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Use of mitochondrial DNA from feces to evaluate the range of secretive species: the case of volcano rabbit

Uso de ADN mitocondrial de heces fecales para evaluar la distribución de especies secretivas: el caso del conejo zacatuche

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The volcano rabbit, *Romerolagus diazi*, is endemic to a small region along the Trans-Mexican Volcanic Belt in central México. Although its distribution in the volcanic fields of the Sierras Nevada and Chichinautzin is not debated, its occurrence in the Nevado de Toluca volcano has been controversial. In this study, we used a species identification tool using DNA isolated from fecal pellets in order to corroborate the occurrence of volcano rabbit in the Nevado de Toluca. Both PCR assays and phylogenetic analysis of fragments of cytochrome b and D-Loop mitochondrial genes provide evidence that although the morphology of collected pellets resemble those of *R. diazi,* they instead correspond to a *Sylvilagus* species. These results support the hypothesis that *R. diazi* is not currently distributed in the Nevado de Toluca.

Key words: DNA; Nevado de Toluca; species identification; volcano rabbit.

El conejo zacatuche es endémico de una pequeña región de la Faja Volcánica Transmexicana en el centro de México. Aunque su distribución en las Sierras Nevada y Chichinautzin no se debate, su presencia en el volcán Nevado de Toluca ha sido controvertida. En este estudio, utilizamos una herramienta de identificación de especies que utiliza fragmentos de los genes mitocondriales citocromo b y D-Loop amplificados de muestras de excretas para corroborar la ocurrencia del conejo zacatuche en el Nevado de Toluca. Tanto los ensayos de PCR como el análisis filogenético de ADN mitocondrial proporcionaron evidencia de que, aunque la morfología de las excretas recolectadas se asemeja a la de las excretas de *R. diazi*, en realidad corresponden a una especie de *Sylvilagus*. Estos resultados soportan la hipótesis de que el Nevado de Toluca no forma parte de la distribución actual de *R. diazi*.

Palabras clave: ADN; conejo zacatuche; identificación de especies; Nevado de Toluca.

The volcano rabbit, *Romerolagus diazi* (Ferrari-Pérez, 1893), known locally as *zacatuche*, is endemic to a small region along the Trans-Mexican Volcanic Belt in central México where it occupies a specialized habitat, in altitudes ranging between 2,900 and 4,250 m (Rizo-Aguilar *et al.* 2016; Velázquez and Guerrero 2019). This range is becoming increasingly fragmented and the area of suitable habitat is decreasing gradually for agriculture, ranching and logging and by forest fires (Uriostegui-Velarde *et al.* 2018). Populations of the volcano rabbit are now at risk and this species is categorized as Endangered, both by the Mexican government (SEMARNAT 2010) and on the IUCN Red List of Threatened Species (Velázquez and Guerrero 2019). The distribution of *R. diazi* in Tláloc, Pelado, Chichinautzin, Monte Tláloc, Iztaccíhuatl and Popocatépetl volcanoes has been documented by collecting, sightings, camera trapping and indirect traces such as fecal pellets identified morphologically (Hoth *et al.* 1987; Velázquez *et al.* 1996; Hunter and Cresswell 2015; Rizo-Aguilar *et al.* 2015, 2016; Uriostegui-Velarde *et al.* 2018). However, its occurrence in the Nevado de Toluca volcano has been controversial (Hoth *et al.* 1987; Cervantes *et al.* 1990; Ceballos *et al.* 1998; Velázquez and Guerrero 2019).

On the basis of such controversy, a recent study attempted to confirm the presence of the volcano rabbit in the Nevado de Toluca both by searching its fecal pellets in 1,807 sites with habitat suitable for the species and by camera trapping (Monroy-Vilchis *et al.* 2020). The authors found fecal pellets attributable to *R. diazi* in only 41 sites but failed to obtain a photographic record of the species. Consequently, they suggest continuing with camera trapping in order to verify the species occurrence in the zone.

Although camera trapping is highly reliable, this direct method is not efficient because it is time consuming and costly, making cameras difficult to deploy. In this study, we used a species identification tool using DNA isolated from pellet samples in order to corroborate the occurrence of the volcano rabbit in the Nevado de Toluca. This non-invasive method has been accomplished for several mammalian species, including rabbits (Waits and Paetkau 2005; Adams *et al.* 2011).

During June to October 2018, we conducted an exhaustive search for pellets of *R. diazi* in 120 sites in the Nevado de Toluca where we had previously identified the presence of bunchgrasses through the analysis of Sentinel 2 satellite images (Figure 1). It was possible to collect 77 pellet samples with similar characteristics to those of the volcano rabbit in only 15 sites. The pellets were put in plastic bags, transported on ice to the laboratory, and stored at -20 °C prior to DNA extraction.

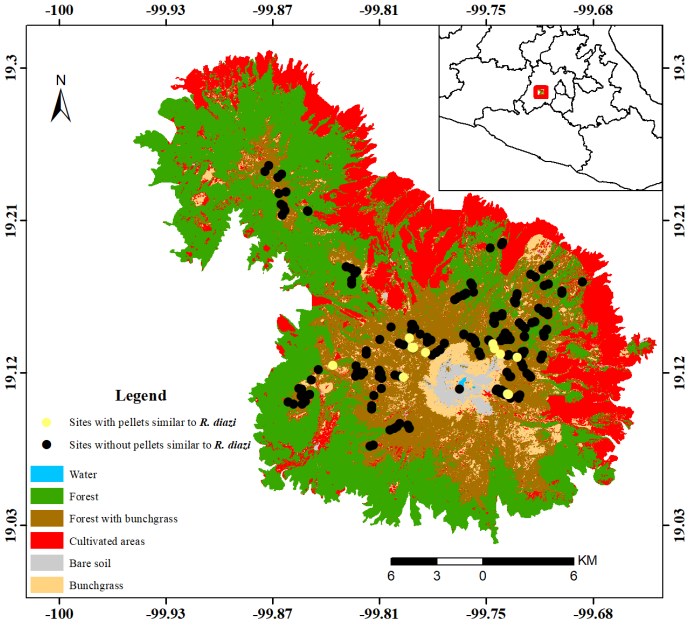

Figure 1. Map depicting the polygon of the Nevado de Toluca and sites surveyed for volcano rabbit pellets.

We attempted to isolate total genomic DNA for all 77 samples with the "ZR Fecal DNA MiniPrep" kit (Zymo research, Irvine, CA, U.S.A.), following manufacturer's instructions, however 17 samples failed to yield quantifiable DNA. Two approaches were then implemented to assess the species identity of the 60 samples that yielded DNA. In the first one, we used the PCR technique as an identification tool of DNA isolated from putative volcano rabbit pellets using a combination of primers that only amplify a 467 base pair fragment of the cytochrome b gene for *R. diazi* (see Osuna *et al.* 2020 for details of primers used). Each PCR reaction contained 1 µl of each 10 µM primer, 12.5 µl of PCR master mix 2X (Promega), 4 µl of DNA extract and 6.5 µl of bidistilled water. PCR amplification was performed in an Eppendorf thermocycler using the following program: a first step of 5 min at 94 °C followed by 34 cycles of 1min at 94 °C, 35 sec at 50 °C, and 40 sec at 72 °C, and a final extension of 10 min at 72 °C. We included two positive controls in all PCR amplification tests. One was obtained from extracted DNA from liver tissue of a road-killed volcano rabbit collected in National Park Iztaccíhuatl-Popocatépetl and the second one was from DNA extracted from a fecal sample of *R. diazi* collected in the locality of Coajomulco, Morelos. These controls had similar conditions (DNA quality and quantity) with respect to the rest of the DNA samples. Negative controls (no template added) were also included to monitor for contamination. All PCR products (25 µl each sample) were checked on 2.0 % agarose gel electrophoresis to confirm the amplification of the desired product.

In the second approach, we amplified and sequenced a 418 base pairs fragment of the control region (D-Loop) in 17 samples that yielded DNA of adequate quality using the primers Pro1 5´-CCACCATCAGCACCCAAAGCT-3 (Mougle 1997) y NC4 5´-AAGAATGGAGTCCCGGTA-3 (Ramírez 2009). PCR and sequencing conditions were described in detail by Osuna *et al.* (2020). Bidirectional sequence reactions were read with a 3500xl genetic analyzer (Life Technology) at the Laboratory of Genomic Sequencing of Biodiversity and Health at the Instituto de Biología, UNAM. The sequences were inspected to correct reading mistakes or ambiguities and aligned with the software Bioedit 7.2.1 (Hall 1999). From the 17 sequenced samples, we obtained only four different haplotypes, which were included in a data matrix containing 31 D-Loop sequences of volcano rabbit obtained from Osuna *et al.* (2020). In addition, we downloaded homologous D-Loop sequences from GeneBank of *Sylvilagus floridanus* (accession number KC923350) and one of *Lepus californicus* (accession number KJ397614) as outgroup. With this matrix, we performed a phylogenetic analysis with the Bayesian inference method using the program MrBayes 3.2.6 (Ronquist *et al.* 2011). The program was run for 1 x 10^7 generations sampling every 10,000 generations and applying a burn-in of 10 %. The molecular evolution model was selected in the program JModelTest 2.1.7 (Posada 2008) using the Akaike information criterion.

PCR profiles of cytochrome b gene showed that none of the 60 putative volcano rabbit samples tested yielded PCR products. Moreover, all positive controls always yielded PCR products of the expected fragment size (Figure 2). Because this reaction is designed to only amplify a 467 bp fragment of cytochrome b if the DNA came from a volcano rabbit, these results suggest that pellets collected in the Nevado de Toluca do not correspond to *R. diazi*. It has been documented that failures in PCR amplifications may occur due to the amount of time the sample is exposed to the environment (Brinkman *et al.* 2010). However, we are certain that DNA degradation is not the reason for the lack of success in obtaining amplicons from pellets collected in the Nevado de Toluca, as the fecal positive control used in all the assays always yielded PCR products of the appropriate size (Figure 2). In addition,

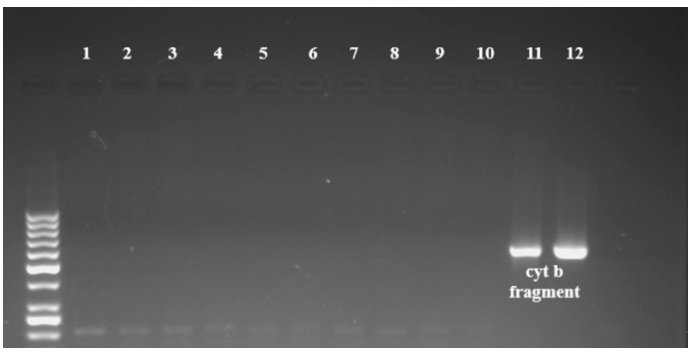

Figure 2. Image of an agarose gel showing resulting PCR assay to identify amplicons of *Romerolagus diazi*. 1-10 correspond to putative volcano rabbit pellet samples, 11-12 are *R. diazi* positive controls.

all the amplifications performed with the D-Loop primers for 17 of the same samples also resulted in amplicons, thus corroborating that the failed cytochrome b PCR amplifications were not due to DNA degradation or a PCR artifact.

The resulting phylogenetic tree showed that the four D-Loop haplotypes of samples attributable to *R. diazi* from the Nevado de Toluca volcano were grouped with the GenBank *S. floridanus* haplotype instead of grouping with any of the 31 previously published haplotypes of *R. diazi* (Figure 3). The haplogroups recovered were strongly supported with posterior probabilities of 1. This result indicates that the pellets are not from *R. diazi*, but from a *Sylvilagus* species. However, we could not definitively assign the identity of the pellets to a particular species of *Sylvilagus* because both *S. floridanus* and *S. cunicularius* are known to occur in the area and there are no available cytochrome b sequences for *S. cunicularius* in GenBank.

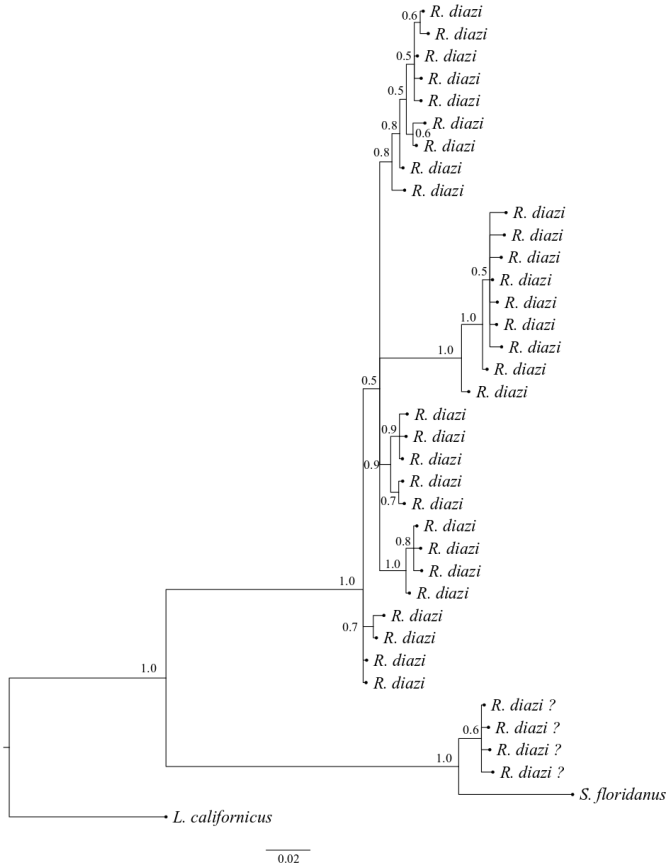

Figure. 3. Bayesian phylogenetic tree of haplotypes of *Romerolagus diazi* and haplotypes obtained from putative samples of *R. diazi*? Numbers in branches represent posterior probabilities.

PCR assays and the phylogenetic analysis based on a fragment of mitochondrial DNA of fecal pellets provide evidence that *R. diazi* does not occur in the Nevado de Toluca. According to Velázquez and Guerrero (2019), native populations of this species have never existed in the Nevado de Toluca. However, at the beginning of the 1970s, a group of volcano rabbits that was confiscated by the General Direction of Flora and Fauna was released in a locality situated at the foothills of the volcano, called "Raíces" (A. Velázquez, personal communication). A few years later, an individual was collected in the same locality and deposited in the mammalian collection of the Instituto Politécnico Nacional (Cervantes *et al.* 1990; Ceballos *et al.* 1998). Since then, there is only indirect evidence where similar pellets to those of the volcano rabbit were adjudicated to the species (Ceballos *et al.* 1998; Monroy-Vilchis *et al.* 2020). Our molecular analysis suggests that fecal pellets that resemble those of *R. diazi* (Cervantes *et al.* 1990) correspond indeed to a *Sylvilagus* species. Consequently, our results support the hypothesis that *R. diazi* is not currently distributed in the Nevado de Toluca (Velázquez and Guerrero 2019). However, more studies are needed in order to confirm our results. Specially because several studies have shown that the use of fecal pellets has been a reliable tool for monitoring the volcano rabbit.

The fact that pellets collected in the Nevado de Toluca were previously misidentified as *R. diazi* pellets is notable, given that in other areas where the species is distributed, the use of pellets has been a reliable tool for monitoring volcano rabbit, as well as to document aspects of habitat use (Velázquez and Heil 1996; Hunter and Cresswell 2015; Rizo-Aguilar *et al.* 2015), diet (Cervantes and Martínez 1992; Martínez-García *et al.* 2012) and physiological stress (Rizo-Aguilar *et al.* 2014), even though *S. cunicularius* and *S. floridanus* are present. Further studies are needed to understand why pellets of *Sylvilagus* converged to the morphology of pellets of *R. diazi* in the Nevado de Toluca.

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