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GENERAL BIOLOGY

Application of Molecular Genetic Characteristics for Reintroduction of the Leopard (*Panthera pardus* L., 1758) in the Caucasus

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Within the framework of the Program of North Persian leopard Reintroduction in the Caucasus, the data on genetic polymorphism in leopard (*P. pardus saxicolor*) have been obtained for the first time from natural populations of the Russian part of the Caucasus (Krasnodar krai, North Ossetia), Transcaucasia (Azerbaijan, Armenia), Iran, Turkmenistan, and Afghanistan. Molecular genetic approaches to subspecies taxonomy of leopards of unknown (potentially hybrid) origin from zoological gardens and breeding centers have been developed. This analysis with the use of 11 microsatellite loci and a fragment of the NADH5 mitochondrial gene was efficiently used for identification of individuals acceptable for breeding and reintroduction in the West Caucasus.

Until recently, the leopard *P. pardus* was rather widespread in the Caucasus and occupied practically all mountainous territories, but its population drastically decreased in the late 19th and early 20th centuries because of intense extermination and disruption of the food resources, to the extent of complete extermination in many regions. In 1950, only single specimens of this species survived in the Caucasus; since the 1960s–1970s, practically no encounters with leopards have been reported.

A special program has been developed and is being implemented for restoration of the Asian leopard population in the Caucasus [1]; along with other measures, it envisages the breeding of animals of this subspecies in captivity followed by their reintroduction. One of the prerequisites of the implementation of the leopard reintroduction program is correspondence of the genetic state of animals intended for reintroduction [2] to that of the animals previously inhabiting this region, with preceding revision of the taxonomic state of leopards from this region.

At present, the situation with the taxonomic name of the North Persian leopard is substantially complicated, and the nomenclature has not been sufficiently developed. A great number of leopard forms the taxonomic position of which causes disagreement have been described within a spacious natural range embracing the most part of Africa and a significant part of South Asia [3–7].

The leopard inhabiting the western part of the range (the south of Central and West Asia including the Caucasus) and being notable for its large size is classified with the subspecies *P. p. tulliana* Valenciennes, 1856 [8], or to *P. p. ciscaucasica* Satunin, 1914 [3], or to *P. p. saxicolor* Pocock, 1927 [9]. In total, seven leopard populations are distinguished in this region of the natural range, which embraces the area from Pakistan (the Indus River) in the east to Turkey (Antaliya) in the west, including most countries of the Arabian Peninsula. These populations were described as independent forms (*tulliana, jarvisi, nimr, ciscaucasica, dathei, saxicolor, sindica*) but recently, based on the results of molecular genetic studies, they have been provisionally combined into a single subspecies *P. p. saxicolor* [5] with a name incorrectly accepted for such interpretation. These authors have shown that the North Persian leopard is genetically different from African forms, on the one hand, and from the Indian *fusca* (and, apparently, *millardi*), on the other hand. The latest data [7] show that the leopard inhabiting the Arabian Peninsula is genetically different from the above-mentioned forms, being an independent subspecies *P. p. nimr* Hemprich et Ehrenberg, 1833. The independent taxonomic position of this form and its isolation from the previously accepted subspecies *P. p. saxicolor* entails, in accordance with the International Code of Zoological Nomenclature [10], the change of name of the group of populations inhabiting the northern part of the range (Iran, Turkmenistan, the Caucasus and West Asia countries) for *P. p. tulliana* Valenciennes, 1856. It should be noted that these molecular genetic studies [5, 7] were performed in the tissues of animals kept in zoological gardens. Among them, only two specimens of the two forms (*sindica* and *nimr*) were of natural origin and other specimens (12 in the first study and 9 in the second study, some of

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Table 1. Characteristics of leopard tissue samples used for analysis

Tissue sample	Place and time of tissue sample taking	Type of tissue	Haplotype, NCBI number
kav150163*	Russia, Krasnodar Krai, Maikop, 1914, collection of the Zoological Museum of Moscow State University, S-150163	Mandibular bone	az2, HQ185544
a6436	Armenia, collection of the Institute of Zoology, National Academy of Sciences of the Republic of Armenia	Pelt	af1, HQ185548
a6437	Armenia, collection of the Institute of Zoology, National Academy of Sciences of the Republic of Armenia	The same	af1, HQ185548
az1	Azerbaijan, Talysh, 2005	Skin	az2, HQ185544
az4*	Azerbaijan, Talysh, 2002	The same	az2, HQ185544
az2*	Azerbaijan, Talysh	Hair	az2, HQ185544
az3*	Azerbaijan, Talysh	The same	az2, HQ185544
ir1*	Iran, Golestan National Park, 2004	Claw	az2, HQ185544
ir2*	Zoo of Teheran, captured in 2002 in the north of Iran in Mazandaran Province	Hair	ir2, HQ185545
ir3*	Wild Life Department of Gurgaon, captured December 1, 2009, 100 km to the north-north-east from the city of Gurgaon	The same	ir2, HQ185545
tu3	Turkmenistan, Nizhny Uzboy, 1989	Claw	az2, HQ185544
tu1*	Turkmenistan, Dogrydere Clove	Hair	az2, HQ185544
tu2*	Turkmenistan, Generalyskoye Clove	The same	az2, HQ185544
dv14264*	Russia, Primorye, Gamov settlement, Peter the Great Bay, February 1, 1929, A. Baturin, collection of the Zoological Museum of Moscow State University, S-150163	Mandibular bone	dv14265, HQ185549
dv14265*	Russia, Primorye, Astafyeva Bay shore, the Pacific coast, January 12, 1924, Baturin, collection of the Zoological Museum of Moscow State University, S-14265	The same	dv14265, HQ185549
dv43794*	Russia, Primorye, Peter the Great Bay, Gamov hunting state farm, October 1, 1945, N. Gorchakovskaya, collection of the Zoological Museum of Moscow State University, S-43794	"	dv14265, HQ185549
dv91361*	Russia, Primorye, Khasansky District, January 1966, U. Polivan', collection of the Zoological Museum of Moscow State University, S-91361	"	dv14265, HQ185549
dv96812*	Russia, Primorye, Nadezhdinsky District, I.D. Chernyshov, collection of the Zoological Museum of Moscow State University, S-96812	"	dv14265, HQ185549
af1	Afghanistan, Kabul	Skin	af1, HQ185548
k16*	Russia, North Ossetia, Fiagdon Clove, 2007	Hair	k5, HQ185547
k1*	Russia, North Ossetia, 2005, zoo	Blood	k1, HQ185546
k21*	Russia, Nalchik zoo	Hair	dv14265, HQ185549
k22*	Russia, Moscow, the nursery in Rechnik settlement, 2010	The same	k5, HQ185547
k23*	Russia, Novosibirsk zoo	Blood	k5, HQ185547
k5*	Russia, North Ossetia, 2005, zoo	Hair	k5, HQ185547
k7*	Russia, Nalchik, 2005, zoo	The same	k5, HQ185547
dv1*	Russia, Primorye, collection of the Primorye State Agricultural Academy, 2008	Muscles	dv14265, HQ185549
dv2*	Russia, Primorye, collection of the Primorye State Agricultural Academy, 2008	The same	dv14265, HQ185549
dv3*	Russia, South Sakhalin zoo, 2010	Blood	dv3, HQ185550

Note: The samples successfully subjected to microsatellite analysis are marked with an asterisk. For other samples, microsatellite analysis is impossible because of DNA degradation.

Table 2. The lengths of alleles (bp) of microsatellite loci used for analysis in different samples

No.	Haplotype	Primer																					
		E7	E7	Fca 304	Fca 304	Fca 43	Fca 43	3E 6F	3E 6F	E21 B	E21 B	Fca 77	Fca 77	Fca 90	Fca 90	Fca 96	Fca 96	Fca 310	Fca 310	Fca 441	Fca 441	Fca 97	Fca 97
1	tu1	172	180	124	124	117	117	168	168	160	164	133	133	114	116	200	204	122	122	156	156	148	150
2	tu2	154	172	124	124	117	117	156	168	160	160	133	143	116	116	204	208	122	122	136	136	140	142
3	ir1	154	154	116	116	115	117	165	165	160	162	137	137	116	116	202	204	122	122	0	0	148	148
4	ir2	154	158	118	118	113	117	151	165	162	162	137	139	116	116	202	202	122	122	148	148	148	152
5	ir3	178	178	116	118	117	117	156	165	160	162	137	137	116	116	202	204	122	122	0	0	148	148
6	az2	158	178	118	126	117	117	165	165	160	160	133	145	114	116	198	204	122	122	0	0	144	152
7	az3	158	178	118	130	117	117	165	165	160	160	133	145	114	116	198	204	122	122	0	0	144	152
8	az4	154	178	118	118	117	117	165	165	160	162	133	137	116	116	202	204	122	122	148	148	144	152
9	k1	156	178	116	128	113	117	159	159	0	0	141	143	104	106	198	198	122	122	0	0	138	144
10	k5	156	160	116	116	113	117	159	159	160	162	135	135	104	106	194	208	0	0	0	0	0	0
11	k7	156	160	116	118	117	117	159	159	160	160	135	143	106	106	194	194	0	0	0	0	0	0
12	k16	160	162	116	122	113	117	159	159	0	0	141	143	104	104	194	208	126	126	156	156	138	138
13	k21	162	162	116	122	107	113	159	162	164	166	141	143	104	108	194	208	118	126	156	156	138	138
14	k22	152	162	116	118	113	117	159	165	160	160	137	143	104	104	194	206	0	0	0	0	148	148
15	k23	156	156	127	127	113	113	162	162	0	0	137	143	104	104	200	200	116	126	0	0	138	148
16	kav150163	172	172	116	128	113	117	162	162	160	160	137	143	104	116	208	208	122	122	0	0	148	148
17	dv1	158	158	116	116	113	113	156	162	160	166	135	135	104	110	200	202	128	128	160	160	150	150
18	dv2	158	158	116	116	113	113	156	162	160	166	129	135	104	110	200	202	128	128	160	160	138	150
19	dv3	160	160	116	116	113	117	156	162	160	160	155	155	0	0	194	209	126	128	160	160	0	0
20	dv14264	156	156	116	116	117	117	156	156	160	166	135	135	104	104	194	194	122	128	148	156	138	148
21	dv43794	158	160	116	116	117	117	156	159	160	160	135	135	104	104	194	202	128	128	152	152	140	148
22	dv14265	154	154	116	116	113	117	156	162	160	160	135	135	108	108	194	208	128	128	160	160	150	150
23	dv91361	158	158	116	116	113	113	156	162	160	160	135	135	104	110	200	202	128	128	156	156	138	138
24	dv96812	158	158	116	118	113	113	0	0	160	160	135	143	104	104	194	202	128	128	164	164	136	150

Note: (0) The samples, for which the data could not be obtained because of DNA degradation.

the specimens being the same) were obtained from the *saxicolor* population bred in zoological gardens. Specimens of *tulliana, jarvisi, ciscaucasica,* and *dathei* were not used at all.

MATERIALS AND METHODS

We have carried out molecular genetic studies of leopards originating from the Russian part of the Caucasus (Krasnodar Krai, North Ossetia), Transcaucasia (Azerbaijan, Armenia), Iran, Turkmenistan, and Afghanistan. Specimens of the Far Eastern leopard from Primorye were analyzed additionally as an external group. Table 1 presents information about the places of taking tissue samples used for analysis. Hair samples were preserved in 96% ethyl alcohol. Blood samples were collected into test tubes with addition of K_3EDTA. DNA was isolated from skin, blood and teeth using a Diatom DNA Prep 200 kit (Izogen Laboratory, Russia) and a QIAamp DNA Mini Kit (Qiagen, United States).

The maternal genetic relationships between specimens presented in Table 1 were determined using the results of analysis of the nucleotide sequences of a fragment of mitochondrial DNA (mtDNA), namely, the NADH dehydrogenase subunit 5 gene. DNA fragments were amplified using reagent kits from Dialat and SibEnzyme (Russia). Primers F and RL4 and the respective cycle were used for the NADH5 gene [7]. DNA sequences was determined in the tested mtDNA fragments using automatic analyzers ABI 310 and ABI 3130 with the respective primers and an ABI PRISM Big Dye Terminator Cycle sequencing kit v. 3.1 (Applied Biosystems, United States). The nuclear DNA was analyzed using 11 microsatellite loci with the primers (E7, Fca304, Fca43, 3E6F, E21b, Fca77, Fca90, Fca96, Fca310, Fca441, Fca97) labeled with fluorescent dyes [11, 12]. The lengths of microsatellite

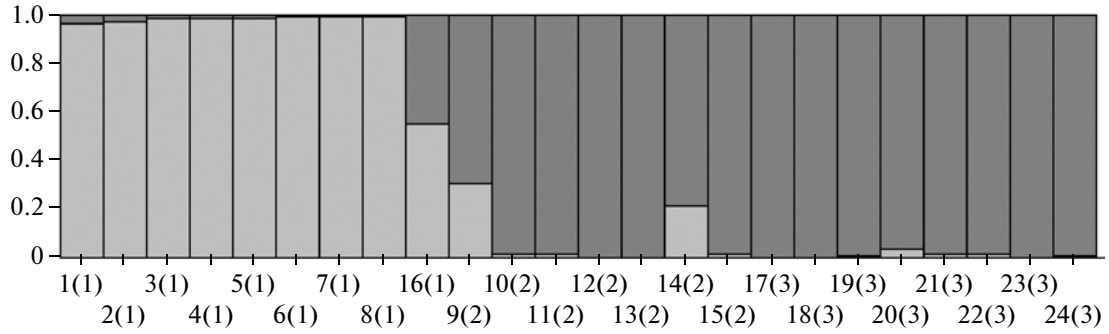

Fig. 1. Distribution of leopard specimens based on the lengths of alleles of microsatellite loci. Constructed in the Structure 2.3.1 software. The number of records per cycle is 50 000; the number of repeats is 10^6. The Y axis: the share of alleles of microsatellite loci of some or other leopard subspecies. The X axis: the order number of an animal in Table 2; taxonomy with one of the following groups is given in parenthesis: *1, P. p. saxicolor*; *2*, leopards of unknown origin; *3*, *P. p. orientalis*.

fragments were determined in an automatic genetic analyzer ABI 3130 with addition of Liz 500 length standard and GeneMapper v. 4.0 (Applied Biosystems, United States). Analysis of molecular variability (AMOVA) for the microsatellite loci and calculation of pairwise genetic distances (Rst) and Rst significance ($p < 0.05$) were performed using the Arlequin 3.5.1.2 software [13]. The subspecies of leopard specimens of unknown origin were determined by means of Bayesian population clustering analysis using the Structure 2.3.1 software [14] and the Admixture model, which presupposes mixing of the two subspecies.

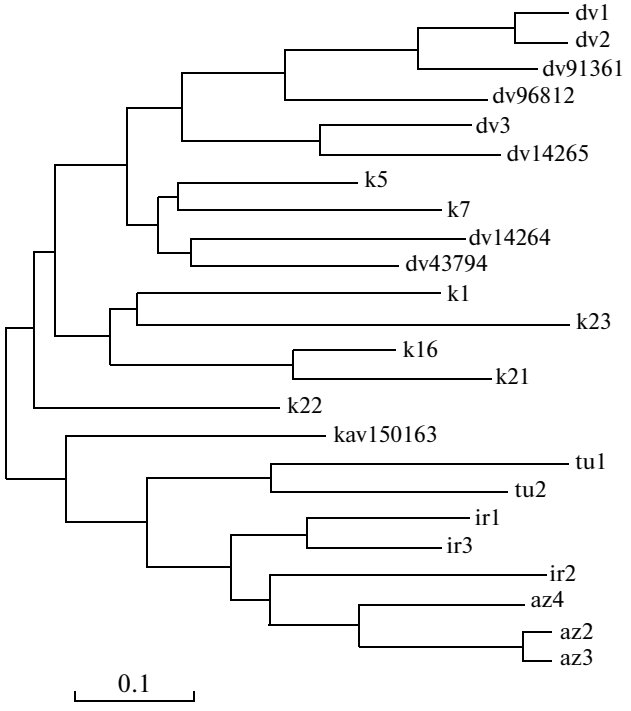

Fig. 2. The Nj tree constructed in Mega 4.0 based on the pairwise genetic distances calculated in MS Tools between all pairs of the samples used for microsatellite analysis.

RESULTS AND DISCUSSION

Comparison of the obtained nucleotide sequences has shown that the specimens under consideration, except for the Far Eastern leopard, are very similar in the given mtDNA fragment (the difference is one or two nucleotides (Table 1)) to the previously described haplotype sax2 (the nucleotide sequence of the ND5 gene fragment, NCBI number AY035278, typical of the North-Persian leopard subspecies stated in the genetic database as *P. p. saxicolor*).

Analysis of nuclear DNA at 11 microsatellite loci (Table 2) in the samples of animals from the Russian part of the Caucasus, Turkmenistan, Azerbaijan, Iran and Primorye using the Structure 2.3.1 software has demonstrated genetic similarity of the animals with haplotypes az2 and ir2 and their difference from the animals with haplotypes dv14265 (identical to the previously described ori2, no. NCBI AY035261, typical of the Far Eastern subspecies *P. p. orientalis*) and dv3 (Fig. 1). The animals of unknown origin from zoological gardens and breeding centers either occupy an intermediate position (k1, k22) or belong to the group of Far Eastern leopards (k5, k7, k16, k23) (Figs. 1, 2). At the same time, judging by their mtDNA, all of them (except for specimen k21) belong to the North Persian subspecies of leopard. It is evidence of their hybrid origin due to crossbreeding of the Far Eastern and North Persian subspecies of leopard. On the contrary, leopard k21 is identical to animals from the sample of Far Eastern leopards in both nuclear and mtDNA. For the purpose of understanding, what groups of leopards from different territories are genetically closer to each other according to nuclear DNA analysis, we have tested different variants of combining these samples into bigger groups using Arlequin 3.5.1.2. At the same time, the animals from zoological gardens of the Caucasus and from the collection of the Zoological Museum of Moscow State University (from the same region) were analyzed independently. The most statistically reliable result ($Fst = 0.407$) is typical of the variant with the total sample divided into two groups:

Table 3. Genetic distances by the method of the sum of squares of the differences (*Rst* value) between the analyzed samples of animals from three groups: 1, tu, az, ir, kav; 2, k; 3, dv

Haplotype	tu	ir	az	k	kav	dv
tu						
ir	0.101					
az	0	0				
k	0.485	0.328	0.36			
kav	0	0.178	0	0.432		
dv	0.586	0.32	0.452	0.055	0.593	

Table 4. The values of *P** for the *Fst* criterion between the analyzed samples of animals (Turkmenistan, Azerbaijan, Iran, Caucasus, and Primorye)

Haplotype	tu	ir	az	k	kav	dv
tu						
ir	0.486					
az	0.748	0.369				
k	0.027*	0.009*	0.018*			
kav	0.991	0.991	0.991	0.991		
dv	0.036*	0.036*	0.018*	0.162	0.234	

* The groups can be reliably considered as genetically isolated at $p < 0.05$.

(1) the leopards from Turkmenistan, Azerbaijan, Iran, and the Russian part of the Caucasus (except for the animals of unknown origin from the zoos) and (2) the leopards of unknown origin from zoological gardens of the Caucasus and Novosibirsk, from a private nursery in Moscow, and the leopards from Primorye. These results are in agreement with clustering in the Structure software (Fig. 1) and show that a considerable part of specimens of unknown origin is similar or identical in the nuclear DNA to the sample of the Far Eastern leopards. In addition, for testing the probability of genetic isolation of leopard from the Russian part of the Caucasus, we have combined the animals of unknown origin from the Caucasus and the animal from Maikop (kav150163) into a single group. Two other groups included the animals of North Persian and Far Eastern subspecies captured in nature. Statistical support for the existence of three groups proved to be much lower (*Fst* = 0.33).

In addition, for clarifying the genetic structure within the groups assigned to the North Persian leopard subspecies, pairwise genetic distances (the *Rst* value) and reliability values *Rst* ($p < 0.05$) between the considered samples of leopards from different territories were calculated (Tables 3, 4). The Nj tree constructed on the basis of pairwise genetic distances (Fig. 2) demonstrated phylogenetic relationships in the analyzed sample.

The presence of the same group of haplotypes (az2, ir2, af1) in all leopards from the Russian part of the Caucasus, Transcaucasia (Azerbaijan, Armenia), Iran, Turkmenistan, and Afghanistan and the similarity of analyzed nuclear DNA loci to each other may be evidence of taxonomy of these animals with a single subspecies. These data confirm the genetic closeness of leopard populations in this region, previously supposed for the Central Asian part of the species range [5].

Our data make it possible to discuss also the nomenclature of leopards inhabiting this region. According to our data, the *ciscaucasica* and *saxicolor* forms should be combined into a single subspecies, suggesting, in accordance with the International Code of Zoological Nomenclature [10], the use of a senior synonym, i.e., *P. p. ciscaucasica* Satunin, 1914. The same conclusion was made from analysis of the craniometric characters of the leopard from this region [15].

The question about the taxonomy and nomenclature of leopard of the whole Caucasus ecoregion, however, needs further study and extension of the analyzed sample: the form inhabiting Turkey and described under the name of *P. p. tulliana* Valenciennesm, 1856, has not yet been studied. If its identity with *ciscaucasica* is shown, the name of this form (*tulliana*) should be extended to all animals of this region.

Thus, our data on the genetics of leopards from natural populations are in agreement with the data obtained previously [5, 7] for the animals kept in zoological gardens worldwide under the name of *saxicolor*. This fact makes it possible to use them for breeding and reintroduction in the West Caucasus. However, the microsatellite loci in different subspecies of the leopard often differ only in frequencies, complicating the identification of hybrid origin of an animal, and further studies of animals from both nature and zoos are necessary.

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REFERENCES

1. Rozhnov, V.V. and Lukarevskiy, V.S., *Programma po vosstanovleniyu (reintroduktsii) peredneaziatskogo leo-*

parda na Kavkaze (The Program on Reintroduction of the West Asian Leopard in the Caucasus), Moscow: KMK, 2008.

2. Rozhnov, V.V., Lukarevskiy, V.S., and Sorokin, P.A., in *Mlekopitayushchie gornykh territorii* (Mammals of Uplands), Moscow: KMK, 2007, pp. 275–278.
3. Geptner, V.G. and Sludskii, A.A., *Mlekopitayushchie Sovetskogo Soyuza*, vol. 2, part 2: *Khishchnye (gieny i koshki)* (Mammals of the Soviet Union, vol. 1, part 2: Carnivores: Hyenas and Cats), Moscow: Vysshaya Shkola, 1972.
4. Jackson, P. and Jackson, A.F*., Les felinns,* Lausanne: Delachaux et Niestle, 1996.
5. Miththapala, S., Seidensticker, J., and O'Brien, S.J., 1996, *Conserv. Biol.*, 1996, no. 4, pp. 1115–1132.
6. Nowell, K. and Jackson, P., *Status Survey and Conservation Action Plan Wild Cats. IUCN/SSC Cat Specialist Group. Gland*, 1996.
7. Uphyrkina, O., Jonson, W.E., Quigley, H., et al., *Mol. Ecol.*, 2001, vol. 10, pp. 2617–2633.
8. Baryshnikov, G.F.,, *Byull. Mosk. O-va Ispyt. Prir. Otd. Biol.*, 1987, vol. 92, issue 4, pp. 21–26.
9. Tylinek, C., Samkove, Z., Selfert, S., and Muller, P., *Das Grosse Buch der wilden Katzen*, Leipzig, 1987.
10. *Mezhdunarodnyi kodeks zoologicheskoi nomenklatury* (The International Code of Zoological Nomenclature), St. Petersburg, 2000, 4th ed.
11. Rozhnov, V.V., Sorokin, P.A., Naidenko, S.V., et al., *Dokl. Biol. Sci.*, 2009, vol. 429, no. 2, pp. 518–522 [*Dokl. Akad. Nauk*, 2009, vol. 429, no. 2, pp. 278–282].
12. Menotti-Raymond, M., David, V., Lyons, L., et al., *Genomics*, 1999, vol. 57, pp. 9–23.
13. Excoffier, L.G., Laval, Schneider S, *Evol. Bioinform. Online*, 2005, vol. 1, pp. 47–50.
14. Pritchard, J.K., Stephens, M., and Donnelly, P., *Genetics,* 2000, vol. 155, pp. 945–959.
15. Khorozyan, I.G., Baryshnikov, G.F., and Abramov, A.V., *Rus. J. Theriol.*, 2006, vol. 5, no. 1, pp. 41–52.