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MOLECULAR GENETIC IDENTIFICATION OF A MEXICAN ONZA SPECIMEN AS A PUMA *(PUMA CONCOLOR)*

PETER A. DRATCH
Laboratory of Viral Carcinogenesis, National Cancer Institute,
Frederick Cancer Research and Development Center,
Building 560/Room 21-105, Frederick, Maryland 21702, U.S.A.
and
National Fish and Wildlife Forensics Laboratory
Ashland, Oregon 96520, U.S.A.

WENDY ROSLUND
National Fish and Wildlife Forensics Laboratory
Ashland, Oregon 96520, U.S.A.

JANICE S. MARTENSON, MELANIE CULVER, AND STEPHEN J. O'BRIEN[1]
Laboratory of Viral Carcinogenesis, National Cancer Institute,
Frederick Cancer Research and Development Center,
Building 560/Room 21-105, Frederick, Maryland 21702, U.S.A.

ABSTRACT: Tissue samples from an alleged Mexican Onza, shot in the western Sierra Madre in 1986, were subjected to several biochemical assays in an attempt to determine the specimen's relationship to felid species of North America. Protein analyses included isoenzyme electrophoresis and albumin isoelectric focusing. Mitochondrial DNA was assayed for restriction fragment lengths with 28 restriction enzymes, and the ND5 gene was sequenced. The resulting protein and mitochondrial DNA characteristics of the Onza were indistinguishable from those of North American pumas.

INTRODUCTION

In the Americas, there are two documented species of large cats: 1) *Puma concolor,* the puma, also called mountain lion, cougar, and panther in different regions of North America, and known as *leon* in Mexico; and 2) *Panthera onca,* the jaguar, or *tigre as* it is known south of the U.S. border.

[1] To whom correspondence should be addressed.

FIG. 1. —Onza shot in 1986 in western Sierra Madre, Mexico. (International Society of Cryptozoology.)

But in the western Sierra Madre of Mexico, locals speak of three species: *leones, tigres,* and *onzas.*

References to a third big cat in the Americas date from 1519 when Bernal Diaz del Castillo, a member of Cortez's conquering army, visited Montezuma's palace, which included a zoo (Greenwell 1987). Diaz del Castillo wrote of seeing "tigers" as well as "lions" of two kinds. Jesuit missionaries of the 18th century also described a cat that was longer and leaner than the puma. Modern hunters say it has the coat color of a puma, a leaner body, longer legs, and a very aggressive nature. Interest in the Onza was rekindled in the 1960's with the publication of a book by Arizona hunter Robert Marshall (1961).

On January 1, 1986, Andres Rodriguez Murillo, a rancher in the state of Sinaloa, northwestern Mexico, shot a cat at night which he at first thought to be a jaguar. Upon examination, he discovered that it was not a jaguar, and seemed different from a puma (Greenwell 1986, 1987). Rodriquez remembered a recent visit to the area by two Americans (Richard Greenwell and Robert Marshall) looking for unusual big cats. He took the cat to a neighbor, Manuel Vega, who, upon examining the animal, immediately identified it as an Onza. The animal had the coloration of a puma, was lean, and had long legs. Vega contacted Ricardo Urquijo, a well-known rancher and an associate of Greenwall, who immediately had the specimen placed in a

fisheries cold storage in Mazatlan. This was reportedly the first complete specimen of a supposed Onza collected in over a decade (Fig. 1).

Greenwell, University of New Mexico mammalogist Troy Best, and graduate student Ned Gentz flew to Mazatlan in February, 1986, and examined the animal at the Regional Diagnostic Laboratory of Animal Pathology of the Mexican Ministry of Agriculture. The specimen was dissected, and tissue samples were removed, preserved, and brought back to the U.S. for analysis.

METHODS

Frozen tissue samples (liver, heart, and blood) from this specimen were sent to Stephen J. O'Brien's laboratory at the National Cancer Institute by Best and Greenwell. DNA was extracted from the heart sample (Sambrook 1989), and was included in a mitochondrial DNA restriction fragment analysis with nine other cat species: lion, tiger, leopard, snow leopard, clouded leopard, jaguar, puma, cheetah, and bobcat. DNA from several individuals of each species (n = 2-10) was used for the analysis. Twenty-eight restriction enzymes were used; southern blots were made and hybridized with a radioactively-labelled molecular clone of domestic cat mitochondrial DNA (O'Brien et al. 1990, Menotti-Raymond and O'Brien 1993).

The mitochondrial NADH dehydrogenase subunit-5 gene (ND5) was amplified by polymerase chain reaction (PCR; Mullis and Faloona 1987) for 30 cycles, using 2.0mM $MgC1_2$ and 49°C annealing temperature, with primers MCND5-1 and MCND5-2 modified by the addition of M13 tails for sequencing. The resulting PCR products were prepared for sequencing using the PRISM dye primer procedure (Applied Biosystems, Inc.). Sequencing was done in the ABI 373A DNA Sequencer.

For isoenzyme electrophoresis, crude protein extracts were made, and isoenzyme electrophoresis was performed according to published protocols (O'Brien 1980, Newman et al. 1985). Isoelectric focusing of tissue homogenates was performed on the Pharmacia PhastGel system using precast gradient gels reconstituted in urea for pH range 5-6 (Hoesch and Dratch 1992), followed by Western blotting using anti-bovine serum albumin (Towbin, Straehelin, and Gordon 1979).

RESULTS

Mitochondrial DNA (mtDNA) is a cytoplasmic chromosome of approximately 17,000 nucleotides that has proven very useful in forensic and genetic identification studies (Avise 1994, Baker et al. 1993, Menotti-Raymond and O'Brien 1993, O'Brien et al. 1990). By digesting mtDNA with several restriction enzymes, each of which recognizes a unique 4-6 nucleotide sequence when it occurs in the mtDNA sequence, a series of "restriction" fragments are identified which are unique to each species. They can be diagnostic for subspecies as well. Individuals of the same species share a large fraction (95—100%) of mtDNA restriction fragments, while individuals of distinct species share fewer identical fragments, largely as a consequence of mutational divergence over time.

TABLE 1.—Mitochondrial DNA fragment sharing with Onza specimen.

Species comparison	Total restriction fragments scored	Number restriction fragments shared	Percent restriction fragments shared
Onza : cheetah	120	34	28.3%
Onza: jaguar	135	24	17.7%
Onza: puma	122	122	100.0%
lion: leopard	148	54	36.4%
lion : puma	131	28	21.3%
lion :jaguar	155	36	23.2%
lion: tiger	158	52	32.9%
tiger: snow leopard	143	60	41.9%

The mtDNA analysis revealed between 120 and 158 mtDNA restriction fragments for each of the felid species involved. An example of the comparison of several species' mtDNA restriction fragment patterns is illustrated below (Fig. 2). Pairwise comparisons of the frequency of restriction fragments shared between species are presented in Table 1.

These results reveal a perfect identity between the mitochondrial DNA restriction fragment pattern of the Onza sample and four North American pumas *(P. concolor)* used in the study. The next closest relationship was between a tiger and a snow leopard (41.9%), followed by lion : leopard (36.4%), and lion : tiger (32.9%).

We chose to sequence the ND5 gene, which is known to be one of the more rapidly evolving genes in the mitochondrial genome (Lopez et al. in preparation), and, therefore, most likely to present sequence polymorphisms between closely related individuals. DNA fragments of 245 base pairs of the ND5 gene were generated by PCR from the Onza DNA, from more than 50 western North American pumas (including two from southern Mexico), from one jaguar, and from one cheetah. The nucleotide sequences were then compared. Fig. 3 shows the alignment of ND5 sequences (all the western North American puma sequences were identical, so only one puma sequence is shown). The Onza sequence was 100% identical to the western North American puma sequence, 87% similar to the cheetah, and 83% similar to the jaguar. Cheetah and jaguar sequences were 85% similar.

Since mitochondrial DNA is maternally inherited, the possibility still existed that the Onza specimen was a hybrid between a female puma and a male jaguar. However, several studies of nuclear-DNA encoded proteins and isoenzymes indicated this was unlikely. Researchers at Texas Tech University had earlier compared the electrophoretic mobilities of 19 isoenzymes, and found no differences between the Onza sample and west Texas pumas

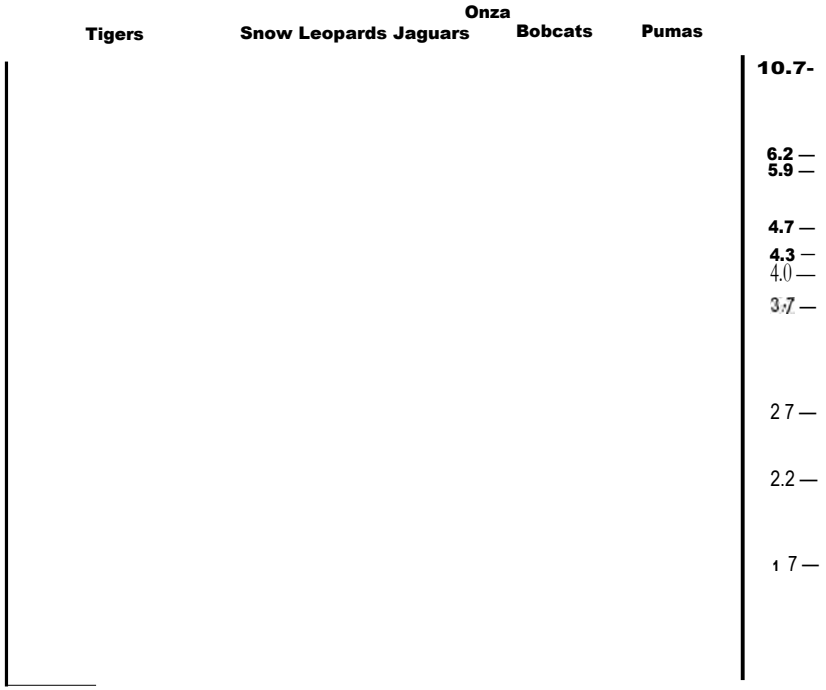

FIG. 2.—Restriction Fragment Comparison: Autoradiograph of mitochondrial DNA fragments produced with the restriction enzyme AccI, one of 28 restriction enzymes used in the band sharing analysis. Species are as indicated. Note intraspecific variation in snow leopards. Molecular weight markers (BamHI/EcoRI digest of adenovirus H DNA) are in the right lane; sizes are in kilobases.

(Richard Greenwell, personal communication). Prior to receiving the Onza sample, an isozyme study performed at the National Cancer Institute had revealed mobility differences for 16 isoenzymes between Texas pumas and jaguars (Stephen O'Brien and Janice Martenson, unpublished observations). If the Onza was a puma x jaguar hybrid, isoenzyme electrophoretic differences (e.g., hybrid protein molecules) would have been detected in the Onza sample by the Texas Tech researchers.

Isoelectric focusing on narrow range urea gels (pH 5-6) and immunoblotting for albumin showed that the Onza had a banding pattern consistent with 40 puma standards, and inconsistent with five jaguar and three cheetah standards (Fig. 4). The albumin isoelectric point of the Onza sample was also different from that of all other cat species analyzed: lion, tiger, snow leopard, clouded leopard, as well as Geoffroy's cat, jaguarundi, serval, fishing cat, lynx, bobcat, and domestic cat.


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puma     ATTCTACTTC TACCTATCAT CATATCCAAT ACCCAATTAT ACAAAAACAG TCTGTATCCC
onza
cheetah  __C____AT  .0.  .T.. O ----G -----  .T...GG..A CT.A..C...
jaguar   _____C___C____ T.0.... C .T. .C.  .T.  .A C____C T

puma     TACTATGTAA AAACTACAGT CTCCTACGCC TTCACCATCA GCATAATCCC AACCATAATA
onza
cheetah  C_____C A_____A_____T_____
jaguar   C--------- ----C---A-  ..T ---------- T-- GG-T---G--

puma     TTTATCTCTT CAGGGCAGGA AGCAGTCATC TCAAACTGAC ACTGACTGTC AATTCAAACC
onza
cheetah  _____T A_____A_____TA_____
jaguar   .....T---- .C..A ----- .A..A.T... ---------- T-A-- ---O......

puma     CTTAAACTGT CATTAAGCTT TAAAATAGAC TACTTCTCAA TCATCTTTAT CCCCGTAGCA
onza
cheetah  ____T A____C_____T_____CC .....
jaguar   ......T...  ....0----- ..T----- G- -------C.. ...T....

puma     CTATT
onza
cheetah  ....
jaguar   ..T.
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FIG. 3. —Alignment of ND5 nucleotide sequences from puma, Onza, cheetah, and jaguar. The dots indicate nucleotide identity to the puma sequence.

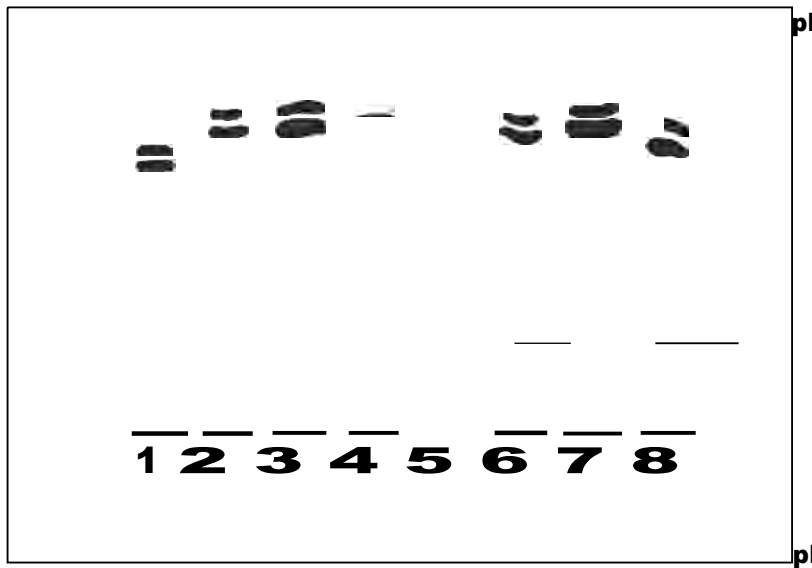

FIG. 4.—Scan of an immunoblot of albumins on narrow range isoelectric focusing gel. Lanes 1 and 5 are cheetah samples, lanes 2 and 6 are puma, lanes 3 and 7 are the Onza, and lanes 4 and 8 are jaguar.

DISCUSSION

Several hypotheses have been proposed on the taxonomy of the Onza (Greenwell 1987): 1) it is a new species closely related to the puma; 2) it is a subspecies of puma distinct from the puma subspecies already described in the region, *Puma concolor azteca;* 3) it is a hybrid between puma and jaguar; 4) as proposed by Helmut Hemmer, it represents a relict population of the Pleistocene North American cheetah, *Acinonyx trumani,* described by paleontologist Daniel Adams and believed to be extinct (Adams 1979, On 1969); and 5) it is a puma, adapted to the particular environment of the western Sierra Madre, or perhaps emaciated.

Greenwell's examination of *A. trumani* skulls demonstrated that th& 1986 Onza specimen, at least, was not a Pleistocene cheetah (Greenwell 1986), eliminating hypothesis four. The albumin isoelectric focusing and the isoenzyme data shows that the Onza specimen was indistinguishable from North American puma samples, and presented no evidence of hybridization, effectively eliminating hypotheses one, two, and three. The mitochondrial DNA studies revealed perfect identity between the mtDNA of the Onza specimen and western North American pumas, again eliminating hypotheses one and two.

Hypothesis five is best supported by our observations that the DNA and proteins obtained from this Onza sample had molecular characteristics indistinguishable from those of western North American pumas. Based upon these cumulative data, we must conclude that the 1986 Onza specimen was a puma, and did not represent a distinct, new, or relict species.

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