Detection of Jaguar (*Panthera onca*) From Genetic Material in Drinking Water

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Jaguar (*Panthera onca*) are of conservation concern and occur at very low densities in the northern portion of their range in northern Mexico and the southwestern United States. Environmental DNA sampling to detect genetic material from drinking water may be an effective approach for jaguar detection in these arid landscapes. Here we develop a qPCR assay for the detection of jaguar mitochondrial DNA, show that large quantities of DNA (mean 66,820 copies/L) can be found in the drinking water of captive animals, and observe detectable levels of DNA (80 copies/L) in a wild habitat with known jaguar populations. We suggest that environmental DNA sampling may represent a useful, complementary sampling tool for detection of rare jaguars, although effective application would require careful consideration of DNA persistence time in the environment.

Keywords: environmental DNA, eDNA, non-invasive genetics, wildlife, monitoring

INTRODUCTION

Jaguar (*Panthera onca*) are of conservation concern, particularly in the northern portion of their range in northern Mexico and the southwestern United States (Brown and González, 2000). Within the United States, the species is Federally protected (U.S. Federal Register 37 FR 6476) and occurs at extremely low abundances, with individuals rarely being detected. Current jaguar monitoring in the United States primarily uses camera traps and genetic testing of scat samples which may be located with the use of detection dogs (Culver, 2016). These approaches can be labor intensive, involving hundreds of cameras and tens of thousands of images (Culver, 2016). Additional sampling tools could help build a better understanding of jaguar distributions at the northern margin of their range and enable more effective protection of rare individuals and management of their habitat.

Environmental DNA (eDNA) sampling—the inference of species presence from genetic material in the environment—has been rapidly adopted for rare aquatic species sampling (e.g., Cristescu and Hebert, 2018; Sepulveda et al., 2020). Recent studies suggest that under the right circumstances, eDNA sampling of water may also be an effective approach for the detection of terrestrial species (e.g., Williams et al., 2018; Franklin et al., 2019). Harper et al. (2019) and Sales et al. (2020) documented the ability to detect terrestrial species from water samples, in some cases with detection rates comparable to camera trapping. Although there are likely multiple routes of transmission, DNA deposited when drinking is likely a major contributor of terrestrial species eDNA in aquatic habitats (Rodgers and Mock, 2015). In arid landscapes, drinking water may be scarce and represent relatively concentrated sources of jaguar eDNA.
Here we describe a hydrolysis assay developed for detection of jaguar mitochondrial DNA in environmental samples, then demonstrate this tool on water samples taken from known and suspected jaguar drinking water sources.

**METHODS**

Using sequence data from the NCBI GenBank database and the R (R Core Development Team, 2020) package DECIPHER (Wright, 2016), we designed and tested candidate quantitative PCR (qPCR) primers in silico, targeting jaguar mitochondrial DNA to the exclusion of 14 other felid species that either potentially co-occur with jaguar in North America or are closely related to jaguar (Table 1). Although only four jaguar mitochondrial genome sequences were included in this initial screen, there are low levels of genetic diversity and structure within the northern range of this species (e.g., Wultsch et al., 2016). We then selected one of these primer sets within the mitochondrial gene ATP6 for hydrolysis probe development using PrimerExpress software (Thermo Fisher Scientific). These primers were selected based on a scan of the entire mitochondrial genome, but this same locus has also been found to have particularly good species discriminatory power across Carnivora (Chaves et al., 2012). We also conducted an in silico analysis of primer specificity through a BLAST (Atschul et al., 1990) search against GenBank to identify any unexpected cross-amplification with non-felids, but we did not attempt to use an in silico approach to evaluate potential cross-amplification of other rare felids which are not expected to be found in North America. We sourced the primers from Integrated DNA Technologies and obtained a FAM-labeled minor groove-binding (MGB) non-fluorescent quencher (NFQ) probe from Thermo Fisher Scientific (Table 2).

We validated assay specificity in vitro by testing tissue-extracted DNA from felids which may be found in the southwestern U.S. (Felis catus, Puma concolor, Lynx rufus, Leopardus pardalis, Puma yagouaroundi) or are closely related to jaguar [Acinonyx jubatus (n = 2), Leptailurus serval (n = 2), Panthera leo (n = 3), Panthera tigris (n = 3)]. These samples were sourced from the collections of partners for other projects and were collected in accordance with any relevant animal care guidelines. We extracted DNA using the DNeasy Blood and Tissue Kit (QIAGEN), quantified DNA using a Qubit fluorometer (Invitrogen), and diluted extracts to approximately 0.1 ng genomic DNA per microliter (0.4 ng gDNA per reaction), then analyzed with qPCR as described below. There was 0.1 ng genomic DNA per microliter (0.4 ng gDNA per reaction), fluorometer (Invitrogen), and diluted extracts to approximately 47 mm diameter, 1.5 micron pore size glass microfiber.

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We tested the ability of the assay to amplify jaguar DNA by analyzing DNA extracted from 10 jaguar scats that were collected in Belize and included in Menchaca et al. (2019; AC; Sackler Institute for Comparative Genomics). We tested the ability to detect jaguar DNA from drinking water by analyzing DNA extracted from six water samples provided to captive animals at the Phoenix Zoo in Phoenix, Arizona (n = 5; Figure 1) and Banana Bank Lodge in Belize (n = 1, Table 3). This sampling required minimal animal disturbance and sampling at the Phoenix Zoo was approved by the Arizona Center for Nature Conservation Research Committee. Finally, we analyzed five water samples from ponds in Belize where wild jaguar are known to occur regionally, but whose recent use of these habitats is unknown. Water samples were collected as described in Carim et al. (2016b). Briefly, for each sample, 5 L of water was drawn through a 47 mm diameter, 1.5 micron pore size glass microfiber filter paper using an electric peristaltic pump at the sampling site. The filter paper was then stored in silica desiccant until received at the lab (<2 weeks) when they were archived at −20°C until extraction. Filters were handled with sterile forceps. Filter cups, forceps, and all other sampling supplies were prepared in a dedicated, restricted-access room at the National Genomics Center for Wildlife and Fish Conservation and were sterilized using a 50% household bleach solution and ultrapure water (4% hypochlorite solution).

We then extracted DNA from half filters using DNeasy Blood and Tissue Kit (QIAGEN) with modifications as described in

### Table 1: List of species and GenBank accession numbers for sequences used in assay development.

| Species       | Latin Name               | GenBank accession number(s) | Assay mismatches |
|---------------|--------------------------|-----------------------------|------------------|
| Jaguar        | Panthera onca           | KM236783, NC022842, KF48564, KP022264 | 0                |
| Domestic cat  | Felis catus              | U20753.1                    | 14               |
| Cougar        | Puma concolor           | NC016470                    | 13               |
| Bobcat        | Lynx rufus               | NC014456                    | 16               |
| Ocelot        | Leopardus pardalis      | NC028315                    | 15               |
| Margay        | Leopardus wiedi          | NC028318                    | 16               |
| Andean        | Leopardus jacobita       | NC028322                    | 15               |
| mountain cat  | Leopardus Geoffroyi      | NC028320                    | 12               |
| Kodkod        | Leopardus guigna         | NC028321                    | 15               |
| Oncilla       | Leopardus tigrinus      | NC028317                    | 15               |
| Pampas cat    | Leopardus colocolo       | NC028314                    | 16               |
| Jaguarundi    | Puma yagouroudni         | NC028311                    | 13               |
| Tiger         | Panthera tigris          | KP022268                    | 13               |
| Leopard       | Panthera pardus          | KP001507                    | 12               |
| African lion  | Panthera leo             | KP001506                    | 12               |

Assay mismatches indicate number of base pair differences between sequence(s) and jaguar assay (primers and probe).

### Table 2: Assay oligonucleotide sequences.

| Oligo         | Sequence                                   |
|---------------|--------------------------------------------|
| Forward       | 5′-AACAACTCGTCTAATCTCACTTCAACAG-3′          |
| Reverse       | 5′-CCAAACAGTTTGGTGACTCAATAGC-3′            |
| Probe         | 5′-FAM-CTTGGGGTCTATACTCT-MGB-NFQ-3′        |
Carim et al. (2016a). In steps 1 and 2, we doubled the amount of ATL and proteinase K, and incubated samples for 48 h. We doubled the amount of AL buffer in step 3 and added 400 µl of ethanol simultaneously. We repeated step 4 loading using a single spin column for each sample until all elution for a given sample had been processed through the spin column. Additionally, we loaded each filter using sterile forceps into a QIAshredder spin column and centrifuged for 2 min × g. The elution from the QIAshredder was also loaded and processed through the corresponding spin column for that sample. Between steps 5 and 6, we added an additional wash of 500 µl ethanol and centrifuged for 2 min at 20,000 × g. In step 6, we increased spin time to 4 min. In step 7, we eluted DNA in 100 µl of 70°C TE and allowed to incubate at room temperature for 10 min before the final centrifuge step. All environmental samples were extracted in a dedicated space where no high concentration sources of DNA are handled.

Quantitative PCRs contained 7.5 µl TaqMan Environmental Master Mix 2.0 (Thermo Fisher Scientific), 900 nM each primer, 250 nM hydrolysis probe (also known as “taqman” probe), 4 µl template DNA, and molecular grade water to a total volume of 15 µl. Environmental and scat samples (described below) also contained an internal positive control template and assay to test for the presence of PCR inhibitors (indicated by a > 1 CT shift in amplification relative to the control samples; TaqMan Exogenous Internal Positive Control Kit from Thermo Fisher Scientific). On the PCR plate with environmental samples, a triplicate no-template control was included to test for contamination. All reactions were run in triplicate. We optimized primer concentrations by testing all possible combinations of forward and reverse primers at 100, 300, 600, and 900 nM concentration (n = 16 combinations) and selecting the combination with the lowest CT value and highest end-point fluorescence (900:900 nM combination).

We also quantified jaguar mitochondrial DNA based on comparison with a standard curve. The standard curve was a dilution series (2, 10, 50, 250, 1,250, 6,250, and 31,250 copies per reaction) of a synthetic jaguar gene covering the target amplicon (gBlock; Integrated DNA Technologies) which was quantified on a Qubit 2.0 fluorometer (dsDNA Broad Range Kit; Invitrogen). Each dilution level was run in six replicates.

### RESULTS AND DISCUSSION

In silico and in vitro testing showed our assay to be highly specific against potential non-target taxa in North America. Additional specificity testing of the assay may be necessary prior to application in the southern portion of the species’ range. Although there was low-level amplification in one African lion tissue sample, we were able to verify that this was due to sample contamination using a synthetic gene covering the target amplicon. In our in silico assessment, the closest non-felid present in North America was Equus caballus (GenBank...
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et al., 2019). Environmental DNA sampling might also become
taxon, where investment in camera traps is impractical (Harper
probability areas, such as at the presumed range periphery of a
that eDNA sampling might be particularly useful in lower
southwestern United States and northern Mexico.

All drinking water samples were strongly positive with a
mean of 6,682 copies/reaction (range 3,251–14,254), or 66,820
copies/L of water. One of the five unknown Belize pond water
samples was also positive, but at much lower concentration (8
copies/reaction or 80 copies/L of water), which is in line with
concentrations of other terrestrial species detections from eDNA
(e.g., Williams et al., 2018). Negative control reactions all showed
no amplification.

The high concentrations of jaguar DNA in captive animal
drinking water sources (over 30,000 copies/L) suggest that eDNA
sampling may have reasonable detection probabilities in more
natural settings, as long as sampling has occurred soon after
the site was visited by an animal. Harper et al. (2019) and Sales
et al. (2020) used eDNA sampling for detection of terrestrial mammals
using a community-wide, metabarcoding approach. They found that low-density, wide-ranging taxa like jaguar
tend to have lower detection rates than common and evenly
distributed taxa. Aquatic organisms, even at low abundances,
provide a constant input source of eDNA. In contrast, terrestrial
species deposit their DNA at a drinking site over a very brief
period of time. Thus, detection probably relies on sampling
within hours or days of visitation—before the DNA has degraded
beyond detectable levels.

In the studies described by Harper et al. (2019) and Sales
et al. (2020), drinking water sources were abundant on the
landscape. For sampling low-density jaguars, a key landscape
characteristic influencing detection might be the number of
drinking water sources within a single home-range. When there
are only several water sources, the mean frequency with which
each water source is visited is relatively high. When there are
many, it could be days or weeks between visits to any one
site. Hence, we see eDNA sampling for rare terrestrial species
as being particularly promising in arid landscapes such as the
southwestern United States and northern Mexico.

Camera trapping, in contrast to eDNA sampling, provides
a more continuous view of habitat use. However, the initial
equipment investment and ongoing maintenance costs may limit
sampling effort over large scales. Other authors have suggested
that eDNA sampling might be particularly useful in lower
probability areas, such as at the presumed range periphery of a
taxon, where investment in camera traps is impractical (Harper
et al., 2019). Environmental DNA sampling might also become
more useful as recent advances in continuous or autonomous
eDNA sampling are refined and provide a more continuous view
of DNA inputs (e.g., Yamahara et al., 2019; Kirtane et al., 2020).

We imagine eDNA sampling being a useful complement to
existing sampling approaches and conveniently added to other
monitoring efforts on the landscape. For example, technicians
performing vegetation surveys in a remote area could also
opportunistically collect water samples without much additional
effort (e.g., <20 min per water sample in this study). Much of
the cost of eDNA sampling, particularly in remote areas, comes
from travel time (Smart et al., 2016). In the arid southwestern
United States the relative cost for sample collection versus
analysis might be even greater. Often in these remote and arid
landscapes, the hydroperiod of ephemeral springs and tanks are
unknown. There is a high level of risk during dry seasons of
hiking into a remote habitat only to find that there is no water.
This risk is mitigated when technicians are performing other
fieldwork simultaneously.

Further savings might be gained when the genetic analyses
for jaguar is conducted on eDNA samples initially collected
to survey for the presence of other species. In this case,
the cost of sample collection and DNA extraction is already
covered. For example, the jaguar qPCR assay described here
might complement a more community-wide survey effort with
metabarcoding. Metabarcoding tends to be less sensitive than
single-species qPCR and even reasonably abundant taxa might
be missed (Harper et al., 2019; Sales et al., 2020). Targeted
analysis used in conjunction with metabarcoding has been found
to be a useful approach for picking up rare-but-important taxa
(Simmons et al., 2016). Alternatively, an analysis for jaguar eDNA
could even be included as part of a larger high throughput qPCR
(HT-qPCR) panel which includes assays for detection of various
aquatic and terrestrial species (Wilcox et al., 2020). In this case,
the expense invested in analyzing samples for the presence
of jaguar is likely <10% of the overall laboratory cost. Generally,
our hope is that this new molecular tool can be leveraged in creative
ways to build cost-effective study designs composed of multiple,
complementary sampling approaches.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online
repositories. The names of the repository/repositories and
accession number(s) can be found in the article/supplementary material.

ETHICS STATEMENT

The animal study was reviewed and approved by Arizona Center
for Nature Conservation Research Committee.

AUTHOR CONTRIBUTIONS

TW, AC, KM, KZ, and MS conceived the study design. TW, JD,
TF, DM, and KZ conducted sampling and analyses. All authors
contributed to writing of the manuscript.
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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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