Short Communication

Presence of the Endangered Amur tiger *Panthera tigris altaica* in Jilin Province, China, detected using non-invasive genetic techniques

Anthony Caragiulo, Yang Kang, Salisa Rabinowitz
Isabela Dias-Freedman, Simone Loss, Xu-Wei Zhou
Wei-Dong Bao and George Amato

Abstract China is home to three subspecies of tiger *Panthera tigris* but there are no estimates of the size of any of the populations. We detected a population of the Endangered Amur tiger *Panthera tigris altaica* in Hunchun Nature Reserve in Jilin Province using both mitochondrial DNA and nuclear microsatellite loci. Four male and one female tigers were detected, indicating the potential for a small breeding group. However, genetic diversity was low overall, with six loci showing a heterozygote deficiency and a mean of 2.55 alleles per locus. This study is the first estimate of the wild Amur tiger population in China to use non-invasive techniques, and the presence of a female tiger indicates this is a potentially viable population. We provide baseline genetic diversity estimates to support monitoring of the population. The small number of tiger scats located indicates the importance of continuing the current conservation efforts for this tiger subspecies in Hunchun Nature Reserve. Such efforts include reducing poaching of tigers and their prey, and implementation of management plans to encourage the persistence and recovery of tigers in this area.

Keywords China, individual identification, microsatellites, mitochondrial DNA, non-invasive scat sampling, *Panthera tigris altaica*, tiger

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Anthony Caragiulo* (Corresponding author), Salisa Rabinowitz, Simone Loss and George Amato Global Felid Genetics Program, Sackler Institute for Comparative Genomics, American Museum of Natural History, Central Park West at 79th Street, New York, NY 10024, USA. E-mail acaragiulo@amnh.org

Yang Kang*†, Xu-Wei Zhou and Wei-Dong Bao (Corresponding author) Department of Zoology, Beijing Forestry University, Haidian, Beijing, China E-mail wdbao@bjfu.edu.cn

Isabela Dias-Freedman Public Health Research Institute, Rutgers University, Newark, USA

*Co-first authors
†Current address: 1229 W 37th Pl Unit 6, Los Angeles, California 90007 USA

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The tiger *Panthera tigris* is the most threatened large felid, with an estimated global population of c. 3,500 individuals (Morell, 2007). Three of nine subspecies have been extirpated by anthropogenic pressures and associated habitat loss (Luo et al., 2004; Dinerstein et al., 2007). Three of the extant tiger subspecies, one being the Amur tiger *P. tigris altaica*, are found in China (Luo et al., 2004). The Amur tiger, categorized as Endangered on the IUCN Red List (Miquelle et al., 2011), has experienced significant reduction of its extensive distribution across northern China as a result of rapid human population growth, habitat loss and poaching (Ma et al., 2005), declining from c. 150 individuals in the 1970s to < 20 in 2000 (Yang et al., 1998; Sun et al., 1999; State Forestry Administration, 2009). Intensive hunting and the capture of cubs for zoos led to a similar decline of the Amur tiger in the Russian Far East during the early 20th century (Heptner & Sludskii, 1972).

Since 1947 the Amur tiger population in the Russian Far East has increased as a result of a prohibition on tiger hunting, reduction in capture of cubs and implementation of management plans for prey. It is estimated there were 428–502 tigers in 2005 (Miquelle et al., 2005), mostly in the Sikhote-Alin Mountains, with a subpopulation of 16–21 in the Russian south-west Primorye (Pikunov et al., 2003) potentially serving as a source population for nearby Jilin and Heilongjiang provinces in China (Sun & Dong, 2005; Chen et al., 2011). Previous studies have focused on population variation and genetic structure of tigers in Russia (Russello et al., 2004; Henry et al., 2009; Miquelle et al., 2010; Sugimoto et al., 2012). The demographic and genetic status of tigers in China is unclear (State Forestry Administration, 2009). In this study we therefore analysed scat samples to assess genotypic variation for identification of individuals of the Chinese tiger population in Jilin Province, and provide baseline estimates of genetic diversity to inform recovery plans for this population.

The field survey was conducted in the 1,087 km² Hunchun Nature Reserve in Jilin Province, China, which occupies a long, narrow strip adjacent to south-west Primorye, Russia (Fig. 1). Eleven scat samples were collected within and adjacent to the reserve during tiger monitoring surveys.

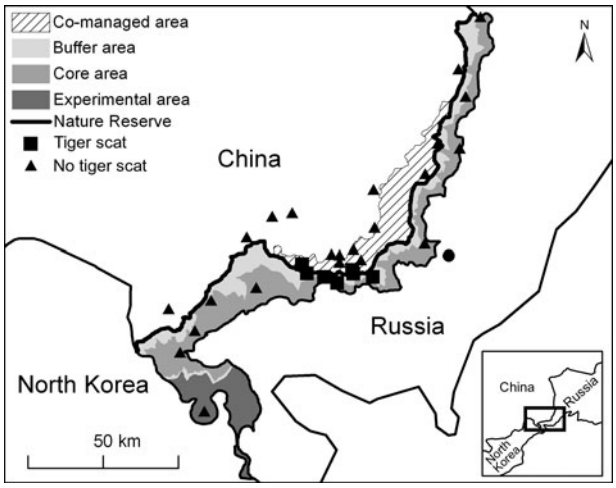

FIG. 1 Location of Hunchun Nature Reserve, showing sampling localities where tiger scats were and were not identified. Some sampling localities were outside the Reserve because there was anecdotal evidence of tigers in these areas. The black circle indicates the nearest tiger population in Russia (Russello et al., 2004) and the inset the location of the main map on the China–Russia border. The three shades of grey indicate different zones within the Reserve.

The surveys covered 1,182 km² and were conducted during 14–23 November 2009, 22 January–2 February 2010, and for 5 days during the winter/spring of 2011. Genomic DNA was extracted using the QIAamp DNA Stool Mini kit (Qiagen, Valencia, USA) following the manufacturer's protocol, with modifications to improve DNA yield and quality (Caragiulo et al., 2014). Extractions were prepared within a polymerase chain reaction (PCR) product-free laboratory reserved exclusively for scat extractions.

Samples were identified to species via PCR and Sanger sequencing using six mitochondrial DNA primer sets spanning four mitochondrial gene regions (Caragiulo et al., 2014). Sequences were edited using *Sequencher v. 4.2* (Gene Codes Corporation, Ann Arbor, USA), and species were identified via neighbour-joining trees (1,000 bootstrap replicates) implemented in *Geneious Pro v. 5.6* (Biomatters, Auckland, New Zealand), constructed using > 2,000 in-house reference sequences spanning multiple animal species. Each identified tiger sequence was accessioned into the BOLD System (Ratnasingham & Hebert, 2007) and GenBank (Benson et al., 2005) when exceeding 200 bp. Generated sequences were compared to references on GenBank for additional confirmation of species identity.

Fluorescently labelled nuclear microsatellite loci were used to identify individuals, and amplified using the QIAgen Multiplex Kit (Qiagen, Valencia, USA) in 20 µl multiplex reactions using 5 µl of template DNA, 10 µl of MasterMix, 2 µl of Q-solution, 0.1–0.8 µM of each 10 µM primer, brought to volume using ultrapure water. A total of 11 microsatellite loci developed from the domestic cat (Menotti-Raymond et al., 1999) were used (Supplementary Table S1). Microsatellite amplifications were analysed using *GeneMapper v. 4.0* (Applied Biosystems, Carlsbad, USA). Genotypes were validated using the quality index (Mondol et al., 2009) to ensure reliable genotyping. Samples amplifying fewer than five loci were removed from further analysis.

The gender of each tiger sample was determined via amplification of the zinc finger regions on the X and Y chromosomes (Wei et al., 2008). Gender-typing PCRs consisted of 25 µl reactions with 0.60 µl of each 0.25 µM primer, 5 µl of template DNA, one illustra puReTaq ready-to-go PCR bead (GE Healthcare, Piscataway, USA), brought to volume with ultrapure water. The two forward primers (ZF-1F and ZFY-2F) were fluorescently labelled for analysis in an ABI 3730xl DNA Analyser (Applied Biosystems) and scored using *GeneMapper*.

Each sample underwent at least four independent PCR reactions per locus and gender-typing reaction using a modified multiple-tubes approach to identify allelic dropout or false alleles, and to confirm allele calls and gender-typing results (Taberlet et al., 1996; Bhagavatula & Singh, 2006; Mondol et al., 2009). *GIMLET v. 1.3.2* (Valière, 2002) was used to determine error rates, *CERVUS v. 3.0* (Kalinowski et al., 2007) was used to identify individual genotypes, and genetic diversity indices were estimated using both *CERVUS* and *GENEPOP v. 4.0* (Rousset, 2008).

Nine scat samples were identified as tiger, and the remaining two as canids. Mitochondrial DNA sequences were accessioned into BOLD (GFG001–GFG009) and GenBank (JQ040982–JQ040983, JQ040929–JQ040937). Recapture samples were found (samples CH004/CH005 and CH007/CH010/CH011 were from the same individual tiger). Samples amplified all loci, except for CH008 and CH009, which amplified 9 loci; CH003 only amplified four loci and was excluded from individual identification analysis. We discerned a total of five individuals, representing four prospective males (CH001, CH004/005, CH007/010/011, CH008), and one prospective female (CH009). The P_{ID} = 0.0001 and the $P_{ID(sib)}$ = 0.013 indicated our loci panel had sufficient statistical power for discerning individuals. Overall, the mean PCR success for all loci was 0.76 (range 0.52–0.88), the mean allelic dropout rate across loci was 0.290 (0.000–0.838), and the mean number of false alleles across loci was 0.008 (0.000–0.025). The mean number of alleles per locus (N_A) was 2.55, the mean expected heterozygosity (H_e) was 0.438, and mean polymorphic information content (PIC) was 0.288 (Supplementary Table S2). Tests for deviation from Hardy–Weinberg equilibrium and linkage disequilibrium could not be performed because of the small sample size. The F_{IS} value was 0.125.

Molecular techniques are widely used to investigate the genetics of threatened species because they provide important insights into taxonomic status, evolutionary history and population genetic structure (Bhagavatula & Singh, 2006).

Such information is especially important for small populations, as they are more susceptible to loss of genetic variation through inbreeding (Frankham et al., 2010). To our knowledge this is the first study to provide population genetic information on wild tigers in China using non-invasive techniques. Based on track surveys, it was estimated previously that there were 3–5 tigers in Hunchun Nature Reserve (State Forestry Administration, 2009). Our analysis identified at least five individual tigers in this area. The presence of at least one female indicates breeding potential but the male-biased sex ratio is not ideal for this. Our genetic diversity indices are not statistically powerful because of the small sample size but are nonetheless valuable because of the scarcity of information on wild Amur tigers in China.

The Amur tiger may be the most genetically impoverished extant tiger subspecies (Luo et al., 2004, 2008; Henry et al., 2009). The sex ratio of Amur tigers in the Russian Far East appears to be female-biased (Sugimoto et al., 2012); the male-bias of the population in Hunchun Nature Reserve could be a result of the dispersal of male tigers from nearby Russian reserves. The protected areas on both sides of the border should therefore be considered a single geographical unit for tiger conservation.

This study provides valuable baseline genetic diversity estimates through non-invasive monitoring for a rare tiger population warranting management. Although we found relatively few tiger scats, our results indicate there is a potentially viable population in Hunchun Nature Reserve, albeit with low levels of observed genetic diversity. Previous studies have shown that small, threatened populations can be managed in the wild to maintain viability (Höglund et al., 2009), and we believe this applies to the Hunchun Amur tiger population. It could be queried whether conservation resources should be invested in a small, peripheral population, but we believe this is appropriate if it contributes to the genetic diversity of the species, especially one as geographically disparate as the tiger. Our findings will aid the development of conservation management strategies for the tiger in China.

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References

Benson, D.A., Karsch-Mizrachi, I., Lipman, D.J., Ostell, J. & Wheeler, D.L. (2005) GenBank. *Nucleic Acids Research*, 33, D34–D38.

Bhagavatula, J. & Singh, L. (2006) Genotyping faecal samples of Bengal tiger *Panthera tigris tigris* for population estimation: a pilot study. *BMC Genetics*, 7, 1–12.

Caragiulo, A., Dias-Freedman, I., Clark, J.A., Rabinowitz, S. & Amato, G. (2014) Mitochondrial DNA sequence variation and phylogeography of Neotropic pumas (*Puma concolor*). *Mitochondrial DNA*, 25, 304–312.

Chen, J., Na, S., Shun, Q., Zhang, L., Tang, J., Lang, J. et al. (2011) Amur tiger and prey in Jilin Hunchun National Nature Reserve, China. *Chinese Journal of Zoology*, 46, 46–52.

Dinerstein, E., Loucks, C., Wikramanayake, E., Ginsberg, J., Sanderson, E., Seidensticker, J. et al. (2007) The fate of wild tigers. *BioScience*, 57, 508–514.

Frankham, R., Ballou, J.D. & Briscoe, D. (2010) *Introduction to Conservation Genetics*. Cambridge University Press, Cambridge, UK.

Henry, P., Miquelle, D., Sugimoto, T., McCullough, D.R., Caccone, A. & Russello, M.A. (2009) In situ population structure and ex situ representation of the endangered Amur tiger. *Molecular Ecology*, 18, 3173–3184.

Heptner, V. & Sludskii, A. (1972) *Mammals of the Soviet Union, Volume II, Part 2 Carnivora (Hyaenas and Cats)*, pp. 83–159. Smithsonian Institution Libraries and National Science Foundation, Washington, DC, USA.

Höglund, J., Johansson, T., Beintema, A. & Schekkerman, H. (2009) Phylogeography of the black-tailed godwit *Limosa limosa*: substructuring revealed by mtDNA control region sequences. *Journal of Ornithology*, 150, 45–53.

Kalinowski, S., Taper, M. & Marshall, T. (2007) Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. *Molecular Ecology*, 16, 1099–1106.

Luo, S.J., Johnson, W.E., Martenson, J., Antunes, A., Martelli, P., Uphyrkina, O. et al. (2008) Subspecies genetic assignments of worldwide captive tigers increase conservation value of captive populations. *Current Biology*, 18, 592–596.

Luo, S.J., Kim, J.H., Johnson, W.E., Walt, J., Martenson, J. & Karanth, U.K. (2004) Phylogeography and genetic ancestry of tigers (*Panthera tigris*). *PLoS Biology*, 2, 2275–2293.

Ma, Y., Zhang, E., Miquelle, D., Wang, T. & Kang, A. (2005) Changes in Amur tiger distribution in northeast China in the past 100 years. In *Recovery of the Wild Amur Tiger Population in China: Progress and Prospect* (eds E. Zhang, D. Miquelle, T. Wang & A. Kang), pp. 164–170. China Forestry Publishing House, Harbin, China.

Menotti-Raymond, M., David, V., Lyons, L., Schäffer, A., Tomlin, J., Hutton, M. & O'Brien, S. (1999) A genetic linkage map of microsatellites in the domestic cat (*Felis catus*). *Genomics*, 57, 9–23.

Miquelle, D., Darman, Y. & Seryodkin, I. (2011) *Panthera tigris altaica*. In *The IUCN Red List of Threatened Species. v. 2014.2*. Http://www.iucnredlist.org [accessed 18 September 2014].

Miquelle, D., Goodrich, J., Kerley, L., Pikunov, D., Dunishenko, Y.M., Aramiliev, V. et al. (2010) Science-based conservation of Amur tigers in the Russian Far East and north-east China. In *Tigers of the World: The Science, Politics, and Conservation of* Panthera tigris (eds R. Tilson & P. Nyhus), pp. 403–423. Elsevier/Academic Press, Oxford, UK.

Miquelle, D., Pikunov, D., Dunishenko, Y., Aramilev, V., Nikolaev, I., Abramov, V. et al. (2005) Amur tiger census. *CAT News*, 46, 11–14.

Mondol, S., Karanth, K.U., Kumar, N.S., Gopalaswamy, A.M., Andheria, A. & Ramakrishnan, U. (2009) Evaluation of non-invasive genetic sampling methods for estimating tiger population size. *Biological Conservation*, 142, 2350–2360.

Morell, V. (2007) Can the wild tiger survive? *Science*, 317, 1312–1314.

Pikunov, D.G., Miquelle, D.G., Abramov, V.K., Nikolaev, I.G., Seryodkin, I.V., Murzin, A.A. & Korkiskho, V.G. (2003) Results of population surveys of leopards (*Panthera pardus orientalis*) and tigers (*Panthera tigris altaica*) in south-west Primorskii Krai, Russian Far East. Final report to The Wildlife Conservation Society and The World Wildlife Fund.

Ratnasingham, S. & Hebert, P.D.N. (2007) Bold: the barcode of life data system (http://www.barcodinglife.org). *Molecular Ecology Notes*, 7, 355–364.

Rousset, F. (2008) GENEPOP'007: a complete re-implementation of the GENEPOP software for Windows and Linux. *Molecular Ecology Resources*, 8, 103–106.

Russello, M.A., Gladyshev, E., Miquelle, D. & Caccone, A. (2004) Potential genetic consequences of a recent bottleneck in the Amur tiger of the Russian Far East. *Conservation Genetics*, 5, 707–713.

State Forestry Administration (2009) *Resources Survey on National Key Terrestrial Wild Animals*, pp. 265–271. China Forestry Publishing House, Beijing, China. [In Chinese]

Sugimoto, T., Nagata, J., Aramilev, V.V. & McCullough, D.R. (2012) Population size estimation of Amur tigers in Russian Far East using noninvasive genetic samples. *Journal of Mammalogy*, 93, 93–101.

Sun, H. & Dong, H. (2005) The wild population monitor of Amur tiger in Heilongjiang Province. *Forestry Science and Technology*, 6, 33–35.

Sun, B., Miquelle, D., Xiaochen, Y., Zhang, E., Hiyai, S., Goshen, G. et al. (1999) *Survey of Amur Tigers and Far Eastern Leopards in Eastern Heilongjiang Province, China, and Recommendations for their Conservation*. A final report to The Wildlife Conservation Society, Bronx, New York, USA.

Taberlet, P., Griffin, S., Goossens, B., Questiau, S., Manceau, V., Escaravage, N. et al. (1996) Reliable genotyping of samples with very low DNA quantities using PCR. *Nucleic Acids Research*, 24, 3189–3194.

Valière, N. (2002) GIMLET: a computer program for analysing genetic individual identification data. *Molecular Ecology Notes*, 2, 377–379.

Wei, K., Zhang, Z., Zhang, W., Xu, X., Liang, X., He, G. et al. (2008) PCR-CTPP: a rapid and reliable genotyping technique based on ZFX/ZFY alleles for sex identification of tiger (*Panthera tigris*) and four other endangered felids. *Conservation Genetics*, 9, 225–228.

Yang, S., Jiang, J., Wu, Z., Li, T., Yang, X., Han, X. et al. (1998) *A Survey of Tigers and Leopards in Eastern Jilin Province, China, Winter 1998*. A final report to UNDP and The Wildlife Conservation Society, Vladivostok, Russia.

Biographical sketches

Anthony Caragiulo's research interests include conservation genetics and using non-invasive molecular techniques to monitor wildlife, especially cougars. Yang Kang is interested in monitoring threatened animal habitat. Salisa Rabinowitz is interested in global conservation of large felids, with the help of non-invasive molecular techniques. Isabela Dias-Freedman's research interests include animal and human genetics, using molecular techniques for wildlife conservation, genetic resistance to emergent diseases, and forensic science. Simone Loss is interested in molecular techniques for wildlife conservation. Xu-Wei Zhou focuses on the molecular ecology of rare mammals. Wei-Dong Bao's research interests include wildlife ecology and felid conservation. George Amato's research interests include non-invasive sampling for threatened species and monitoring the trade in threatened species products, using DNA-based forensic science.