ISSN 0012-4966, Doklady Biological Sciences, 2009, Vol. 429, pp. 518–522. © Pleiades Publishing, Ltd., 2009.
Original Russian Text © V.V. Rozhnov, P.A. Sorokin, S.V. Naidenko, V.S. Lukarevskiy, H.A. Hernandez-Blanco, M.N. Litvinov, A.K. Kotlyar, V.G. Yudin, 2009, published in Doklady Akademii Nauk, 2009, Vol. 429, No. 2, pp. 278–282.

GENERAL BIOLOGY

Noninvasive Individual Identification of the Amur Tiger (*Panthera tigris altaica*) by Molecular-Genetic Methods

V. V. Rozhnov[a], P. A. Sorokin[a], S. V. Naidenko[a], V. S. Lukarevskiy[a], H. A. Hernandez-Blanco[a], M. N. Litvinov[b], A. K. Kotlyar[b], and V. G. Yudin[c]

Presented by Academician D.S. Pavlov July 15, 2009

Received July 16, 2009

DOI: 10.1134/S0012496609060118

The procedure of noninvasive individual identification of tigers by molecular-genetic methods was developed within the framework of the Program of the Amur Tiger Research in the Russian Far East. Using this procedure, the number, sex, and relationships in a group of tigers in Ussuri Nature Reserve, Far East Branch, Russian Academy of Sciences was defined. The comparison of the results of isolation, amplification, and analysis of nuclear DNA fragments from different samples (blood, hair, and feces) showed their significant similarity in the microsatellite fragment lengths. This indicates the possibility to use feces and hair collected in the Amur tiger habitats for noninvasive individual identification of these animals.

The range of the Amur tiger (*P. tigris altaica*) is the most northern and most geographically remote from the ranges of other mainland tiger subspecies. As a result, it has been isolated for a long time [1]. The long-term isolation and catastrophic drop in abundance under the influence of anthropogenic factors are believed to be the causes of a very low genetic diversity of the modern Amur tiger population. The analysis of variable fragments of mitochondrial and microsatellite DNA revealed their lowest variability among all tiger subspecies [2, 3].

At such a low genetic diversity and abundance of the Amur tiger, it is important to determine the possibility of using molecular-genetic methods for noninvasive individual indentification of these predators in nature. Feces, urine, and hair are successfully used in molecular-genetic studies on wild animals [4–6]. The presence in feces of predatory mammals of both their own DNA and the DNA of their prey determines the specifics of molecular-genetic analysis. To determine the DNA of a test animals, only the colorectal epithelial mucus is used, which is collected from the surface of feces [7].

To develop the procedures for noninvasive individual identification of the Amur tiger in the Far East of Russia, samples of blood, hair, and feces of animals were collected. The material for analysis was collected in the Zoological Center of the Institute of Biology and Soil Sciences, Far East Branch of the Russian Academy of Sciences (Gaivoron village, Spassk raion, Primorskii krai); in the Sysoev Priamurskii Zoo (Khabarovsk krai); and in the Komarov Ussuri Nature Reserve, Far East Branch of the Russian Academy of Sciences. Blood and feces were conserved in 96% ethanol. Blood samples were collected in tubes containing K3EDTA. DNA was isolated from feces using the QIAamp DNA Stool Mini Kit (Qiagen, United States); from blood and hair, using the QIAamp DNA Mini Kit (Qiagen, United States). The species and sex were determined by the polymerase chain reaction (PCR) with two pairs of primers—ZFX-PF (5'-TACCGAGCGATATAGCTCCAG-3') and ZFX-PR (5'-GTGTTCCTACGTTAAGCTATTG-3') for the X chromosome and DBY7-PF (5'-CTCATGAAGCCCTATTTTTGGTTG-3') and DBY7-PR (5'-ACGGCGTCCGTATCTTCCA-3') for the Y chromosome with subsequent visualization in 3% agarose gel [8]. To perform species identification in an ABI 3130 automatic genetic analyzer using the Big Dye kit (Applied Biosystems, United States), the nucleotide sequence of a fragment of one sex chromosome was determined. Individual identification of animals was performed using six most informative microsatellite loci with fluorescently labeled primers (D10, Fca43, Fca304, E21B, 3e6f, and e7) [9, 10]. The lengths of microsat-

[a] *Severtsov Institute of Ecology and Evolution, Russian Academy of Sciences, Leninskii pr. 33, Moscow, 119071 Russia*

[b] *Komarov Ussuri Nature Reserve, Far East Branch, Russian Academy of Sciences, Ussuriisk, Russia*

[c] *Institute of Biology and Soil Sciences, Russian Academy of Sciences, Far East Branch, pr. 100-letiya Vladivostoka 159, Vladivostok, 690022 Russia*

Table 1. Characteristics of animals and samples used in molecular-genetic analysis

Tiger name	Origin	Coded notation of animal	Sex	Analyzed sample type (coded notation)	Sample collection date
Nyurka	Zoological Center of the Institute of Biology and Soil Sciences, Russian Academy of Sciences, Far East Branch	*tn*	f	Blood (b) Hair (h) Excrements (e)	Apr. 29, 2008, Nov. 5, 2008
Kucher	Zoological Center of the Institute of Biology and Soil Sciences, Russian Academy of Sciences, Far East Branch	*tk*, *tk* (30 min), *tk* (4 days)	m	Blood (b) Hair (h) Excrements (e)	Apr. 29, 2008, Nov. 5, 2008
Almaz (the son of Kucher and Nyurka)	Zoological Center of the Institute of Biology and Soil Sciences, Russian Academy of Sciences, Far East Branch	*tal*	m	Blood (b) Hair (h) Excrements (e)	Apr. 29, 2008, Nov. 4, 2008
Araliya (the daughter of Kucher and Nyurka)	Sysoev Priamurskii Zoo (Khabarovsk krai)	*ta*	f	Blood (b)	Aug. 26, 2008
Volya	Sysoev Priamurskii Zoo (Khabarovsk krai) (caught in nature near Inokent'evka village, Khabarovsk krai)	*tb*	f	Blood (b) Hair (h)	Aug. 26, 2008
Ser'ga	Ussuri Nature Reserve (Russian Academy of Sciences, Far East Branch)	*t002*	f	Blood (b) Hair (h) Excrements (e)	Nov. 1, 2008

ellite fragments were determined in an ABI 3130 automatic genetic analyzer using the Liz 500 standard and the GeneMapper 4.0 software (Applied Biosystems, United States). To increase data significance, PCR with DNA from feces was performed at least four times.

As a result, we obtained sets of lengths of six microsatellite loci of three Amur tigers from the Zoological Center (samples *tn*, *tal*, and *tk*), two tigers from the Sysoev Priamurskii Zoo (samples *ta* and *tb*), and one tiger from the Ussuri Nature Reserve (sample *t002*) (Table 1).

It was found that the use of sets of lengths of obtained microsatellite loci makes it possible to discriminate between close relatives (samples *tal*, *ta*, *tn*, and *tk*) (Table 2).

To select the storage procedure, we performed analysis of samples collected at different time intervals after defecation. Before being conserved in ethanol, the samples were kept under natural weather conditions at an ambient air temperature of approximately 20°C. Analysis of samples obtained from the same animal that were stored for different time before conservation (*tk*, 30 min after collecting and *tk*2, 4 days after collecting) showed that feces collected within 3 or 4 days after defecation are still suitable for genotyping. However, in this case, the risk of loss of information from certain microsatellite loci and sex chromosomes significantly increases. For example, for this reason we failed to obtain data for any microsatellite locus for seven samples collected in the Ussuri Nature Reserve (Table 3). For the remaining 28 samples, partial information was obtained (for two to five loci), which in most cases was suitable for animal identification (Table 3).

The comparison of the results of genotyping of different samples (blood, hair, and feces) and the results of several PCR analyses of the same DNA (Tables 2–4) showed their considerable similarity.

Thus, the developed method makes it possible to perform noninvasive individual identification of Amur tigers on the basis of feces left by them in natural conditions.

As a result of genotyping of 35 samples of feces of the Amur tiger, collected on the territory of the Ussuri Nature Reserve during field research, we determined the number and sex of the animals that use this territory all year round. These were an adult female tiger, which was caught and tagged with a GPS Argos transmitter collar, and its three offspring, which carried one maternal allele in each microsatellite locus. In addition, we managed to genotype a male tiger, possibly the father of these three cubs, which was seen in the reserve in the period from the spring of 2008 to the spring of 2009. We also identified one (possibly two) tiger(s) that was not in kinship relationships with the above-mentioned animals. In total, as a result of molecular-genetic analysis of Amur tiger feces, we have identified six or seven animals on the territory of the reserve.

Thus, the results of this study demonstrate the principal possibility of using molecular-genetic analysis methods for noninvasive individual identification of the Amur tiger by feces left by them. However, because of a very small amount of nuclear DNA

Table 2. Allele length (bp) of microsatellite loci for different types of samples (b, blood; h, hair; and e, excrements) obtained from the tigers from the Zoological Center of the Institute of Biology and Soil Sciences (Far East Branch, Russian Academy of Sciences), Sysoev Priamurskii Zoo (Khabarovsk krai), and the Ussuri Nature Reserve (Far East Branch, Russian Academy of Sciences) in April 2008–May 2009

Sample	Locus, primer, dye								
	1, e7, tamra (yellow)			2, fca 304, rox (red)			3, fca 43, fam (blue)		
	b	h	e	b	h	e	b	h	e
tn	152/152	152/152	152/152	136/136	136/136	136/136	123/127	123/127	123/127
tn (repeated)	–	–	141/153	–	–	136/136	–	–	123/127
tal	152/156	152/156	153/157	136/136	136/136	136/136	127/127	127/127	127/127
tal (repeated)	152/156	*	152/156	136/136	*	136/136	*	–	127/127
tk	152/156	152/156	152/156	134/136	*	*	123/127	123/127	123/127
tk (repeated)	152/156	*	152/156	134/136	–	134/136	123/127		123/127
tk (30 min)	–	–	152/156	–	–	134/134	–	–	123/127
tk2 (4 days)	–	–	156/156	–	–	*	–	–	123/127
t002	152/152	152/152	152/152	128/136	128/136	128/136	119/123	119/123	119/123
tb	152/152	152/152	–	128/136	128/136	–	123/123	123/123	–
ta	152/156	–	–	136/136	–	–	123/123	–	–
Sample	Locus, primer, dye								
	4, 3e6f, r6g (green)			5, e21b, r6g (green)			6, d10, tamra (yellow)		
	b	h	e	b	h	e	b	h	e
tn	153/153	153/153	153/153	160/160	160/160	160/160	149/151	149/151	149/151
tn (repeated)	–	–	*	–	–	160/160	–	–	*
tal	153/153	153/153	153/153	160/160	–	–	151/151	151/151	151/151
tal (repeated)	*	–	153/153	–	–	160/160	151/151	–	151/151
tk	153/156	153/156	153/156	160/160	160/160	160/160	149/151	149/151	149/151
tk (repeated)	153/156	–	*	160/160	–	160/160	149/151	–	149/151
tk (30 min)	–	–	*	–	–	160/160	–	–	*
tk2 (4 days)	–	–	*	–	–	160/160	–	–	*
t002	153/156	153/156	153/156	160/164	160/164	160/164	149/149	149/149	149/149
tb	153/156	*	–	162/162	162/162	–	149/149	149/149	–
ta	153/153	–	–	160/160	–	–	149/149	–	–

Note: Here and in Tables 3 and 4, asterisks mark the samples data for which were not obtained because of DNA degradation. Dash, not determined.

obtained from feces, the processes of DNA isolation and its analysis by PCR become largely stochastic and may sometimes give erroneous results. To minimize such errors, it is necessary to perform experiments in several replicates. DNA preservation in collected samples is also of great importance. It is difficult to make an unambiguous conclusion on the suitability of various samples for analysis; however, it is advisable to use feces left no longer than two or three days ago. In addition, our data testify to the unique possibility to use molecular-genetic methods for determination of paternity and kinship relationships in natural groups of Amur tigers. The obtained data will be entered into the general genetic database for the Amur tigers based on noninvasive method of material collection in different areas.

In addition to solving ecological problems, the use of these methods of analysis makes it possible to solve

Table 3. Allele length (bp) of microsatellite loci 1, 2, 3, 4, 5, and 6 (pooled data of several PCR procedures) for the tigers from the Ussuri Nature Reserve (Far East Branch, Russian Academy of Sciences) obtained by analysis of feces

Sample no.	Microsatellite loci						Sex chromosomes	Sex
	1	2	3	4	5	6		
4w	152/152	134/134	119/127	*	160/160	149/151	*	*
Pt1	152/152	*	123/127	*	*	*	150/200	m
Pt4	152/152	134/134	123/127	*	160/160	149/149	*	*
E004z	152/152	134/134	123/127	153/156	*	149/149	150/200	m
E057z	152/152	*	127/127	*	*	*	*	
E016z	152/152	*	123/127	*	*	*	150/200	m
E043l	152/152	134/134	*	153/156	*	149/149	150/200	m
E019n	152/152	134/134	123/127	153/156	160/160	*	150/200	m
T002	152/152	128/136	119/123	153/156	160/164	149/149	200/200	f
E008n	152/152	134/136	123/127	*	160/160	*	150/200	m
E009n	152/152	134/136	119/123	153/153	160/160	149/149	200/200	f
E011z	152/152	134/136	119/123	*	160/160	*	200/200	f
E021n	152/152	128/136	119/123	*	160/164	149/149	200/200	f
Teo4n	152/152	*	123/123	*	*	*	150/200	m
R003, E011n	*	*	*	*	*	*	200/200	f
E001h, t045l	*	*	*	*	*	*	*	*
E002n, E009z	*	*	*	*	*	*	150/0	m
E005h	*	*	*	*	*	*	150/200	m
E004z	152/152	134/134	123/127	153/156	*	149/149	150/200	m
E0157z	152/152	*	123/127	*	*	*	150/200	m
E016z	152/152	*	123/127	*	*	*	150/200	m
E043l	152/152	134/134	123/127	153/156	*	149/149	150/200	m
E001z	*	*	127/127	153/156	*	*	150/200	m
T038n	152/152	128/128	127/127	*	–	151/151	150/200	m
E32l	*	*	127/127	*	*	*	150/200	m
E032n	152/152	128/128	127/127	153/153	160/164	151/151	150/200	m
E005z	*	134/136	123/127	*	160/164	*	*	*
E071l	152/152	128/134	119/123	*	*	149/149	150/200	m
E017n	*	128/134	127/127	*	*	*	200/200	f
E010z	152/152	128/134	123/123	153/156	*	149/149	150/200	m
E014z	*	134/134	*	*	160/160	149/149	150/200	f
T012n	152/152	128/136	*	153/156	160/164	149/149	150/200	f

Table 4. Sex determined for five studied tigers for different types of samples

Animal coded notation	Sex	Sample			Animal sex determined by molecular-genetic analysis
		blood	hair	excrements	
tn	f	200	200	200	f
t002	f	200	200	200	f
tal	m	150/200	150/200	150/200	m
tb	f	200	200	–	f
tk	m	150/200	150/200	150/200	m

forensic and nature-conservation problems regarding the tiger, a species listed in the Red Data Book of the Russian Federation. In particular, we performed such analysis on the basis a request from the Ministry of Internal Affairs of the Russian Federation for species identification of samples collected as evidence as well to confirm that different samples belonged to the same animals.

ACKNOWLEDGMENTS

We are grateful to researchers with the Ussuri Nature Reserve (Far East Branch, Russian Academy of Sciences) and the Sysoev Priamurskii Zoo for their help in collecting the material used in this study.

This study, performed within the framework of the Program of the Amur Tiger Research in the Russian Far East, was supported by the Konstantinovsky International Charity Fund, the OAO Transneft Oil Transportation Company, and the OAO Tekhsnabexport Company.

REFERENCES

1. Kitchener, A.C. and Dugmore, A.J., *Animal Conserv.,* 2000, vol. 3, pp. 113–124.
2. Luo, S.-J., Kim, J.-H., Johnson, W.E., et al., *PLoS Biol.*., 2004, vol. 2, pp. 2275–2293.
3. Henry, P., Miquelle, D., Sugimoto, T., et al., *Mol. Ecol.*, 2009, pp. 1–12.
4. Murphy, M.A., Kendall, K.C., Robinson, A., and Waits, L.W., *Conserv. Gen.*, 2007, vol. 8, pp. 1219–1224.
5. Ernest, H.B., Boyce, W.M., and Bleich, V.C., *Conserv. Gen.*, 2003, vol. 4, pp. 353–366.
6. Fernando, P., Vidya, T.N.C., and Rajapakse, C., *J. Heredity*, 2003, vol. 94, no. 2, pp. 115–123.
7. Wehausen, J.D., Ramey, IIR.R., and Epps, C.W., *J. Heredity*, 2004, vol. 95, no. 6, pp. 503–509.
8. Sugimoto, T., Nagata, J., Aramilev, V.V., et al., *Conserv. Gen.*, 2006, vol. 7, pp. 799–802.
9. Bhagavatula, J. and Singh, L., *BMC Gen.*, 2006, p. 7.
10. Menotti-Raymond, M., David, V., Lyons, L., et al., *Genomics*, 1999, vol. 57, pp. 9–23.