygmaeus</i> | COZ                     | 21       | Cozumel Island, MX    |
| <i>Procyon lotor</i>    | Y1-4                    | 9        | Yucatan Peninsula, MX |
| <i>P. lotor</i>         | WMX1-WMX2               | 2        | Western MX            |
| <i>P. lotor</i>         | NE1                     | 5        | New York, USA         |
| <i>P. lotor</i>         | NE2                     | 2        | Massachusetts, USA    |
| <i>P. lotor</i>         | TN1-2                   | 2        | Tennessee, USA        |
| <i>P. lotor</i>         | NMX <sup>a</sup>        | 2        | New Mexico, USA       |
| <i>Nasua nelsoni</i>    | COZ1, COZ2 <sup>b</sup> | 2        | Cozumel Island, MX    |
| <i>Nasua narica</i>     | Y1-Y4                   | 13       | Yucatan Peninsula, MX |
| <i>N. narica</i>        | ARZ                     | 2        | Arizona               |
| <i>N. narica</i>        | PAN                     | 2        | Panama                |
| <i>N. narica</i>        | BZ1-BZ2                 | 2        | Belize                |

<sup>a</sup>NMX represents a sample obtained from Museum of South western Biology, University of New Mexico (specimen # MSB11178 and MSB 64943).

<sup>b</sup>COZ2 represents a tanned skin from which only a partial sequence was obtained (University of Kansas Natural History Museum Specimen #KU876).

three field sites which represent the primary remaining populations (McFadden, 2004). Institutional tissue archives and samples from unrelated raccoon and coati studies were used for populations inside and outside of the Yucatan region. Most populations were represented by multiple individuals; when only one individual was available, several sequences were compared from independent extractions of the same sample and with independent polymerase chain reaction (PCR) amplifications. Seven *P. pygmaeus* from each of three trapping sites ( $n = 21$ ) on Cozumel were sequenced. Only a single *N. nelsoni* was captured in 3 years on Cozumel reflecting the extreme rarity of these taxa. We supplemented this sample using three tanned *N. nelsoni* museum specimens, one of which yielded usable DNA (see 'Results'). DNA was extracted from museum specimens in an ancient DNA laboratory at the American Museum of Natural History (AMNH) where strict contamination protocols were implemented. DNA from these extractions was sequenced multiple times, and always in a separate laboratory and with separate reagents/tools to prevent contamination by extant samples.

DNA was extracted from tissue with the QIAamp DNA purification kit (Valencia, CA, USA) and then the mitochondrial DNA (mtDNA) control region was amplified by PCR using universal primers (Kocher *et al.*, 1989) and primers designed from homologous regions from our samples (McFadden, 2004; see Supplementary Material). Amplification was carried out in 50  $\mu$ L reactions using 1  $\mu$ L of genomic DNA, 2.5 mM MgCl<sub>2</sub>, 0.2 mM of each dNTP, 1.0  $\mu$ L of each primer,  $\times$  1 PRC buffer, and 0.2 U *Taq* DNA polymerase (Promega, Madison, WI, USA). PCR conditions included an initial denaturation at 95 °C for 1 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 63 °C for 1 min and extension at 72 °C for 1 min. Following the last cycle, an additional step at 72 °C for 7 min was performed. PCR products were purified using

Qiagen QIAquick PCR Purification Kits (Valencia, CA, USA) and sequenced using an ABI 3730xL capillary sequencing machine (Foster City, CA, USA). Sequencing reactions were performed using the Big Dye Terminator Ready Reaction Mix version 3.0 and 3.1 (PE Applied Biosystems, Foster City, CA, USA) and purified following the manufacturer's instructions.

Sequence data were aligned using Clustal X version 1.7 (Thompson, Higgins & Gibson, 1994) and Sequencher version 4.0 (Gene Codes Corporation, 1999, Ann Arbor, MI, USA). Program Modeltest 3.6 (Posada & Crandall, 1998) was used to determine the most likely evolutionary model for the data. Modeltest, using maximum likelihood criterion, determined that the HKY +  $\Gamma$  (Hasegawa, Kishino & Yano, 1985) was the most appropriate model for both *Procyon* and *Nasua* sequence data. Haplotype diversity ( $H$ ; Nei, 1987), the number of nucleotide substitutions per site ( $K$ ), and nucleotide diversity ( $\pi$ , Nei & Gojobori, 1986) estimates for each population were calculated in Arlequin version 3.0 (Schneider, Roessli & Excoffier, 2000) (Table 2) and DnaSP (Rozas *et al.*, 2003). Pairwise genetic distances (HKY +  $\Gamma$ ) were calculated in PAUP\* 4.0B10 (Swofford, 2002) (Table 3).

Owing to low resolution in our initial phylogenetic tree-based analyses, we used haplotype networks to explore the genetic relationships between island and mainland sister taxa. A haplotype network was constructed using TCS v.1.2 (Clement, Posada & Crandall, 2000) which implements statistical parsimony (Templeton, Crandall & Sing, 1992). For comparison, patterns of genetic variation between identified haplotypes were also depicted using Median Joining networks (Bandelt, Forster & Röhl, 1999) as implemented in Network (<http://www.fluxus-engineering.com>).

We tested for population differentiation between groups of individuals from Cozumel (COZ), and from the mainland, including for *Nasua*: the Yucatan (Y), Belize (BZ), Arizona (ARZ) and Panama (PAN), and for *Procyon*: the Yucatan (Y), Western Mexico (WMX) and the Continental US (USA). The total variance between populations was partitioned into hierarchical components using analysis of molecular variance (AMOVA; Excoffier, Smouse & Quattro, 1992) in Arlequin 3.0 (Schneider *et al.*, 2000). This provides estimates of the per cent of total variance accounted for within populations, among populations and among regions. Statistical significance of the variance components was determined by > 5000 permutations of genotypes. The geographic variation of mtDNA d-loop haplotype frequencies was measured by calculating pairwise  $F_{st}$  and  $\Phi_{st}$  for each pair of populations using Arlequin. Probability values for individual comparisons were derived by generating 10 000 distributions in which haplotypes were permuted between populations.  $\chi^2$  statistics may have higher power than sequenced based statistics for detecting population structure (Hudson, Slatkin & Maddison, 1992), so we tested the extent of geographical heterogeneity in haplotype frequency distributions with a  $\chi^2$  analysis using DnaSP (v. 3.0) (Rozas *et al.*, 2003). Arlequin was also used to test for a random distribution of haplotypes between pairs of populations using an exact test of population differentiation, which identifies non-random associations of

**Table 2** Genetic diversity indices for each population of mainland and island raccoons (*Procyon*) and coatis (*Nasua*),  $n$  is the sample size,  $H(n)$  is the number of haplotypes,  $H_d$  is the haplotypic diversity ( $\pm$  standard deviation, sd),  $N_s$  is the number of transitions, and  $N_v$  the number of transversions,  $K$  is the mean number of pairwise differences between individuals and  $\pi$  is nucleotide diversity

| Population     | $n$ | $H(n)$ | $H_d \pm \text{SD}$ | $N_s$ | $N_v$ | $K \pm \text{SD}$   | $\pi \pm \text{SD}$ |
|----------------|-----|--------|---------------------|-------|-------|---------------------|---------------------|
| <i>Procyon</i> |     |        |                     |       |       |                     |                     |
| Yucatan        | 9   | 4      | 0.782 $\pm$ 0.069   | 10    | 2     | 4.972 $\pm$ 2.668   | 0.007 $\pm$ 0.004   |
| Cozumel        | 21  | 1      | 1.000 $\pm$ 0.500   | 0     | 0     | 0                   | 0                   |
| USA            | 11  | 7      | 0.900 $\pm$ 0.161   | 28    | 1     | 13.000 $\pm$ 7.077  | 0.015 $\pm$ 0.009   |
| <i>Nasua</i>   |     |        |                     |       |       |                     |                     |
| Yucatan        | 13  | 4      | 0.782 $\pm$ 0.069   | 3     | 0     | 1.051 $\pm$ 0.744   | 0.001 $\pm$ 0.001   |
| Cozumel        | 2   | 2      | 1.000 $\pm$ 0.500   | 6     | 0     | 7.000 $\pm$ 5.291   | 0.014 $\pm$ 0.015   |
| Range          | 6   | 4      | 1.000 $\pm$ 0.176   | 24    | 5     | 18.166 $\pm$ 10.278 | 0.030 $\pm$ 0.020   |

Range represents all individuals from Arizona, Panama and Belize.

haplotypes among populations for both global and pairwise comparisons (Raymond & Rousset, 1995).

To test whether sequences conformed to neutral expectations and could therefore be used for coalescent analyses, we used DnaSP to compute Tajima's (1989)  $D$ -values, and Fu & Li's (1993)  $D^*$  and  $F^*$  test statistics. Because population structure are probably not in genetic equilibrium between the forces of mutation, genetic drift and migration, we conducted coalescent analyses to estimate population divergence times between the island and mainland taxa using the program MDIV (Nielsen & Wakely, 2001) and the HKY model of sequence evolution to correct for multiple substitutions at sites. MDIV is based on a coalescent model that jointly estimates the divergence time and migration rates among pairs of populations using DNA sequence data. MDIV uses a likelihood approach to estimate divergence time, expressed in units of effective population size, and migration between populations that are assumed to have diverged from a common ancestor. Program MDIV uses Bayesian inference where estimates are the mode of their respective posterior distributions, and credibility intervals of the estimates are the shortest intervals that contain 95% of the respective posterior distribution. Using MDIV, maximum likelihood estimators were generated for  $\theta$  (where  $\theta = 2N_e\mu$ ) and  $T$  (the time since divergence in mutational time, where  $T = t/2N_e$ ). Accordingly,  $t$  (number of years since population divergence) is equal to  $T(\theta/2\mu)$ , where  $\mu$  is divergence between sequences per year per nucleotide.

To convert coalescent times to ybp an estimated mutation rate ( $\mu$ ) is required. This rate is variable across carnivores and depending on the rate used, may significantly change estimates of divergence time. Therefore, we used three substitution rates to calculate divergence: Stoneking *et al.*'s (1992)  $1.18 \times 10^{-7}$  substitutions site $^{-1}$  year $^{-1}$ , which is derived from humans; Ho, Kolokotronis & Allaby's (2007)  $5.84 \times 10^{-7}$  substitutions site $^{-1}$  year $^{-1}$  based on arctic fox ( $6.25 \times 10^{-7}$ ) and brown bear ( $5.43 \times 10^{-7}$ ); Pesole *et al.*'s (1999) estimate of  $8.9 \times 10^{-9}$  substitutions site $^{-1}$  year $^{-1}$  based on examination of several mammals. MDIV requires a per locus estimate ( $\mu$ ) so we multiplied the mutation rate per site for the d-loop by the sequence length and by generation time ( $g$ ) to obtain  $\mu$ . Generation time for both taxa was set at 2 years. Analyses were run five times for each

comparison with different random seeds to assess the stability of results. The analysis was carried out using the HKY model with  $t_i/t_v$  estimated from the data. A Markov chain length of  $5 \times 10^6$  steps was used, discarding the first 500 000 steps as burn-in, and prior distributions of  $M$  (migration rate) and  $t_{\text{pop}}$  (scaled divergence time) between 0 and 30 were used.

We applied the program IM (Hey & Nielsen, 2004) to the samples from our Cozumel–Yucatan populations. IM was used in this instance in addition to MDIV because it allows for asymmetric rates of gene flow and different effective population sizes of the source populations. IM uses a MCMC approach to simultaneously estimate the following parameters:  $\theta$ ,  $m1$  (number of migrants from population 1 to population 2),  $m2$  (number of migrants from population 2 to population 1) and  $t$ . The program was run initially for 50 000 000 steps using five chains, following a 500 000 step burn-in. To ensure the program was performing well, we ran the program five times with different random seeds. Across independent runs, we also explored varying priors and heating scenarios (linear, two-step and geometric).

## Results

Analyses of 43 *Procyon* specimens yielded of 893 bp of mtDNA sequence data. There was no significant heterogeneity in base frequencies across taxa ( $\chi^2 = 6.824$ , d.f. = 36,  $P = 0.989$ ). Average base composition was 25.49% C, 26.88% T, 30.47% A and 17.16% G. The best-fit model determined by ModelTest was HKY +  $\Gamma$  (Hasegawa *et al.*, 1985) and assumed unequal base pair frequencies (0.307, 0.252, 0.166), a transition/transversion ratio of 15.03, Gamma shape of 0.094 and two substitution types. Mean number of pairwise differences for *P. lotor* (Yucatan + USA) was  $8.27 \pm 4.08$  and nucleotide diversity was  $0.019 \pm 0.010$ . A single haplotype was found for all Cozumel *P. pygmaeus* individuals, while 11 haplotypes were identified for *P. lotor*. Of the 11 *P. lotor* haplotypes, 26 polymorphic sites were identified, all of which were substitutions (24 transitions, two transversions).

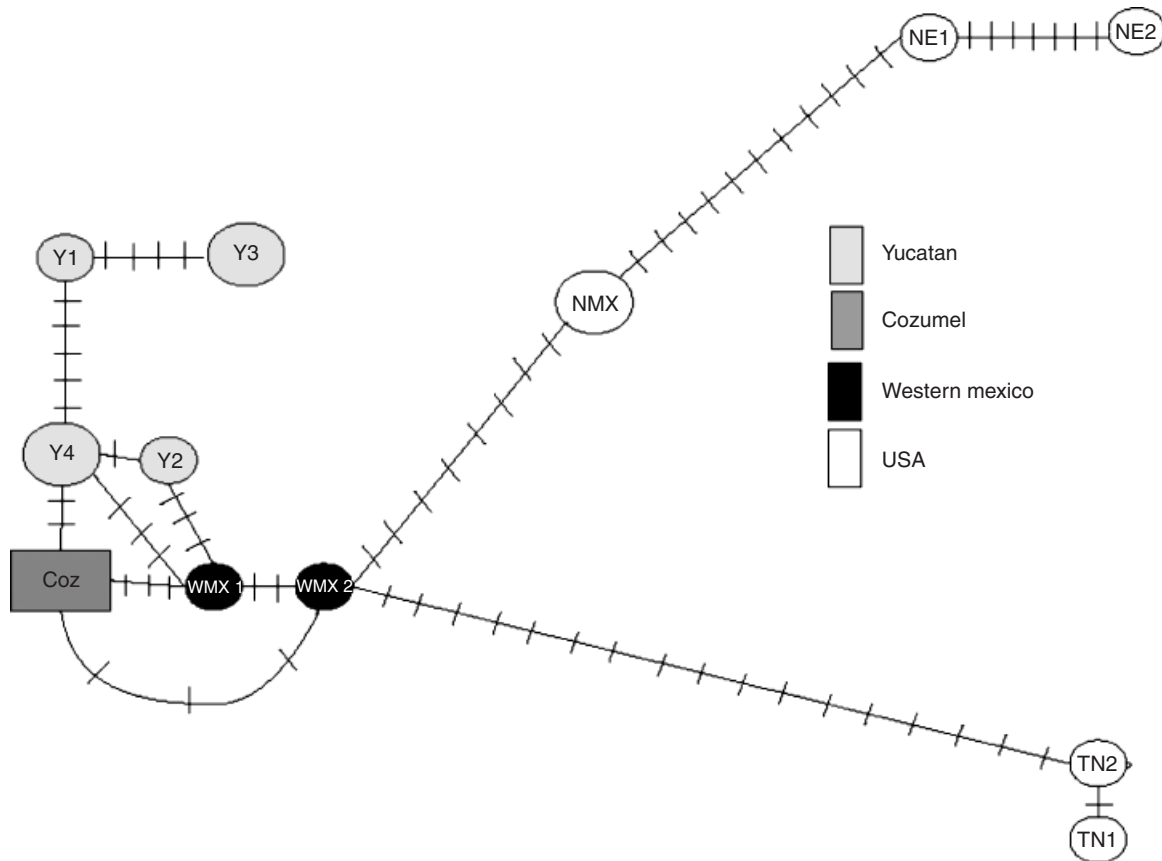
For *Nasua*, we obtained 631 bp of DNA sequence data from 21 *Nasua* specimens, including 450 bp for a museum

skin (COZ 2). There was no significant heterogeneity in base frequencies across taxa ( $\chi^2 = 2.73$ , d.f. = 30,  $P = 1.00$ ). Average base composition 25.48% C, 29.40% T, 27.83% A and 18.19% G. Of a total of 37 polymorphic sites there were 29 sites characterized with substitution and eight sites characterized with insertions/deletions. The best-fit model determined by ModelTest was HKY +  $\Gamma$  and assumed unequal base pair frequencies (0.283, 0.259, 0.182), a transition/transversion ratio of 3.28, Gamma shape of 0.014 and two substitution types. Mean number of pairwise differences for *N. narica* was  $5.75 \pm 2.89$  and 1.0 for *N. nelsoni*. The 21 specimens included eight *N. narica* haplotypes and two *N. nelsoni* haplotypes, with average pairwise sequence divergence of 1.38% (range 0.168–4.00%, Table 4). The sequence divergence (based on HKY +  $\Gamma$  distance) between the mainland and island taxa was 0.506%. The per cent divergence between the four mainland subpopulations was *c.* 2.4 times higher than the divergence between insular *N. nelsoni* and Yucatan *N. narica*.

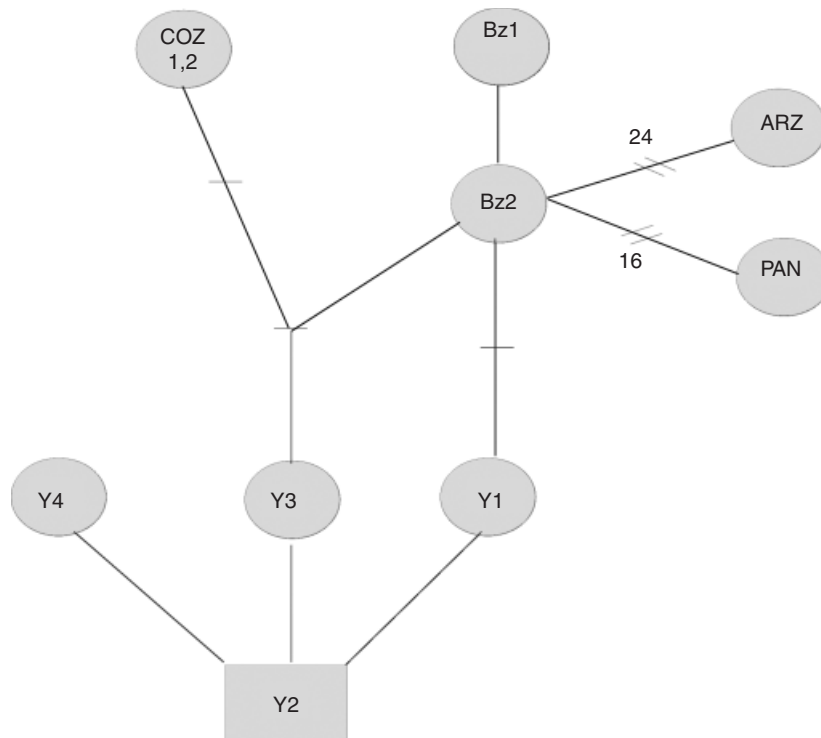
Median joining and statistical parsimony networks show comparable spatial patterns of genetic diversity, with the latter shown in Fig. 2. A single *Procyon* haplotype network was reconstructed within which all haplotypes had a 95%

probability of being parsimoniously connected. The haplotype network identified four distinct lineages which corresponded to the geographical regions of sampling. The haplotypes included one distinct Yucatan group (haplotypes Y1–Y4), a United States grouping (NE1, NE2, NMX, TN1, TN2), a Western Mexico group (WMX1 and WMX2) and a Cozumel lineage (COZ) (Fig. 2). The Cozumel haplotype was linked to the Yucatan and Western Mexico groups, and the latter also linked the two branches of the United States.

For *Nasua*, a single haplotype network was reconstructed within which all haplotypes had a 95% probability of being parsimoniously connected (Fig. 3). Two haplotypes were identified from *N. nelsoni* and six haplotypes were identified from *N. narica*. The haplotype network identified three distinct lineages among the eight haplotypes, which corresponded to broad geographical regions. The haplotypes included one distinct Yucatan grouping (haplotypes Y1–Y4), a Belize grouping (BZ1, BZ2) and a Cozumel group (COZ) (Fig. 3). The Cozumel haplotype was linked by both Belize and the Yucatan. A single Yucatan haplotype (Y2) was independent of all other Yucatan haplotypes in the network.



**Figure 2** The 95% statistical parsimony network of Cozumel and mainland *Procyon* spp. Each line in network represents one mutational change in sequenced control region mtDNA. Size of circle represents the relative frequency of this haplotype among individuals sampled in that region. Each line in network represents one mutational change in sequenced control region mtDNA. Varying shapes correspond to network groupings. mtDNA, mitochondrial DNA.



**Figure 3** The 95% statistical parsimony network of Cozumel and mainland *Nasua* spp. Each line in network represents one mutational change in sequenced control region mtDNA. mtDNA, mitochondrial DNA.

**Table 3** Pairwise fixation indices calculated for the different populations

|                | USA              | Yucatan          | Cozumel        |
|----------------|------------------|------------------|----------------|
| <i>Procyon</i> |                  |                  |                |
| USA            | –                | 0.07524          | <b>0.58621</b> |
| Yucatan        | 0.07778          | –                | 0.46221        |
| Cozumel        | <b>0.55000**</b> | <b>0.52778**</b> | –              |
|                | Range            | Yucatan          | Cozumel        |
| <i>Nasua</i>   |                  |                  |                |
| Range          | NA               | 0.13521          | 0.0622         |
| Yucatan        | <b>0.10897**</b> | NA               | 0.16568        |
| Cozumel        | 0.08890          | <b>0.10986**</b> | NA             |

$F_{st}$  values are above diagonal and  $\Phi_{st}$  values below diagonal. Statistically significant values ( $P < 0.05$ ) are in bold. Double asterisks (\*\*) below the diagonal indicate significant values ( $P < 0.05$ ) for the exact test of population differentiation. Individuals are categorized based on geographic origin. For *Nasua*, range represents all individuals from Arizona, Panama and Belize, and Yucatan represents only haplotypes from the Mexican Yucatan.

For both species, the among-group component of the AMOVA analyses was significant when both haplotype frequencies and molecular distances were considered (*Procyon*:  $F_{st} = 0.3585$ ,  $P < 0.002$ ;  $\Phi_{st} = 0.2802$ ,  $P < 0.030$ ; *Nasua*:  $F_{st} = 0.131$ ,  $P < 0.030$ ;  $\Phi_{st} = 0.222$ ,  $P < 0.046$ ). The  $\chi^2$  tests also supported significant differentiation (*Procyon*:  $\chi^2 = 108.22$ ,  $P < 0.042$ , d.f. = 17; *Nasua*:  $\chi^2 = 11.499$ ,

$P < 0.007$ ). Pairwise comparisons showed significant population structure between *P. pygmaeus* and all *P. lotor* populations when both the haplotype frequencies and molecular distances were considered (Table 3). The Yucatan *Procyon* populations did not strongly differ from those found in the USA ( $F_{st} = 0.075$ ,  $P = 0.064$ ;  $\Phi_{st} = 0.077$ ,  $P = 0.082$ ). An exact test of differentiation also supported a similar pattern of differentiation (Table 3). *Procyon* populations did not have significant values ( $P > 0.10$ ) of Tajima's  $D$ -values or Fu and Li's  $D^*$  and  $F^*$  statistics, as expected under selective neutrality of the control region sequences.

For *Nasua*, pairwise comparisons also showed significant population structure between Yucatan *N. narica* and all other mainland coati populations (Belize, Panama, Arizona) when haplotype frequencies and molecular distances were considered (Table 3). The Yucatan populations of coatis (*Nasua narica yucatanica*) did not statistically differ from those found on Cozumel Island according to the  $F_{st}$  ( $F_{st} = 0.166$ ,  $P = 0.061$ ) but did have significant  $\Phi_{st}$  values ( $\Phi_{st} = 0.089$ ,  $P = 0.050$ ); the global AMOVA also indicated Cozumel populations significantly differed from other mainland populations ( $F_{st} = 0.108$ ,  $P = 0.050$ ). The exact test of differentiation found significant differentiation among Cozumel Island coatis and Yucatan coatis ( $F_{st} = 0.109$ ,  $P = 0.015$ ; Table 3). *Nasua* populations did not have significant values ( $P > 0.05$ ) of Tajima's  $D$ -values or Fu and Li's  $D^*$  and  $F^*$  statistics, as expected under selective neutrality of the control region sequences. We conclude that the *Procyon* and *Nasua* sequences are effectively neutral,

and therefore the use of coalescent modeling of divergence time of the mainland and island populations is appropriate.

Average sequence divergence between the Yucatan *P. lotor* and *P. pygmaeus* was 0.47%. Average sequence divergence between all *P. lotor* haplotypes was 1.82%. The Yucatan region was represented by four haplotypes with average sequence divergence of 0.39%. The Cozumel haplotype was most similar to the Y2 and Y4 haplotypes and differed from these haplotypes by 0.29%. The Western Mexico haplotype was more similar to Cozumel haplotypes (0.74%) than haplotypes from the Yucatan (1.14%) (Table 4). A population aggregation analysis of the *Procyon* mtDNA identified no fixed diagnostic nucleotide positions distinguished *P. pygmaeus* from *P. lotor*. Based on our limited dataset, fixed diagnostic nucleotide positions distinguish Y1 and Y3 from other Yucatan haplotypes, the two Yucatan haplotypes Y2 and Y4 are the more similar to the Cozumel haplotypes than are haplotypes Y1 and Y3 (Supplementary Material).

**Table 4** Average HKY+ $\Gamma$  pairwise distances for mtDNA sequence data between haplotypes of *Procyon* and *Nasua*

|                | Cozumel | Yucatan | USA    |
|----------------|---------|---------|--------|
| <i>Procyon</i> |         |         |        |
| Cozumel        | –       |         |        |
| Yucatan        | 0.0039  | –       |        |
| USA            | 0.0234  | 0.0218  | –      |
| Western Mexico | 0.0074  | 0.0114  | 0.0290 |
|                | Cozumel | Yucatan | Belize |
| <i>Nasua</i>   |         |         |        |
| Cozumel        | –       |         |        |
| Yucatan        | 0.0051  | –       |        |
| Belize         | 0.0056  | 0.0052  | –      |
| Range          | 0.0239  | 0.0290  | 0.0315 |

Haplotypes described as range include those from Panama and Arizona and were grouped because of low sample sizes and because each population had a single haplotype.  
mtDNA, mitochondrial DNA.

For *N. nelsoni* and Yucatan *N. narica*, average sequence divergence was 0.51%. Average sequence divergence between all *N. narica* haplotypes was 1.60%. The Yucatan region was represented by four haplotypes with an average sequence divergence of 0.24%. The Cozumel *Nasua* were especially divergent from the Panama (3.34%) and Arizona (1.4%) populations (Table 4). A single diagnostic polymorphism at bp 232 differentiated *N. nelsoni* from the Yucatan *N. narica* (Supplementary Material). One Cozumel sample (COZ2) came from a museum skin and is missing a 200 bp fragment. Nonetheless, this sample also contains the single diagnostic polymorphism. Although our sample size is too small to be conclusive, several polymorphisms (bp #213 and 617) found in *N. nelsoni* also indicate a close relationship to the Belize haplotypes.

We initially used IM to estimate parameters of the two population isolation model of species divergence for our sequence dataset for *Procyon* (the larger of our datasets). Although we were able to achieve adequate mixing of the Markov chains and relatively stable estimates for some of the parameters of  $m$ , we were unable to obtain stable estimates of the full posterior density and the scaled population splitting time  $t$ . Thus we viewed all parameter estimates from our IM analyses as unreliable. Given the low levels of variation in our data, it was not surprising that we had little power to estimate all the parameters of this complex model.

We were, however, able to use the simpler model implemented in MDIV to derive a coalescent estimate of mtDNA divergence between island and mainland carnivores. The posterior probability distribution of  $\theta$  was well resolved. Coalescent modeling of the *Procyon* sequences in the mainland and Cozumel populations using 5 million generations and a burn in of 2 million generations provided good estimates of the parameters  $\theta$ ,  $M$ ,  $T$  and TMRCA. The modal value of  $\theta$  in the Bayesian posterior distribution was 2.715 for Cozumel versus mainland Yucatan populations of the raccoon. The minimum population divergence time ( $T = 0.097$ ) was estimated at 1525 generations or *c.* 3050 ybp based on a mutation rate per locus of

**Table 5** Likelihood based estimation  $\theta$  ( $2N_e\mu$ ) and standardized estimates of the divergence times ( $T$ ) between *Procyon* and *Nasua* populations\*

| Populations    | $\theta$ ( $4N_e\mu$ ) | $T$ ( $t/\mu$ )   | $t$ ( $\mu_1 = 2.11 \times 10^{-4}$ ) | $t$ ( $\mu_2 = 1.59 \times 10^{-5}$ ) | $t$ ( $\mu_3 = 1.04 \times 10^{-3}$ ) |
|----------------|------------------------|-------------------|---------------------------------------|---------------------------------------|---------------------------------------|
| <i>Procyon</i> |                        |                   |                                       |                                       |                                       |
| PP_YUC         | 2.715 (0.55–11.03)     | 0.097 (0.22–4.72) | 15 093                                | 200 111                               | 3050                                  |
| PP_WMX         | 1.882 (0.62–6.90)      | 2.196 (0.27–4.61) | 19 613                                | 260 037                               | 3963                                  |
| WMX_USA        | 7.016 (0.40–17.88)     | 0.952 (0.08–4.85) | 31 694                                | 420 218                               | 6404                                  |
| PP_USA         | 11.501 (5.97–25.78)    | 0.256 (0.13–4.38) | 13 825                                | 183 302                               | 2793                                  |
| YUC_USA        | 10.616 (0.64–27.90)    | 0.717 (0.09–4.87) | 36 144                                | 479 214                               | 7303                                  |
| <i>Nasua</i>   |                        |                   |                                       |                                       |                                       |
| NN_BZ          | 0.797 (0.07–3.18)      | 2.236 (0.09–4.85) | 11 970                                | 158 705                               | 2418                                  |
| NN_YUC         | 0.891 (0.09–3.83)      | 1.123 (0.09–4.85) | 6252                                  | 82 896                                | 1263                                  |
| YUC-BZ         | 1.724 (0.14–6.39)      | 1.188 (0.09–4.58) | 13 760                                | 182 438                               | 2780                                  |

Values of  $t$  were calculated in years before present (ybp). Values in parentheses are 95% credibility intervals. A generation time of 2 years was used to translate divergence times from generations into ybp. Subscripts for  $\mu$  refer to source of substitution rate: 1, Stoneking *et al.* (1992); 2, Pesole *et al.* (1999); 3, Ho *et al.* (2007).

\*PP, *Procyon pygmaeus*; NN, *Nasua nelsoni*

$1.04 \times 10^{-3}$  substitutions locus<sup>-1</sup> generation<sup>-1</sup> (Ho *et al.*, 2007), with alternative estimates of 15 093–200 111 ybp (Table 5).

For *Nasua*, coalescent modeling yielded a modal value of  $\theta$  in the Bayesian posterior distribution of 0.891 for Cozumel versus mainland populations. Minimum population divergence time ( $T = 1.123$ ) was estimated at 2526 generations or *c.* 1263 ybp, with alternative estimates of 6252 and 82 896 ybp (Table 5). The Belize population appears to have diverged from both the Cozumel Island population and Yucatan populations on very similar time frames (Table 5).

## Discussion

Genetic structuring of the Cozumel *Nasua* and *Procyon* was detectable on a population level, but as anticipated, phylogenetic analysis was precluded due to low levels of sequence divergence (0.39–2.39%). Sequence data indicate that although *P. pygmaeus* has no intrapopulation variation, it is genetically distinct from the Yucatan *P. lotor* ( $F_{st} > 0.05$ ). In general however, the intraspecific variation within the examined *Procyon* was low. This is consistent with other carnivores (Vila *et al.*, 1999; Cassens *et al.*, 2000; Davison *et al.*, 2001; Walker *et al.*, 2001; Michaux *et al.*, 2004).

Our data suggest that *P. pygmaeus* likely colonized Cozumel only somewhat before the arrival of *N. nelsoni*. The divergence between the island–mainland group is estimated to have occurred 3050–200 111 ybp for *Procyon*, and 1263–82 896 ybp for *Nasua*. Two of the three selected substitution rates provide time frames well before Mayan peoples colonized the island (earliest estimates are  $\sim$ 2500 ybp, Hamblin, 1984). However, using substitution rates derived from Ho *et al.* (2007), we can not definitively reject the hypothesis that these species colonized before Mayan colonization of Cozumel Island. Clearly additionally samples within the Yucatan and the island, along with microsatellite analyses, would allow for a more precise estimate of divergence dates between the island and the mainland taxa.

As is expected with a limited number of samples, our credibility intervals were broad and when lower values of  $\theta$  and/or  $T$  (within the 95% CI) are used to calculate  $t$ , much more recent estimates are obtained, many of which fall within the last 2500 years. However, it should be noted that both the lower (1263 ybp) and upper (200 111 ybp) bound modal values for our divergence estimates are not biologically realistic dates. The lower bound modal estimate for *N. nelsoni*, as well as many of the lower 95% CIs, predict divergence dates well after procyonids were known (Hamblin, 1984) to have inhabited Cozumel Island. Additionally, upper bound modal estimates, and some of the upper 95% CIs, also put the divergence date of island carnivores at a time when the island was known to still be submerged during the Pleistocene glacial melt (Spaw, 1978; Ward, 1985). The moderate level of variance surrounding the divergence estimates and the biological implausibility of some of the upper and lower modal values highlights the need to avoid using any of the divergence estimates as absolute times.

Thus, we are unable to definitively determine if the colonization of Cozumel by procyonids was a natural (non-anthropogenic) event. However, two of three substitution

rates yielded divergence dates consistent with a natural colonization of Cozumel. In any case, such historical biogeographic scenarios rely heavily on the molecular dating of divergence times. The use of single locus (mtDNA) sequence data to calculate divergence times can be problematic because of large standard errors due to coalescent stochasticity (Edwards & Beerli, 2000; Jennings & Edwards, 2005) and because of difficulties in calculating locus-specific mutation rates (Ho *et al.*, 2005, 2007). However, even if the timing of the mainland–Cozumel divergence is inexact, results of our analyses suggest that the pygmy raccoon likely predates human arrival on the island and thus should be treated as distinct taxonomic and more importantly, management unit. Furthermore, even if the taxa are the result of a more recent colonization event than suggested by our analyses, population genetic analyses nonetheless indicate that both the *P. pygmaeus* and *N. nelsoni* are distinct from their mainland conspecifics (i.e. significant  $F_{st}$  and  $\Phi_{st}$ ). Our results show significant quantitative evidence for population level distinction between pygmy raccoons and those located in the Yucatan. A single haplotype represented all sampled *P. pygmaeus* indicating a recent population bottleneck, perhaps associated with the founder event, or with the existence of frequent catastrophic hurricane events. Both species are also geographically (and thus reproductively) isolated and morphologically distinct (Helgen & Wilson, 2003, 2005; McFadden, 2004); specifically, pygmy raccoons exhibit as much as a 14% size reduction since their divergence from mainland raccoons (McFadden, 2004). Therefore, we believe that the body of evidence supports continuing previously developed species-level recognition. Such recognition is similar to the well accepted recognition of the Channel Island fox *Urocyon littoralis* as a distinct species/management unit despite a human-assisted divergence from mainland *Urocyon cinereoargenteus* *c.* 10 000–16 000 ybp (Gilbert *et al.*, 1990; Goldstein *et al.*, 1999).

Generally, morphologic and genetic variation in island populations is lower than in similarly sized mainland populations (Van Valen, 1962; Soulé, Yang & Myers, 1975), reflecting the degree of isolation, the effective population size, and the number of generations since founding (Wright, 1969). This explain the lack of genetic variability in *P. pygmaeus*. Identification of a single *P. pygmaeus* haplotype also suggests either a high level of gene flow between the three examined subpopulations or results of periodic bottlenecks due perhaps to the catastrophic decadal destructive hurricanes that occur in the region and are suspected to directly influence Cozumel's animal populations (Cuarón *et al.*, 2004). However, it should be noted that morphology of insular mammals often evolve at extremely rapid rate (e.g. Lister, 1989; Millien, 2006) and so a species level designation based on morphological criteria alone, or in conjunction with variable genetic data, should be interpreted with caution.

Given the extreme rarity of *N. nelsoni* on Cozumel (Cuarón *et al.*, 2004; McFadden, 2004) and in museum collections, and the associated small sample size of *N. nelsoni* available for this study, it is difficult to make conclusions about this population's haplotype diversity or

species level uniqueness. However, our analyses suggest that the Cozumel taxa are most closely related to their Yucatan and Belize conspecifics, and although population differentiation tests ( $F_{st}$ ) do not unequivocally support the recognition of distinct island–mainland groupings, the global AMOVA and exact test do show population differentiation between the Yucatan and the island populations. Based on the incongruence between these tests, we believe small sample size and low level of population differentiation are responsible for the insignificant  $F_{st}$  value. Nonetheless, other tests for differentiation indicate that the populations are distinct, and coalescence times for these taxa indicate it likely has existed on Cozumel for >1500 years, and perhaps >100 000 years. Based on these findings, combined with the extreme rarity of this taxon, we believe that a continued strong conservation focus on these taxa is scientifically supported. Although *N. nelsoni*'s estimated divergence time using Ho *et al.*'s (2007) substitution rate yields a divergence estimate around the same time as the Mayans colonized Cozumel, the standard errors surrounding  $\theta$  and  $T$  preclude us from definitively ruling out that *Nasua* colonized Cozumel before Mayan colonization.

Justifying species or subspecies designation of recently (e.g. <100 000 years) isolated populations is fraught with difficulties, in part for reasons such as incomplete lineage sorting which may be at work in the Cozumel taxa, and in part because of unclear criteria for designating the level of uniqueness necessary for placement within a particular taxonomic category. It is therefore important in such cases that a holistic perspective be used to evaluate the population in question. In the case of the Cozumel procyonids, this requires considering not solely the presence or absence of unique alleles (especially when only examining one gene) but also examining data on genetic differentiation, estimated divergence times of sequences, and data on the morphology of the animals. Based on their smaller physical stature and cranial characteristics, Merriam (1901) concluded that *P. pygmaeus* was worthy of species level designation, a conclusion also supported by recent analyses by McFadden (2004) and by Helgen & Wilson (2005). For *N. nelsoni*, evidence of greatly reduced stature is also noted by several researchers (Merriam, 1901; Thomas, 1901; Jones & Lawlor, 1965), although whether the extent of morphometric differentiations justified species-level designation has been questioned (Decker, 1991). Therefore, although our data do not unequivocally suggest unique species status, we believe that the body of evidence does suggest a precautionary approach which allows these taxa (allopatric populations or species) to be managed as distinct management units.

Significant genetic structure between populations is one indication that populations may be recognized as separate management units (Avice 1995, 2000; Frankham, 1995; Michaux *et al.*, 2004). A management unit is 'any population that exchanges so few migrants with others as to be genetically distinct from them normally' and thereby 'demographically independent at the present time' (Avice, 2000). Based on results which indicate levels of significant genetic differentiation, as well as associated findings of a unique

haplotype in *P. pygmaeus* and a fixed character in *N. nelsoni*, we recommend that the island populations be managed separately from coati and raccoon populations on the mainland. In particular, immediate management strategies must be put in place to limit release of mainland animals on Cozumel, with its associated risk of introgression.

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## Supplementary material

The following supplementary material is available for this article online:

**Table S1.** Amplification and sequencing primers for the control region for *Procyon* (P) and *Nasua* (N). Primers designed to be species specific are noted as follows: *Nasua* (N), *Procyon* (P), Carnivores (C) and not specific (NS); the corresponding strand is listed as heavy (H) and light (L).

**Table S2.** Polymorphic sites observed in a 893 bp sequence in the d-loop region of the mitochondrial DNA from Cozumel (*P. pygmaeus*) and mainland (*P. lotor*) raccoons. Position numbers match the sequence numbers where the polymorphism occurred.

**Table S3.** Polymorphic sites observed in a 631 bp sequence in the d-loop region of the mitochondrial DNA from Cozumel (*N. nelsoni*, COZ) and continental (*N. narica*) coatis; numbers match the sequence numbers where the polymorphism occurred. Only a partial sequence for COZ2 is available.

This material is available as part of the online article from <http://www.blackwell-synergy.com/doi/abs/10.1111/j.1469-7998.2008.00461.x>

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