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Antimicrobial resistance genes in wild coyotes (*Canis latrans*) from Guanacaste and Central conservation areas of Costa Rica

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A B S T R A C T

Effective antimicrobial resistance (AMR) surveillance and mitigation efforts require locally adapted approaches that consider both ecological and public health dimensions. The coyote (*Canis latrans*) is widely distributed in Costa Rica, especially in buffer zones between conserved ecosystems and human settlements, and frequently interacts with local communities and domestic animals. In this study, we investigated the potential of *C. latrans* as a sentinel species for environmental AMR monitoring in Costa Rica. Fecal samples were opportunistically collected from two conservation areas, Guanacaste (ACG) and Central (ACC), between March and August 2022. Species confirmation was achieved through mitochondrial D-loop sequencing. We evaluated the prevalence and spatial distribution of nine antimicrobial resistance genes (ARGs) in microbial DNA extracted from coyote scat deposition. Multidrug-resistant microbiomes were detected in 74% of the samples (26/35). ARG prevalence was higher in regions with greater human population density, with detection rates of 23% in the ACC compared to 13% in the ACG. Analysis of the most frequently detected genes (*sul*II, *tet*Q, *tet*Y, and *cat*AI) revealed significant differences in gene abundance and distribution between conservation areas (PERMANOVA, F = 3.944, p = 0.0084). Additionally, host genetic analysis using coyote mtDNA provided the first preliminary insight into population genetics in Costa Rica, revealing low haplotype (n = 3), haplotype diversity (Hd = 0.606), and nucleotide diversity (π = 0.0099). This study contributes baseline data to guide conservation and public health policy in the region, providing valuable information for controlling AMR in Costa Rica.

1. Introduction

The coyote (*Canis latrans*) is a widely distributed species inhabiting border zones, buffer areas between conserved ecosystems, and human settlements. It exhibits remarkable plasticity across a wide range of environments, including those affected by anthropogenic changes [1]. In Costa Rica, coyotes have expanded into new territories, frequently occurring near urban areas where deforestation and the expansion of monocultures and livestock production have facilitated the incorporation of domestic animals into their diet [2]. As a result, today it can be found across all ecosystems in the country [3].

Habitat fragmentation due to urban expansion, degradation of the natural environment, expansion of agricultural production, and the decline of apex predators have increased coyote presence in buffer zones and human-modified landscapes [1]. Such environmental pressures can alter population dynamics and reduce genetic diversity in wildlife populations [4]. In addition, ecological disturbances modify trophic interactions, increasing coyotes' reliance on lower-quality food resources, which contribute to immunosuppression, alterations in nutrient flow within the biological community, and an increased predisposition

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to disease acquisition [5].

The movement of coyotes between natural and modified environments increases their interactions with domestic animals and humans [6]. These interactions may facilitate the circulation of pathogens and antimicrobial resistance within wildlife populations and surrounding ecosystems [7–9]. In addition, coyotes can contribute to the dissemination of antibiotic resistance across ecosystems once resistant bacteria or resistance genes are present in their microbiota, potentially spreading through trophic interactions and environmental exposure [10,11].

Numerous global studies have documented the widespread occurrence of ARGs in wildlife, emphasizing the need for continuous monitoring of antibiotic resistance in diverse ecosystems [12]. In this context, Costa Rica has emerged as a leader in Central America in antimicrobial resistance research, with several studies reporting the presence of ARGs in wildlife and across multiple environments [12–17]. Because coyotes frequently move between natural habitats and human-modified landscapes, interacting with domestic animals and anthropogenic resources, they may serve as effective terrestrial sentinel species for monitoring emerging diseases and antimicrobial resistance in conserved ecosystems [18,19].

Evaluating antimicrobial resistance (AMR) in environmental contexts is important because ecosystems and wildlife can serve as reservoirs and dissemination pathways for antibiotic resistance genes (ARGs) [11,20]. Furthermore, understanding the genetic diversity of species and their connected populations provides phylogenetic information that supports future studies of biodiversity and population structure [21]. From a conservation medicine perspective, integrating pathogen surveillance with population genetic analyses provides valuable insights into ecosystem health and wildlife resilience. However, studies simultaneously evaluating antimicrobial resistance and population genetic structure in wildlife remain limited, particularly in Neotropical ecosystems.

To address this gap, this study analyzed noninvasive fecal samples from *C. latrans* in two key conservation regions of Costa Rica: the Guanacaste Conservation Area (ACG) and the Central Conservation Area (ACC). Polymerase chain reaction (PCR) based molecular techniques were applied to perform a preliminary assessment of population structure and to detect the presence of antibiotic resistance genes (ARGs) associated with sulfonamides (*sul*I, *sul*II), phenicols (*cat*AI, *cat*AII), beta-lactams (bla_{TEM}), tetracyclines (*tet*Q, *tet*W, *tet*Y), and quinolones (*qnr*S). Subsequently, we analyzed whether ARG concentrations differed within and between the two conservation areas and explored potential associations between ARG concentrations and landscape variables.

To our knowledge, this is the first study in Central America to integrate ARG surveillance with genetic characterization of a generalist mammalian species. The results establish a molecular baseline that can inform conservation planning, guide ecosystem protection measures, and support the integration of conservation medicine principles into wildlife management strategies. Ongoing monitoring of genetic diversity and ARG prevalence is important for optimizing antibiotic stewardship and supporting adaptive ecosystem management. This integrative approach underscores the interconnectedness of human, animal, and environmental health, aligning with the principles of One Health and reinforcing the need for cross-sectoral conservation efforts [22].

2. Methods

2.1. Study area

The sampling was conducted in two conservation areas managed by the Costa Rican government through the National System of Conservation Areas (SINAC). In the ACG, the sample sectors included: Santa Elena, Santa Rosa, Pocosol, and Horizontes. Samples were simultaneously collected in the Costa Rican high-altitude forests: Turrialba Volcano, Irazú Volcano, and the Cascajal de Vázquez de Coronado in the ACC. Both regions are characterized by great biological diversity, including multiple ecosystems such as tropical forests, cloud forests, and rainforests, with the ACG also containing dry forests. The distance between the two conservation areas is approximately 200 km. The closest urban area to ACC is San José, the capital of Costa Rica, located approximately 18 km away, whereas the nearest urban center to ACG is the city of Liberia, located approximately 30 km away. Distances between conservation areas and between each conservation area and the nearest urban settlements were estimated using straight-line measurements in Google Earth Pro (Google LLC, Mountain View, CA, USA). The two regions differ in surrounding human population pressure, with ACC located closer to the Central Valley, where most of Costa Rica's population is concentrated. These areas have been subjected to human activities, including intensive use of agrochemicals and fertilizers, diversion of river courses, and contamination of water sources [14]. These variables enable an investigation of the relationship between the presence of antibiotic resistance genes (ARGs) and the proximity of human activities, as mediated by the niche of the mesopredator coyote (Fig. 1).

2.2. Sample collection

Potentially distinguishable scats from terrestrial carnivorous species were collected opportunistically between March and August 2022 ($n = 53$, 28 samples from ACG and 25 from ACC). All samples were collected and analyzed with authorization from the Costa Rican government (R-CM-UNA-004-2022-OT-CONAGEBIO) and in compliance with the ethical requirements of the Universidad Nacional (UNA) for research and funding support. Sample identification and selection were based on previously reported habitat and activity patterns [23], as well as on the physical characteristics of scats, associated footprints, and camera-trap data, which confirmed the presence of coyotes in the area. Each fecal sample was described according to its morphological components, including fruit, hair, bones, and seeds (Supplementary Material, Fig. 1S, Table 1S), and was individually stored in 50 mL conical tubes, appropriately labeled with identification (ID), date, and GPS coordinates. The samples were then transported, maintaining the cold chain at 4 °C, to the Regional Institute for Studies in Toxic Substances (IRET) for storage at −80 °C until their subsequent analysis at the Genomic Analysis Laboratory (LAGen) of the School of Biological Sciences, Universidad Nacional, Costa Rica. Species identity was validated through mitochondrial DNA (mtDNA) analysis. A chi-square test was used to assess the relationship between sampling location and the occurrence of dietary items (fruit, bone, grass, and hair) (Supplementary Material, Table 1S).

2.3. Fecal DNA extraction

DNA extraction was performed using the QIAamp DNA Stool Mini Kit (Qiagen), according to the manufacturer's instructions. The concentration of extracted DNA was determined using a UV–visible microvolume spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific). DNA integrity was evaluated through electrophoresis by 1% *w/v* agarose gel, using GelRed (Biotium) and GeneRuler 1 kb DNA Ladder (Thermo Fisher Scientific Inc.).

2.4. PCR amplification for species identification

To confirm the species identity of the collected feces as *C. latrans*, segments of the D-loop mtDNA control region were amplified by endpoint PCR using forward primer SIDL (5′-TCTATTTAAAC-TATTCCCTGG-3′) and reverse primer H3R (5′CCTGAAG TAGGAACCAGATG-3′) [17]. PCR amplification was performed in a 25 µL reaction containing 12,5 µL of Dream Taq Hot Start Master Mix (2×) (Thermo Fisher Scientific Inc.), 0.8 µL of each primer, 2.5 µL of template DNA, and 8,4 µL nuclease-free water to complete the final volume. The

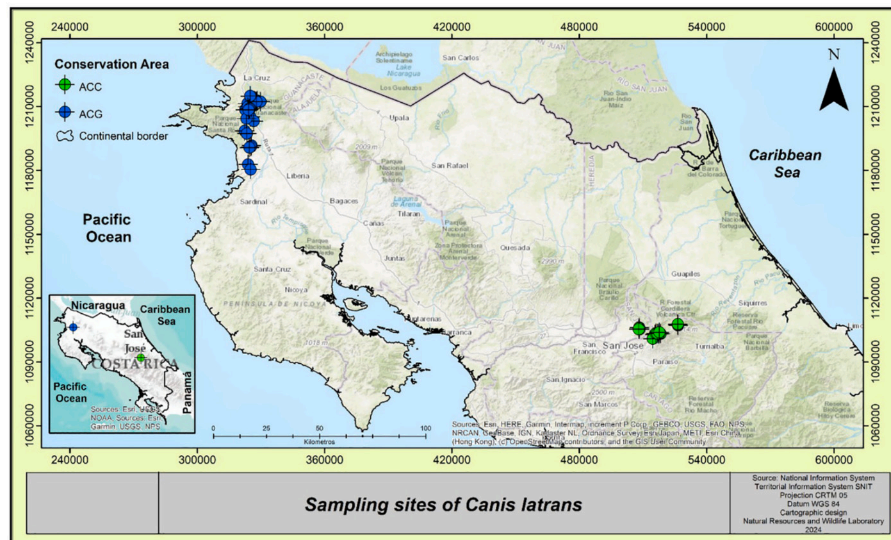

Fig. 1. Geographic distribution of the sampling sites ($n = 53$) within the two conservation areas of Costa Rica included in this study. A. Guanacaste Conservation Area (ACG), including the Santa Elena, Santa Rosa, Pocosol, and Horizonte's sectors. B. Central Conservation Area (ACC), including the Turrialba Volcano, Irazú Volcano, and Cascajal de Vázquez de Coronado sectors. Green and blue dots indicate the specific locations where fecal depositions were collected in each sector. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

amplification conditions are detailed in Table 2S (Supplementary Material). PCR amplicon sizes of 315 to 401 base pairs (bp) were considered potentially positive for carnivorous species. These positive samples were purified by absolute ethanol precipitation and subsequently confirmed for molecular identity by Sanger sequencing on a 3500 Genetic Analyzer (Applied Biosystems). Sequencing was performed with the same SIDL-H3R primers [24] using BigDye Terminator v3.1 chemistry and a BigDye XTerminator purification kit, following the manufacturer's instructions. The sequences obtained were manually edited in Geneious Prime® (v.2023.2.1, Biomatters Ltd.). Electropherograms were visually reviewed, ambiguous base calls were corrected when possible, and low-quality bases at the 5′ and 3′ ends were trimmed to retain only high-confidence base calls.

2.5. Antibiotic resistance genes (ARGs) detection

The presence of bacterial DNA was confirmed by amplifying the 16S rDNA gene with primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) [25] in all positive fecal samples from *C. latrans*. PCR amplification was performed in a 25 μL reaction containing 12.5 μL of DreamTaq PCR Master Mix (2×) (Thermo Fisher Scientific Inc.), 0.8 μL of each primer, 1.5 μL of template DNA (2,8 dilution), and nuclease-free water to complete the final volume. Thermal cycling conditions are provided in Table 2S (Supplementary Material). The amplification was considered positive for bacteria if the amplicon size was 1000–1400 bp. Then, fecal DNA extracts were analyzed by PCR for the presence of genes encoding resistance to sulfonamides (*sul*I), phenicols (*cat*AI, *cat*AII), beta-lactams (bla_{TEM}), and tetracyclines (*tet*Q, *tet*W, and *tet*Y) (Table 2S, Supplementary material). Finally, representative PCR products from each amplified ARG were selected and purified to confirm their identities by Sanger sequencing. A total of 38 PCR products were sequenced, including *tetQ* ($n = 6$), *tetY* ($n = 5$), bla_{TEM}($n = 6$), *tetW* ($n = 7$), *sulI* ($n = 7$), and *catA1* ($n = 7$). Sequencing reactions were performed using the same primers employed for PCR amplification.

2.6. Quantitative PCR

ARGs were screened using quantitative PCR (qPCR) on a CFX96 Real-Time System (Bio-Rad, USA). The reaction mixture consisted of 5 μL of PowerTrack™ SYBR Green Master Mix (Thermo Fisher Scientific), 0.4 μL of each forward and reverse primer (10 μM) (Invitrogen), 0.25 μL of Yellow Sample Buffer (Thermo Fisher Scientific), 2.95 μL of RNase-free water, and 1 μL of template DNA, for a final volume of 10 μL. Negative controls were included in each run by replacing template DNA with RNase-free water.

The 16S rRNA gene was amplified to confirm the presence of bacterial genetic material in each sample. Samples were considered validated when a 10-fold dilution yielded a cycle threshold (Ct) value <25 [26]. The specific primers and amplification conditions for each ARG are detailed in Table 3S (Supplementary material). MacVector software (v18.6) was used to analyze CARD (Comprehensive Antibiotic Resistance Database) control sequences and generate and test potential primer pairs, known primer pairs, and their optimal PCR conditions [27]. An approximation for the number of ARG copies was performed for each triplicate analysis, where the average was used in the formula [28]:

$$\log_{10}(\text{ARG\%}) = 2 + 0,33^{*}(\text{Ct16s} - \text{CtgenARG})$$

Ct16SrRNA was the cycle threshold for bacterial determination, CtARG was the cycle threshold for each gene, and 0.33 was the mean slope for all the genes tested. The results were expressed as the log10 of the hypothetical percentage of bacteria that each gene represents in the percent load of ARG, because the exact time since deposition of the fecal samples is unknown, variations in DNA degradation and bacterial abundance may occur. Therefore, ARG abundance was estimated by relative quantification, normalized to the bacterial load in each sample. Then, samples were also classified into “multiresistant microbiome,” defined as at least three ARGs encoding resistance to different groups of antimicrobials, and “non-multiresistant microbiome” [29].

2.7. Taxonomic assignment by molecular approach

A phylogenetic reconstruction analysis was performed as a taxonomic assignment approach to confirm species identity by aligning the sequences obtained in this study (33 sequences with >80% high-quality base pairs). In addition, mitochondrial D-loop reference sequences retrieved from the GenBank database (http://www.ncbi.nlm.nih.gov/genbank) belonging to closely related species within the genus Canis (*C. lupus*, *C. lycaon*, and *C. rufus*) from North America were included to provide a comparative framework and ensure accurate taxonomic

assignment of the environmental sequences. For the molecular detection of ARGs in fecal samples identified as *C. latrans*, a phylogenetic reconstruction was also conducted using sequences selected based on the closest BLAST hits available in the NCBI database, together with curated ARG sequences retrieved from the Comprehensive Antibiotic Resistance Database (CARD, https://card.mcmaster.ca). Sequence matrices were initially generated for each gene (mitochondrial D-loop or ARGs) through multiple sequence alignment using MAFFT v7.490 [30]. Conserved regions were then selected using less-stringent parameters in Gblocks v0.91, eliminating divergent or uninformative sites from each alignment. IQ-TREE (https://www.hiv.lanl.gov/content/sequence/IQTREE/iqtree.html). A multicore program was used to construct phylogenetic trees using the maximum likelihood (ML) algorithm. The best-fit substitution models were selected based on the Akaike Information Criterion (AIC): HKY + F + G4 for the D-loop dataset (AIC = 2398.2137) and TVMe+G4 for the ARG dataset (AIC = 13,575.677). The parameters used for phylogenetic inference of both trees were free-rate heterogeneity and node support calculated using the ultrafast bootstrap method with 10,000 replicates [31]. The FigTree v1.4.4 program was used to visualize and edit the topology inference.

2.8. Genetic diversity analysis

The genetic variation was evaluated through the determination of the number of haplotypes (h), haplotype diversity (Hd), and nucleotide diversity (π) [23]. This information provided a preliminary estimate of genetic diversity in coyotes in Costa Rica. A dataset was obtained using Geneious Prime® software with an MAFFT v7.490 alignment, employing the iterative refinement method (G-INS-i) and the 1PAM parameter k = 2 [30]. Subsequently, DnaSP software was used to analyze DNA sequence variation in the *C. latrans* population in this study [32].

2.9. Distribution patterns of ARGs

To identify the most prevalent and representative resistance genes, a heat map was generated. Principal Component Analysis (PCA) was performed to identify the genes that contribute most to the overall variance and to reduce potential multicollinearity among variables. Based on the eigenvalues, the genes with the greatest weight were selected to subsequently assess whether their concentrations differed within and between the two conservation areas. Additionally, a canonical correspondence analysis (CCA) was conducted to assess associations between various types of antibiotic resistance genes (ARGs) and landscape variables, including forest cover area, river and road lengths, and the extent of agricultural activities specifically cattle ranching, poultry farms, and pig farming farming within a 37 Km^2 buffer surrounding each sampling site. All variables were expressed in Km^2 and subsequently log10-transformed to achieve appropriate scaling. River and road lengths represent the distance from each sample to the nearest river or road, while river density corresponds to the total length of rivers per Km^2. Livestock density was calculated as the number of animals per Km^2, based on data from the national census conducted by the National Service of Animal Health (SENASA) in 2018. The previously mentioned variables were transformed to log10 to achieve scaling. Differences in ARG concentrations between conservation areas, as well as among ARGs within the same area, were evaluated using Permutational Multivariate Analysis of Variance (PERMANOVA) and Dunn's test. These tests were performed because the data did not follow a normal distribution. Statistical analyses and graphical visualizations were carried out in R software (v4.3.2), employing the *ggplot2* package (v3.4.4) and the ggdist package (v 3.3.2).

3. Results

3.1. Fecal morphology

The fecal samples of *C. latrans* exhibited a variety of components upon morphological description (Supplementary material, Fig. 1S, Table 1S). According to chi-square analysis, ACG samples had a 60% probability of containing nance fruit (*Byrsonima crassifolia*), giving them a yellowish-brown colour. In contrast, ACC samples had a 72% probability of containing hairs predominantly very dark brown (df = 1, p = 0.05).

3.2. Molecular identification of C. latrans in non-invasive scat sampling

Of the 53 samples collected, 35 belonged to *C. latrans* (66%, 35/53), with a product size of 360 bp. Five came from domestic dogs (*Canis lupus familiaris*) (9%, 5/53) and four from gray foxes (*Urocyon cinereoargenteus*) (8%, 4/53). It was impossible to amplify the mitochondrial D-loop housekeeping gene in nine samples (17%, 9/53), likely due to the presence of PCR inhibitors in the feces, such as bile salts and complex polysaccharides [33]. An average of 96% to 99% molecular identity was found in the GenBank database for each species, as determined by BLAST searches (Supplementary material, Table 5S).

The phylogenetic reconstruction was performed as a taxonomic assignment approach to confirm species identity. The analysis included reference *C. latrans* sequences from North America retrieved from GenBank, together with the mitochondrial D-loop fragments generated in this study, confirming the identification of the Costa Rican fecal samples as *C. latrans*. This reconstruction was therefore interpreted strictly as a species-level assignment rather than as an inference of evolutionary relationships. The analysis revealed three clusters; the first comprises most samples from ACC, with one sample from ACG showing 97% branch support. The second cluster included sequences predominantly from ACG, four sequences from ACC, and one sequence from Louisiana, with a branch support of 73. Finally, the third cluster comprised samples from the ACG and was associated with one sample from North Carolina with a branch support of 95. The *C. latrans* samples from the ACC were more closely related to those from Texas, Ohio, and Manitoba, Canada. In contrast, the ACG samples showed greater affinity to individuals recorded in Louisiana, North Carolina, and the Great Lakes region (Fig. 2).

Of the 35 samples that belonged to *C. latrans*, only 33 were analyzed in DnaSP due to sequence quality constraints in two samples. The genetic variation observed in these samples indicated that the two sampled habitats shared a total of three haplotypes (h = 3), with a haplotype diversity (*Hd*) of 0.606, a variance of 0.0024, and a nucleotide diversity (π) of 0.0099 (Table 1).

3.3. Detection and prevalence of ARGs in C. latrans fecal samples

In total, 33 fecal samples from *C. latrans* were used for ARG detection. Eighteen samples identified as dogs, foxes, or unamplified samples were excluded, as well as two *C. latrans* sequences due to low sequence quality. End-point PCR amplification of the seven ARGs evaluated showed that 97% of the assessed samples (n = 35: ACG n = 19 and ACC n = 16) amplified at least one of the ARGs analyzed. The bla_{TEM} gene was detected in most of the samples (97%), while *cat*AII was not identified. According to the MDR (multi-drug resistance) classification [34], only two samples (M24, M40) were not resistant to three or more of the four antibiotic classes tested. The results are shown in Tables 2 and 4S.

In the quantitative analysis, all samples showed amplification of at least three of the nine genes evaluated. Four samples (11%) amplified all

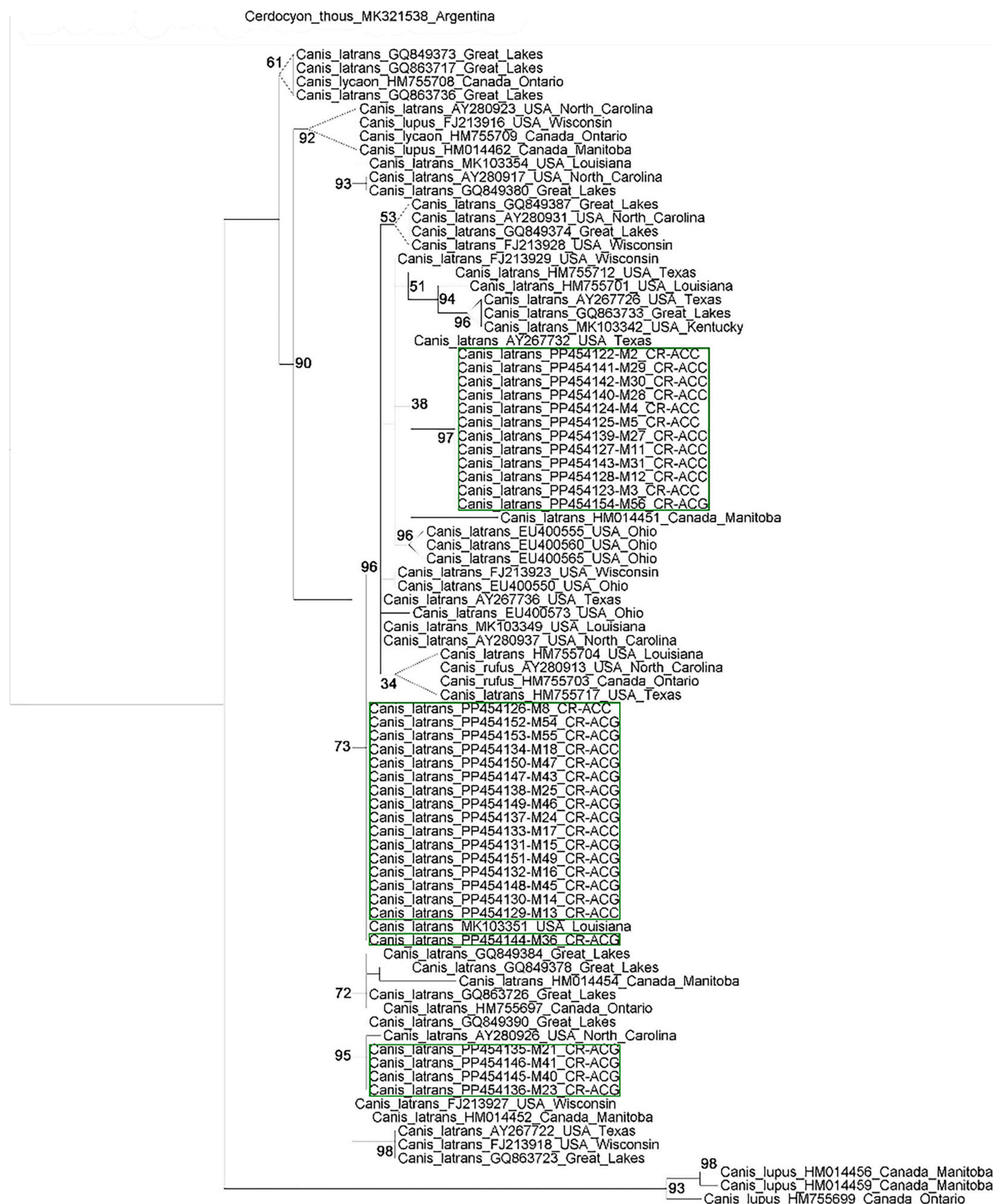

Fig. 2. Taxonomic assignment tree based on mitochondrial D-loop fragment and maximum likelihood (ML) approach, confirming the molecular identity of the fecal DNA. Sequences from this study are highlighted in green (NCBI accession numbers are provided in Supplementary Table 5S). Closely related *Canis* species were included to provide phylogenetic context for species-level identification. *Cerdocyon thous* (MK321538) was used as an external group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

targeted genes. Overall, 74% of the samples exhibited a multidrug-resistant microbiome, with 23% of positives in the ACC ($n = 34/144$) and 13% in the ACG ($n = 23/171$). All samples tested positive for *tet*W, *tet*Q, and *sul*I. The highest amplification signal was recorded for *tet*Q (6.44) in sample M54 from the ACG. A heatmap was created to visualize the relative abundance of ARGs across individual samples, with colour intensity representing the percentage of each gene in each sample and white indicating the absence of amplification. This analysis revealed variability in ARG profiles among samples; however, consistent with PCR results, *tet*W, *tet*Q, and *sul*I remained the most abundant genes (Fig. 3).

3.4. Molecular detection of ARGs in fecal samples

A phylogenetic reconstruction was generated using ARG sequences retrieved from the NCBI and the CARD database. Reference sequences were selected based on the closest BLAST matches to validate the identity of ARGs detected by end-point PCR and qPCR. The resulting topology showed clustering according to the six ARG families analyzed (Fig. 4). Sequences corresponding to tetracycline resistance genes (*tet*Q, *tet*W, and *tet*Y) clustered with homologous ARG sequences retrieved from the NCBI and CARD databases, which have been widely reported in environmental and gastrointestinal bacterial communities. The β-lactamase gene bla_{TEM} was grouped with reference sequences commonly detected in members of the family *Enterobacteriaceae*. Similarly, the sulfonamide resistance gene sulI clustered with previously reported ARG

Table 1
List of *C. latrans* individuals that support each mitochondrial *D-loop* fragment haplotype. The absolute frequency in the sample (Fr) and geographic distribution of haplotypes is also indicated. ACG: Guanacaste Conservation Area, and ACC: Central Conservation Area.

	Location	Fr	Sample
Haplotype 1	ACC	11	M2, M3, M4, M5, M11, M12, M27, M28, M29, M30, M31.
	ACG	1	M56.
Haplotype 2	ACC	4	M8, M13, M17, M18.
	ACG	13	M14, M15, M25, M45, M46, M47, M49, M54 M16, M24, M55, M36, M43.
Haplotype 3	ACG	4	M21, M23, M40, M41.

Table 2
Prevalence of evaluated ARGs in the samples collected from the Guanacaste Conservation Area (ACG) and the Central Conservation Area (ACC) in Costa Rica.

Antibiotic resistance gene	Sampled area		Prevalence	
	ACG	ACC	n	%
bla$_{TEM}$	18	16	34	97%
*sul*I	17	16	33	94%
*tet*W	16	16	32	91%
*tet*Y	7	16	23	66%
*tet*Q	7	13	20	57%
*cat*AI	7	11	18	51%
*cat*AII	0	0	0	0%

sequences associated with diverse Gram-negative bacteria. The phenicol resistance gene *cat*AI was grouped with homologous sequences reported in environmental and commensal bacterial communities. Overall, the phylogenetic placement of the sequences obtained in this study supports the molecular identity of the amplified ARG fragments detected in environmental samples of coyote feces from the Guanacaste and Central Conservation Areas of Costa Rica.

3.5. Distribution patterns of ARGs

Principal Component Analysis (PCA) of ARG profiles identified two principal components, which explained 98.77% of the total variance. The first component (PC1; 95.33% variance) was primarily influenced by *cat*AII and *sul*II, while the second component (PC2; 3,44% variance) was mainly associated with *tet*Q and *tet*Y. These findings suggest distinct grouping patterns among samples based on specific ARGs (Table 3).

Significant differences in antimicrobial resistance gene (ARG) concentrations were observed between the two conservation areas (PERMANOVA, F = 3.944; p = 0.0084) (Fig. 5). In the case of *sul*II gene, concentrations were higher in the ACC compared to the ACG, while *tet*Q exhibited similar distributions across both areas, suggesting a broader environmental dissemination. *cat*AII showed the lowest abundance across all samples. Meanwhile, *the sulII and tetY* genes show differences in concentration between the two conservation areas (Dunn's post hoc test, $p < 0.038$), indicating different exposure to antibiotics.

Canonical Correspondence Analysis (CCA) was performed to explore the associations between ARGs and environmental variables. The first two canonical axes accounted for 87% of the variance (57.61% for the first axis and 29.39% for the second), indicating strong explanatory power. The genes *tet*Y, *tet*W, *cat*AI, and *cat*AII were associated with proximity to roads, whereas *sul*II was linked to riverine environments. Additionally, *bla*$_{TEM}$ showed a weak and indirect association with livestock- and pork-dominated areas, whereas *tet*Q and *sul*1 exhibited broader distributions across the ordination space. These results suggest heterogeneous drivers underlying ARG distributions, reflecting both environmental persistence and anthropogenic influences (Fig. 6).

4. Discussion

Antimicrobial resistance (AMR) is a growing concern with substantial public health implications [35]. However, its impact on natural ecosystems, particularly in protected areas, remains poorly understood, despite previous reports suggesting a link between its occurrence in environmental bacterial populations and levels of human activity [12]. Hence, identifying a key sentinel species for environmental monitoring is fundamental. The coyote is a suitable candidate due to its ecology, meso-carnivore status, opportunistic feeding habits, wide territorial range, and high mobility. These traits enable coyotes to traverse diverse ecosystems, including urban, rural, and wild areas, making them well-suited for environmental monitoring [36].

This study explores the potential of using coyotes as a sentinel species. Although ARGs naturally occur in the environment due to production by certain microorganisms, their presence may also indicate prior exposure to antibiotics through contact with pharmaceutical residues derived from human activity, agricultural practices, or contaminated food sources [37]. Once colonized, coyotes could act as reservoir hosts, naturally amplifying antibiotic resistance genes (ARGs) within their microbiota. These genes can subsequently be transmitted between bacterial populations, both within and across species, through horizontal gene transfer mechanisms [11].

4.1. Molecular identification and haplotype assignment in coyote populations

Given the difficulties in capturing wild animals, it is necessary to adopt more passive methods for collecting environmental samples. Fecal samples and DNA, for example, are easier to obtain and do not require

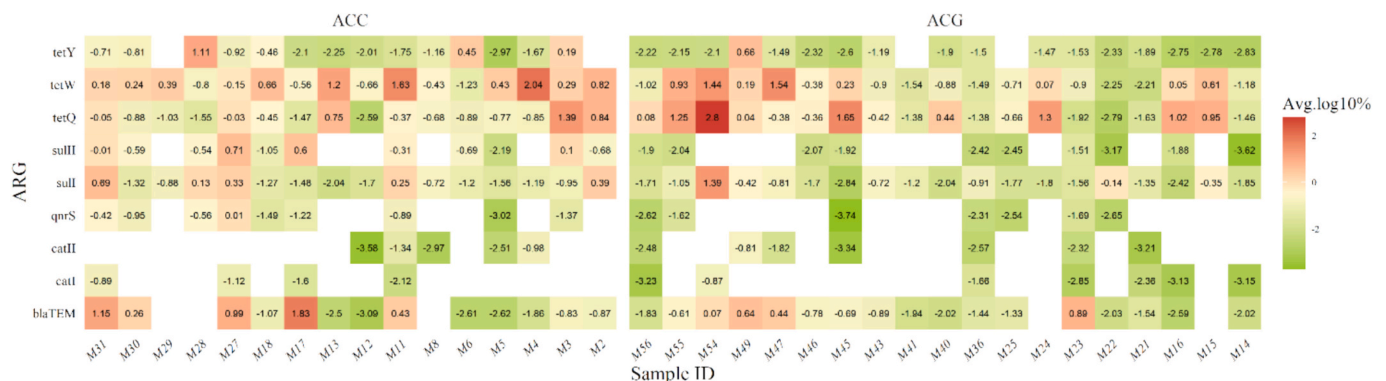

Fig. 3. Antibiotic resistance genes (ARGs) in fecal samples of coyotes (*C. latrans*) from Costa Rica collected from the Central Conservation Area (ACC) and the Guanacaste Conservation Area (ACG). The scale indicates the relative concentration of each gene. The white colour represents the absence of ARGs.

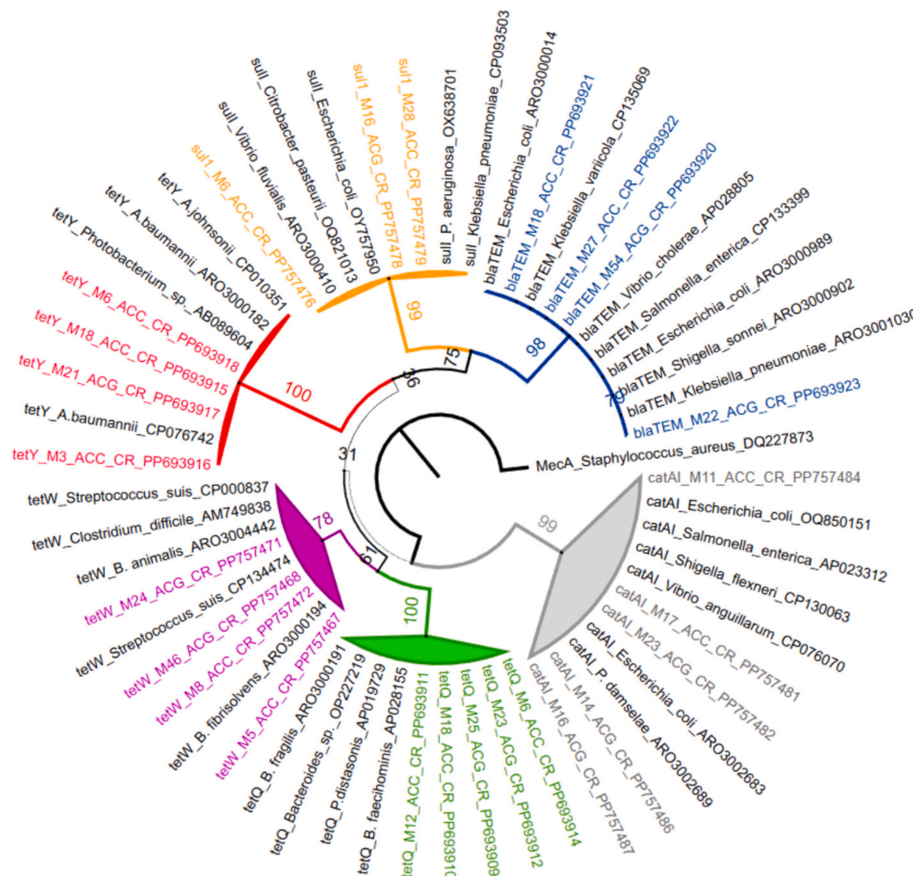

Fig. 4. Maximum likelihood (ML) phylogenetic tree of antimicrobial resistance genes (ARGs) detected in *C. latrans* fecal samples. Colors indicate the ARG families analyzed (orange: *sulI*, pink: *catAI*, blue: *blaTEM*, gray: *catA1*, green: *tetQ*, purple: *tetW*, and red: *tetY*). A total of 57 sequences were included in the analysis, comprising 38 sequences generated in this study and deposited in GenBank (PP693909 to PP693925, PP757467 to PP757487, Table 5S), as well as 21 reference sequences retrieved from the NCBI and 11 from the CARD database. *Staphylococcus aureus* clone 249 *mecA* (DQ227873) was used as the outgroup. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Eigenvalues of principal component (PC) analysis for antimicrobial resistance genes (ARGs) of coyotes (*C. latrans*) from Costa Rica. The genes with the highest weight are in bold.

ARG	PC1	PC2
bla_{TEM}	0.155140	−0.283977
*cat*AI	−0.051282	−0.364123
*cat*AII	**0.440568**	−0.013508
*qnr*S	0.439747	−0.057981
*sul*I	0.433581	−0.042573
*sul*II	**0.440954**	−0.013560
*tet*Q	0.045060	**−0.628346**
*tet*W	0.439847	−0.005182
*tet*Y	−0.088861	**−0.621599**

animal capture [38]. This approach also facilitates the characterization of their diet by identifying fruit residues, hair, bones, and seeds, which are consistent with the diverse diet documented in coyotes. As omnivorous generalist predators, coyotes adjust their foraging behavior to the available resources in their habitat, consuming a wide variety of foods, including rodents, lagomorphs, domestic mammals, birds, fruits, vegetables, arthropods, and ungulate carcasses [39].

On the other hand, amplification and sequencing of the mitochondrial D-loop region confirmed the taxonomic assignment of the collected feces [24], identifying 35 scat samples as *C. latrans*. Samples corresponding to domestic dogs (9%) were more frequent in the ACC, potentially due to human activities in the surrounding area, such as tourism and agriculture [40]. This confirms and validates the efficacy of the fecal collection methodology employed, ensuring the non-invasive collection of samples for *C. latrans*. This finding corroborates existing literature, underscoring the extensive distribution and adaptability of this meso-carnivore in multiple ecosystems [41].

This study reveals, for the first time, preliminary genetic variability in *C. latrans* populations from two locations in Costa Rica. The phylogenetic reconstruction revealed some genetic differentiation between the two populations, with samples forming separate clusters in the mtDNA D-loop based taxonomic assignment. This pattern could be explained by geographic distance and habitat fragmentation, as well as by the coyote's adaptive capacity and remarkable ecological versatility. In the evaluation of genetic diversity, heterozygosity is generally considered beneficial, as it promotes greater genetic variability and adaptability, leading to individuals with enhanced vigor, fertility, and disease resistance [42]. Our preliminary genetic analyses revealed a potential degree of consanguinity in the sampled population, which contrasts with previously reported data from the Mexican population [43]. However, mitochondrial DNA reflects maternal inheritance; the patterns observed here should be interpreted as preliminary signals of population structure. Therefore, the current sample size is insufficient to draw definitive conclusions [43,44].

4.2. ARG detection, prevalence, and distribution patterns in coyote scat

Regarding the AMR results, it is important to note that 74% of the evaluated samples exhibited a multi-resistant microbiome [29,45]. This high prevalence suggests that antimicrobial resistance genes are widely distributed in the studied environments. Although previous studies have

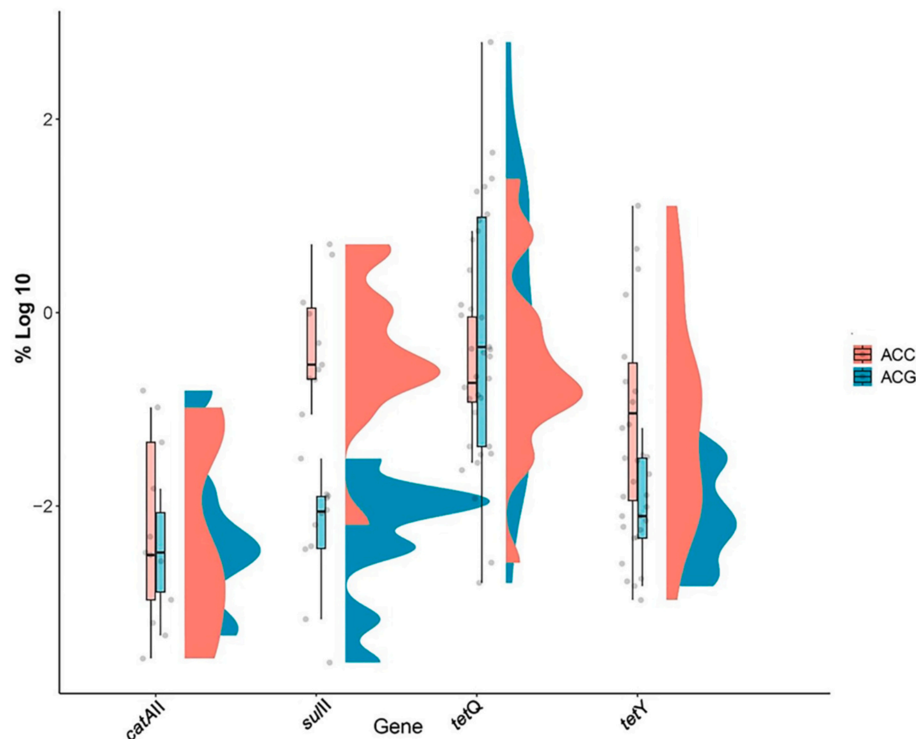

Fig. 5. Comparison of antimicrobial resistance gene (ARG) concentrations between the Central Conservation Area (ACC) and the Guanacaste Conservation Area (ACG) in *C. latrans* fecal samples. Boxplots show medians and interquartile ranges; violin plots illustrate the distribution of $\log_{10}$-transformed concentrations. Significant differences based on PERMANOVA ($F = 3.944$, $p = 0.0084$) and post hoc tests ($p < 0.05$) were detected for *sul*II and *tet*Y.

reported associations between anthropogenic activities and increased ARG occurrence [46], our results indicate that these genes are present across both conservation areas, highlighting the complex ecological dynamics that influence ARG distribution in natural ecosystems. Previous studies in Costa Rica indicated that genes conferring resistance to tetracyclines, beta-lactamases, and sulfonamides are among the most frequently detected across diverse environments, including wildlife, synanthropic animals, intensive crops, livestock, aquaculture farming, soil, water, and food sources [12–14,16,17,45,47]. Our findings are consistent with these results, showing that these ARG families were also present in the coyote samples.

The high prevalence of multidrug-resistant (MDR) microbiomes observed in the coyote fecal samples may be attributed to their opportunistic feeding behavior. Coyotes forage across a wide range of environments, including rural, peri-urban, and urban areas, exposing them to diverse microbial communities that carry antimicrobial resistance genes [1,36]. Previous studies have similarly documented the presence of MDR bacteria in wildlife, underscoring the role of these species in shaping the ecology of antimicrobial resistance [7,10].

Our data highlight the importance of evaluating each ecosystem individually, even in a small country like Costa Rica, as the persistence of antimicrobial compounds and resistant bacteria may be influenced by factors such as antimicrobial use, host microbial communities, and geographic, environmental, and climatic conditions [28]. The sequential use of PCR and qPCR is a response to the methodological work involved in screening and quantifying ARGs. Consequently, two strategies for detecting ARGs were standardized to expand the potential of AMR environmental monitoring.

Furthermore, in this study, we estimated the relative abundance of ARGs in samples from two different environments. Canonical correlation analysis of geographic variables suggested correlations between specific genes and road, water, and agricultural activities. The high frequency of genes encoding tetracycline resistance could be explained by the use of tetracycline in livestock and agriculture [48]. This exposure may be attributable to the use of roads by both cattle, which defecate along them, and coyotes. This suggests an elevated risk of ARG transmission at the human-wildlife interface, intensified by the proximity of ACC to the Greater Metropolitan Area, which is characterized by extensive human activity and accounts for nearly 50% of the national population [49]. The genes *tet*Y and *tet*W were associated with areas near roadways, supporting previous findings on the role of human infrastructure in facilitating the spread of ARGs. This pattern aligns with growing evidence that proximity to anthropogenic activity increases the risk of ARG acquisition [14,47]. Other authors suggested that tetracycline resistance is typically associated with human fecal samples, making their presence a marker of human/animal fecal environmental contamination [50]. Furthermore, this study is consistent with evidence showing that coyotes, along with other wildlife such as raccoons (*Procyon lotor*), *Virginia opossums* (*Didelphis virginiana*), and golden jackals (*Canis aureus*), exhibit a higher prevalence of tetracycline-associated ARGs among medium-sized carnivores (Canidae) [51].

The bla_{TEM} gene showed a weak and indirect association with areas characterized by livestock and swine production, as indicated by its position away from the main anthropogenic gradients in the CCA ordination. This location in the biplot suggests that the variance in its detection is not directly explained by local livestock densities, but rather by a widespread presence across the landscape. Such a pattern is consistent with studies that report frequent detection of bla TEM at the wildlife-livestock interface [52]. Similar results have been documented in Costa Rican wildlife, including *Tapirus bairdii* [15], and in surface water samples from regions inhabited by wild felids [13], suggesting long-term environmental persistence of this resistance gene regardless of immediate livestock pressure.

For sulfonamide genes, both were amplified in samples with high abundance in ACC and ACG. However, canonical correspondence analysis revealed that *sul*II was the sole gene that demonstrated differential amplification between the two conservation areas and was associated with riverine environments. Other studies carried out in the

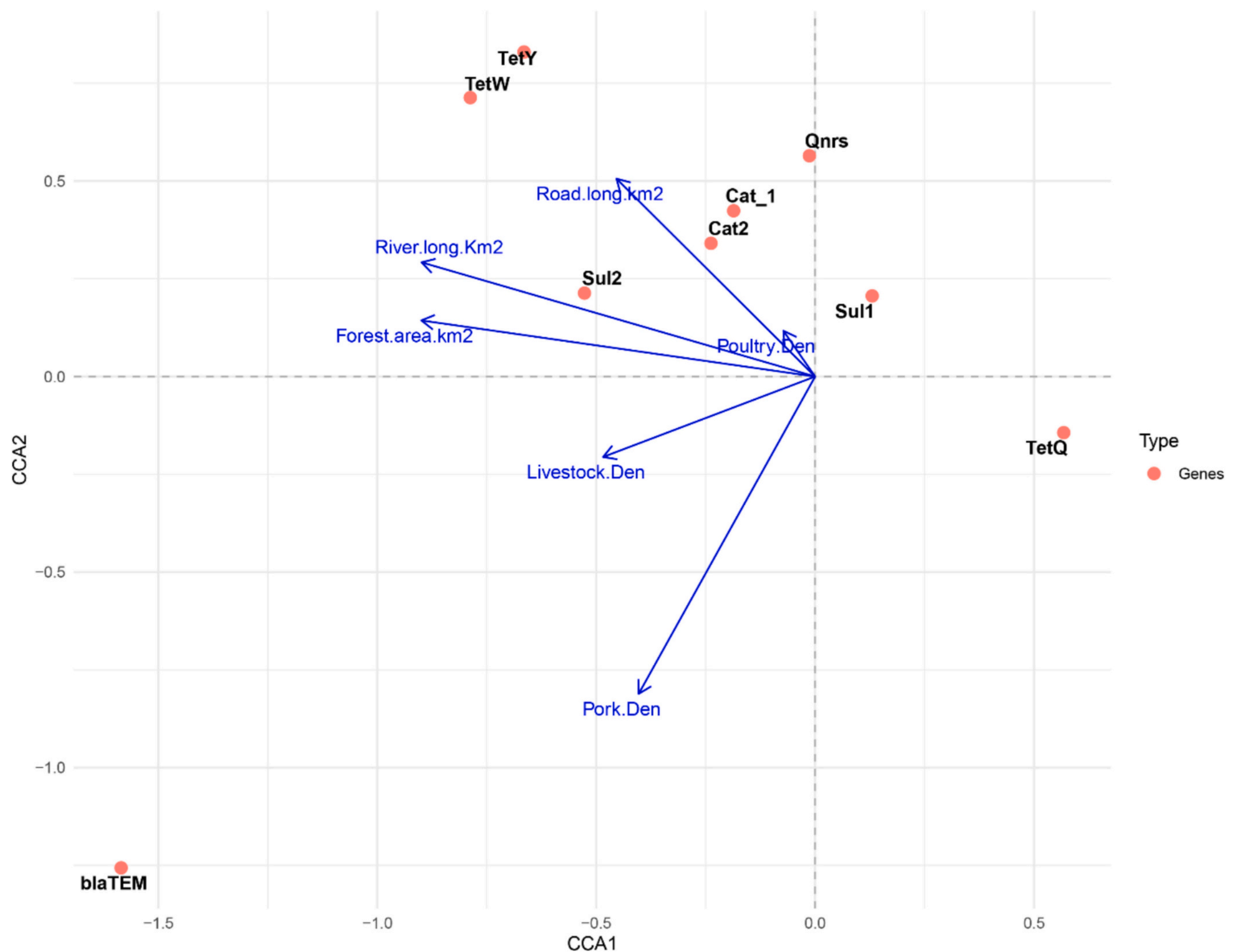

Fig. 6. Canonical Correspondence Analysis (CCA) showing associations between antimicrobial resistance genes (ARGs) and environmental variables in coyote (*C. latrans*) fecal samples from Costa Rica. Red points represent ARGs and blue arrows represent environmental variables (Long: length in square kilometers km^2, and Den: density of livestock farms). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

country have reported the presence of both genes in synanthropic wildlife and feline fecal samples, highlighting the environmental circulation of sulfonamide resistance determinants [14]. This relationship reflects ecological differences, particularly the markedly higher annual rainfall differences in the Central Valley compared to Guanacaste (2500–5500 mm) [49]. Given that water bodies are recognized as major vehicles for the dissemination of antimicrobial resistance, these findings raise important questions about the role of hydrological factors in ARG distribution [53].

The presence of genes encoding resistance to phenicol was identified in both conservation areas. The prevalence of antibiotic resistance in these species remains a concern, particularly given that chloramphenicol use has been banned in food-producing species in the country [54,55]. The possible association between phenicol genes and roads, as well as the previously reported *catAI* in urban pigeons in 2021 [45], underscores the notion that historical antibiotic use and environmental contamination could sustain the circulation of resistance genes independently of current antibiotic practices. In Costa Rica, chloramphenicol had a historical role in both human medicine and veterinary practice as documented in early studies evaluating pharmaceutical formulations in Central America [56], with official product registrations in the National Service of Animal Health, SENASA, during the early 2000s [54]. However, concerns regarding its hematological toxicity led to a progressive prohibition in the livestock sector. By 2009, chloramphenicol was already listed as a drug not authorized for use in animals [57], in line with international food safety regulations by Codex Alimentarius and the European Union. This evidence demonstrates that restrictions on animals were consolidated before 2009, although the general ban was reinforced in 2021 [58], which may explain why the continued detection of the catA1 gene in environmental matrices could reflect the persistence of selective pressure from its historical use. It is also possible that this result is related to the use of other phenicols, such as florphenicol, in both human and veterinary practice [59].

5. Conclusion

This study presents the first evidence of antimicrobial resistance genes (ARGs) in coyotes from two conservation areas in Costa Rica and provides a preliminary mitochondrial D-loop analysis of this species in the country. Furthermore, mitochondrial D-loop analysis revealed low but detectable genetic differentiation between the two regions. Future studies incorporating larger sample sizes and nuclear markers such as microsatellites or SNPs would provide a more comprehensive understanding of population structure than mitochondrial D-loop sequences alone. The presence of multidrug-resistant microbiomes was confirmed, with the highest prevalence observed in one region, influenced by

proximity to densely populated urban areas. These findings offer important baseline data on coyote population structure, and together, support the value of coyotes as sentinel species for environmental AMR monitoring, given their adaptability and frequent contact with human-altered landscapes. Therefore, continued monitoring of ARGs and population genetics in wildlife can support integrated conservation efforts in Costa Rica.

Key findings and implications from this study include:

Coyotes (*C. latrans*) can serve as effective sentinel species for tracking antimicrobial resistance at the human-wildlife interface, especially in areas subject to land-use change, where ARG diversity may increase. In this context, mitochondrial D-loop sequences were used for the first time to characterize maternal lineages in Costa Rica coyote populations, providing baseline genetic data for the species in the country.

Furthermore, this study also provides the first documented evidence of antimicrobial resistance genes in *C. latrans* in Costa Rica and Central America, revealing notable ARG diversity. A high proportion of samples exhibited multidrug resistance, with the highest prevalence observed in the ACC region, underscoring the need for public initiatives that promote the prudent use of antibiotics across the human, veterinary, and agricultural sectors. Finally, despite the prohibition on chloramphenicol use in food-producing animals, the detection of phenicol resistance genes suggests environmental reservoirs and highlights the need for further investigation into potential sources of contamination.

CRediT authorship contribution statement

Lina Puentes-Sánchez: Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. **Rodolfo Umaña-Castro:** Writing – review & editing, Writing – original draft, Visualization, Data curation. **Kinndle Blanco-Peña:** Writing – review & editing, Writing – original draft, Funding acquisition. **Carolina Sáenz-Bolaños:** Writing – review & editing, Project administration. **Victor Montalvo-Guadamuz:** Writing – review & editing. **Kevin Lloyd-Alcock:** Writing – review & editing. **Daniel Sánchez-González:** Writing – review & editing, Investigation. **Kari Brossard-Stoos:** Writing – review & editing. **Denis Salas-González:** Formal analysis, Data curation. **Francisco Quesada-Alvarado:** Writing – review & editing, Formal analysis, Data curation.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the authors used DeepL and ChatGPT to improve readability and language. After using this tool/service, the authors reviewed and edited the content as needed and took full responsibility for the published article.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.onehlt.2026.101431.

Data availability

Data will be made available on request.

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