Original Investigation

Siberian tiger's recent population bottleneck in the Russian Far East revealed by microsatellite markers

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ARTICLE INFO

ABSTRACT

Because size reduced, or bottlenecked, populations are more prone to adverse events, the detection of genetic bottleneck signatures in wildlife species is highly relevant for conservation. Here we applied 11 microsatellite markers to the endangered Siberian tiger (*Panthera tigris altaica*) using tissue and blood samples of animals from the Primorsky region of the Russian Far East. Excess heterozygosity and mode shift in allele frequencies tests were positive, while the M-ratio test was negative, indicating the likelihood of a contemporary rather than a historical population bottleneck. The recent genetic population bottleneck could be attributed to the well-documented demographic collapse of the Siberian tiger population in the 1940s, when population size hit bottom with 20–30 surviving animals. The mean effective population size N_e was 14 Siberian tigers (CI$_{95}$: 12–25 animals), and the effective population size/census size ratio (N_e/N ratio) was 0.028. This is the first molecular evidence of a recent Siberian tiger population bottleneck, which is of great interest for further conservation and management plans of the highly endangered largest felid species, while the worryingly low effective population size challenges the optimism for the recovery of the huge Siberian cat.

Introduction

With only a few hundred individuals remaining, the Siberian or Amur tiger (*Panthera tigris altaica*) is listed as endangered in the IUCN Red List (Miquelle et al. 2010a). By biogeographical and molecular genetic means the establishment of the Siberian tiger population was estimated to have occurred less than 10,000 years ago (Kitchener and Dugmore 2000; Luo et al. 2004). *P. t. altaica* was formerly distributed across a vast territory of the southern regions of the Russian Far East, in northern China, and on the Korean peninsula (Heptner and Sludskii 1972; Miquelle et al., 2010b). Due to hunting, poaching and habitat destruc-

tion nowadays 502 adult/subadult tigers are still roaming the woods of the Russian Far East (Miquelle et al. 2007), about twenty tigers (immigrants from Russia) live in North-eastern China, and most likely none survived in Korea (Matyushkin et al. 1999; Miquelle and Pikunov 2003; Miquelle et al. 2007; http://www.iucnredlist.org/apps/redlist/details/15956/0). The current Russian population of *P. t. altaica* is divided in two subpopulations of which the larger one (∼79%) lives in Primorski Krai, the southern and central Sikhote-Alin region, whereas the other, smaller one (∼21%) lives in its northern part, Khabarovski Krai (Miquelle et al. 2007).

The current proportion of strictly protected areas in the Russian Far East is estimated at 3.4%, 10,300 km^2 (Miquelle et al. 2005). These areas are insufficient to prevent extirpation of the Siberian tiger, because of expanding forestry and external demand for tiger parts (Miquelle et al. 1999; Carroll and Miquelle 2006). Hence, the urgency of identifying areas outside the current protected areas, which is critical for the persistence of this threatened felid species (Carroll and Miquelle 2006; Miquelle et al. 2007).

Genetic analyses have shown that Siberian tigers are the most genetically impoverished of all extant tiger subspecies (Russello et al. 2004; Luo et al. 2004, 2008). To make matters worse, the

Fig. 1. Map showing approximate sample collection sites in the Russian Far East. ALT is the international abbreviation for *Panthera tigris altaica*.

actual effective population size in the Sikhote-Alin mountain range is estimated to be as low as 28 individuals (CI$_{95}$: 19–48) (Henry et al. 2009).

Based on the well-documented 20th century decline of the Siberian tiger population, a study was undertaken to find traces of it in the genetic footprint of the species (Henry et al. 2009) by genotyping scat samples across 8 microsatellite loci. However, it failed to find evidence of a recent population bottleneck, although genetic signatures of a historical contraction were detected (Henry et al. 2009). This disparity in signal was argued to be due to several reasons, including historical paucity in population genetic variation associated with postglacial colonization, potential gene flow from a now extirpated Chinese population, and/or potentially insufficient power of the loci used to detect the recent demographic contraction (Henry et al. 2009).

The aim of our study was to test for presence and extent of a recent Siberian tiger's population bottleneck using a larger number of microsatellite loci applied to tissue and blood samples from *P. t. altaica* belonging to the tiger population of Primorski Krai of the Russian Far East.

Material and methods

Sample collection and DNA extraction

A total of 15 wild-born Siberian tiger individuals were sampled from the distribution range of Primorski Krai of the Russian Far East (Fig. 1) between 1999 and 2000. Blood samples were taken from seven immobilized wild Siberian tigers in time of radio-collaring and were provided for genetic analysis by Russian collaborators of the Russian-Chinese Wildlife Conservation Society (WCS). One tissue sample came from a tiger that had drowned in Khanka Lake and was provided by the Khanka Lake Nature Reserve. Other tissue samples were taken from seven poached animals that had been seized by the staff of the Tiger State Inspection Service of the Russian Far East.

The DNA was extracted from blood or tissue samples following the standard phenol/chloroform procedures (Sambrook et al. 1989). The DNA extractions were carried out in a separate laboratory exclusively used for samples expected to have a low DNA concentration. Two blanks (reagents only) were included in each extraction to monitor for contamination (Handt et al. 1994).

Fluorescent-based polymerase chain reaction analysis of microsatellite DNA

Twenty microsatellite loci (FCA01, FCA06, FCA14, FCA24, FCA69, FCA82, FCA85, FCA96, FCA97, FCA 136, FCA139, FCA161, FCA 191, FCA211, FCA249, FCA261, FCA310, FCA391, FCA441 and FCA719) originally designed for the domestic cat (*Felis catus*) (Menotti-Raymond et al. 2003) were amplified by PCR using fluorescent dye-labelled primers in 6 multiplex reaction (Table 1). PCR was conducted using 1 μl DNA (25–50 ng/μl), 1 μl of each multiplex primer mix (see Table 1 for primer concentrations), 5 μl 2× Multiplex PCR Master Mix and 2 μl 5× Q-solution (both QIAGEN®) in a final volume of 10 μl. Samples were subjected to the following

Table 1
Characterization of 18 loci for the Siberian tiger (*Panthera tigris altaica*) based on 15 individuals from the Russian Far East. Panel: PCR multiplex panel.

Locus	Fluorescent dye	Primer concentration (μM)	Panel	N_A	Fragment size (bp)	H_O	H_E	$H_{EQ\ TPM}$	P_{HWE}
FCA14[a]	FAM	0.15	I	4	152–158	0.538	0.680	0.596	0.111
FCA69[b]	HEX	0.15	I	3	96–102	0.667	0.605	0.458	0.844
FCA249[a]	HEX	0.25	I	1	238	NC	NC	NC	NC
FCA24[a]	HEX	0.2	II	2	216–218	0.533	0.515	0.285	1.000
FCA85[a]	FAM	0.1	II	4	127–135	0.733	0.692	0.577	0.800
FCA391[b]	FAM	0.1	II	1	210	NC	NC	NC	NC
FCA96[b]	FAM	0.3	III	2	201–207	0.133	0.405	0.272	0.020[c]
FCA139[b]	HEX	0.1	III	2	132–134	0.357	0.516	0.275	0.314
FCA719[a]	HEX	0.1	III	4	175–187	0.533	0.674	0.590	0.549
FCA06[a]	HEX	0.2	IV	2	184–188	0.267	0.331	0.267	0.458
FCA97[a]	FAM	0.2	IV	1	140	NC	NC	NC	NC
FCA136[a]	FAM	0.5	V	1	250	NC	NC	NC	NC
FCA161[b]	HEX	0.2	V	3	180–188	0.571	0.579	0.456	0.664
FCA191[a]	HEX	0.3	V	4	138–150	0.400	0.646	0.466	0.020[c]
FCA211[b]	FAM	0.1	V	1	109	NC	NC	NC	NC
FCA01[a]	HEX	0.3	VI	2	123–125	0.286	0.349	0.281	0.485
FCA261[a]	FAM	0.1	VI	4	196–204	0.643	0.685	0.591	0.617
FCA310[b]	HEX	0.1	VI	2	124–206	0.286	0.349	0.275	0.492

N_A, number of alleles. H_O, observed heterozygosity. H_E, expected heterozygosity. H_{EQ}, H_E under mutation-drift equilibrium for TPM mutation models. NC, not calculated. P_{HWE}, P-value for Hardy-Weinberg equilibrium.

[a] Loci tested for cross-species amplification in this study.
[b] Loci tested for cross-species amplification by Luo et al. (2004).
[c] Locus deviated from HWE.

thermal profile for amplification in a CFX96 thermal cycler (Bio-Rad): 15 min at 95 °C (initial denaturing), followed by 35 cycles of three steps of 30 s at 94 °C (denaturation), 30 s at 55 °C (annealing) and 30 s at 72 °C (extension), before a final elongation of 30 min at 60 °C. PCR blanks (reagents only) were included in each PCR experiment. PCR products were sized together with ROX® size standard on an ABI 3130xl Genetic Analyzer (Applied Biosystems). Allele sizes were determined using GeneMapper 3.7 (Applied Biosystems) followed by manual proofreading. To avoid genotyping errors, all PCRs and sample analyses were repeated 3 times.

Microsatellite analysis

To calculate the expected (H_E) and observed (H_O) heterozygosity, to test for linkage disequilibria (LD), and to test for Hardy-Weinberg equilibrium (HWE) we applied the program GENEPOP (v.3.4; Raymond and Rousset 1995). Deviations from HWE and tests for LD were evaluated using Fisher's exact tests and sequential Bonferroni corrections. Detection of possible genotyping mistakes (scoring error due to stuttering, large allele dropout) were made with the software MICROCHECKER (Oosterhout et al. 2004). To avoid biasing of results by Null-alleles, their frequencies were estimated using ML-NULLFREQ (Kalinowski and Taper 2006).

Theory predicts that in recently bottlenecked populations, observed heterozygosity will be higher than would be expected at mutation-drift equilibrium (H_{EQ}) (Piry et al. 1999). Therefore we assessed the genetic signature of Siberian tigers using (i) tests to determine heterozygote excess such as sign test, standardized differences test and Wilcoxon sign-rank test, and (ii) the mode-shift test, i.e. distortion of the typical L-shape distribution (all implemented in the software package BOTTLENECK; v.1.2.02; Piry et al. 1999). Significance was assessed by 10,000 iterations in a one-tailed Wilcoxon sign rank tests. We used the intermediate, two-phase mutation model (TPM) with a proportion of the stepwise mutation model (SMM) of 5% as recommended (Piry et al. 1999). TPM had been shown to deliver the most realistic results of mutational events in microsatellite loci (Di Rienzo et al. 1994).

We also calculated the *M* statistics of Garza and Williamson (2001) (http://swfsc.noaa.gov/textblock.aspx? Division=FED&id=3298), where *M* here is defined as the ratio of number of alleles *k*, to their range in size *r*, applying parameter values from the two-phase model (TPM) specified by the authors ($\theta[4N_e\mu] = 1$–10, $p_s = 0.9$, $\Delta_g = 3.5$) using the program M_P_VAL (Garza and Williamson 2001). During a bottleneck rare alleles are lost quickly by genetic drift thus reducing the number of observed alleles *k* faster than the size range *r* of those alleles (*r* is only reduced by the loss of the largest or the smallest allele), thus resulting in a reduced *M*-ratio ($M = k/r$). Critical *M* values were determined by applying the program CRITICAL_M (http://swfsc.noaa.gov/textblock.aspx?Division=FED&id=3298) based on 1000 simulations and additional parameters as implemented by Henry et al. (2009): marker mutation rate $\mu = 5 \times 10^{-4}$, and pre-bottleneck N_e ranging from 500 to 5000. The *M*-test is particularly suited to detect historical bottlenecks (Garza and Williamson 2001).

To check for possible sub-population structure among the collected samples we used the Bayesian assignment test of the software STRUCTURE (v.2.3.3; Pritchard et al. 2000). To determine the appropriate burn-in and run lengths for accurate parameter estimates of *P* and *Q*, we set *K* = 1 and watched for the likelihoods to converge under various burn-in and run lengths. The final burn-in and run lengths of Markov chains were then both 10^5. We ran 20 independent runs for each *K* (for *K* = 1–10) and its associated parameter set to verify the consistency of estimates across runs.

Siberian tiger effective population size (N_e) was estimated based upon the use of summary statistics in an approximate Bayesian computation framework (Tallmon et al. 2004), using the software ONESAMP (Tallmon et al. 2008). As priors we considered N_e values ranging between 2 and 500 (Henry et al. 2009). All input file preparations were made using CONVERT (v. 1.31; Glaubitz 2004).

Results and discussion

Most populations fluctuate in size and range over time. If the fluctuations are pronounced enough, populations may become very small, experiencing a so-called bottleneck or they may become extinct (Morris and Doak 2002). Although we had only a limited number and scope of samples, our tiger samples at least yielded high-quality DNA from wild individuals, which was not available in previous studies. In our study, we originally used 20 cat (*Felis*

Table 2
Parameters and results for M-ratio and bottleneck analyses used to detect significant reductions in effective population size of Siberian tiger population from the Russian Far East.

Bottleneck tests		M-ratio test	
	TPM mutation model		
Sign test	Exp = 5.93	M	0.835
	Obs = 11	Mc (θ = 1)	0.492
	P = 0.001	Mc (θ = 2)	0.434
Standardized differences test	T2 = 2.744	Mc (θ = 10)	0.398
	P = 0.003		
Wilcoxon's signed rank test	P < 0.001		

Fig. 2. Shifted distribution mode of allele frequencies at 11 polymorphic microsatellite loci applied to the Siberian tiger population from the Russian Far East.

catus; FCA) microsatellite loci. Out of these 18 yielded PCR signals in the Siberian tiger and 13 where polymorphic. FCA82 and FCA441 failed to be amplified. FCA01, FCA06, FCA14, FCA24, FCA82, FCA85, FCA97, FCA136, FCA191, FCA249, FCA261, and FCA719 were cross-species amplified for the first time in the Siberian tiger, while the other 8 loci (FCA69, FCA96, FCA139, FCA161, FCA211, FCA310, FCA391, and FCA441) had been tested previously (Luo et al. 2004). The mean number of alleles per locus was N_A = 2.92, ranging from 2 to 4 (Table 1). Loci FCA96 and FCA191 deviated from HWE and were excluded from the analyses, which were based on the remaining eleven loci. For the 11 loci examined there was neither evidence of LD (P > 0.05), nor was there evidence for scoring errors due to stuttering, large allele dropout, or null alleles. The high quality of the DNA extracted from Siberian tiger tissue samples permitted the correct microsatellite scoring without significant error rates.

Besides a low mutation rate, the low number of alleles per locus could also be the first indicator of a chronic bottleneck that lasted over multiple generations (Garza and Williamson 2001). It is consistent with earlier analyses considering *P. t. altaica* the most genetically impoverished of all extant tiger subspecies (Luo et al. 2004, 2008; Russello et al. 2004; Piry et al. 1999).

Bottleneck analyses rely, substantially, on the absence of population structure (Busch et al. 2007; Broquet et al. 2010). Population structure and sampling scheme could present a false signal of population bottleneck (Wakeley 1999; Chikhi et al. 2010).

Using Bayesian assignment test of the software STRUCTURE, ln Pr($X|K$) for the likely number of populations K, decreased with increasing K. The mean ln Pr($X|K$) values and their standard deviations for the first five K based on 20 iterations were −342.92 ± 0.22 (for K = 1), −344.43 ± 0.74 (K = 2), −344.09 ± 0.65 (K = 3), −346.48 ± 1.24 (K = 4), and −345.32 ± 1.60 (K = 5). As ln Pr($X|K$) was highest for K = 1, it indicated that no evidence of sub-structuring exists among the collected samples.

We estimated the mean effective population size N_e to be 14 animals (CI$_{95}$: 12–25) Considering an upper bound estimate of the Russian Far East tiger population of 502 individuals (Miquelle et al. 2007; Henry et al. 2009), the effective population size/census size ratio (N_e/N ratio) was 0.028, which is about half of the 0.054 obtained by Henry et al. (2009). In comparison with other threatened animals (Palstra and Ruzzante 2008) N_e in Siberian tigers is extremely low, challenging the optimism that the population of the huge cat is in recovery.

The mean expected heterozygosity of our data set was H_E = 0.540 (Table 1), but our data displayed an excess of heterozygosity relative to the number of alleles expected at mutation–drift equilibrium (H_{EQ}). Using BOTTLENECK we detected a significant heterozygosity excess (P < 0.01 for Sign test, standardized differences test, and Wilcoxon's signed rank tests), indicating a contemporary population bottleneck (Table 2). A bar plot, displaying allele categories (X-axis) versus percentages of alleles in each category (Y-axis) showed a shifted mode for allele frequency distribution (Fig. 2), also indicating a contemporary population bottleneck. Populations with historically relatively stable population sizes will generate L-
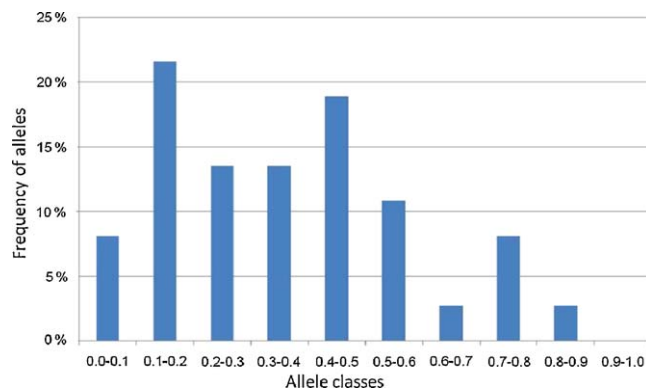
shaped graphs in such category plots (Piry et al. 1999; Cristescu et al. 2010), while bottlenecks cause alleles at low frequencies (<0.1) to become less abundant than alleles in one or more intermediate allele frequency classes (Luikart et al. 1998), thereby generating the shifted mode for allele frequency distribution observed in the Siberian tiger.

The M-ratio which tests for a historical population bottleneck was consistently above the critical value thresholds (Table 2), regardless of the assumed pre-bottleneck N_e. It was also above the critical value of 0.68 proposed by Garza and Williamson (2001). All test results indicate a recent rather than a historical Siberian tiger population bottleneck (Williamson-Natesan 2005).

The three different tests for significance used in our study provided statistical evidence of population decline and recovery in two different time scales (recent and historical). While heterozygosity excess and allele frequency distributions recover relatively quickly, the M-ratio is expected to have a long recovery time (Garza and Williamson 2001; Williamson-Natesan 2005).

The results did not change significantly when we included the two loci at which allele frequencies deviated from HWE (data not shown).

A historical bottleneck had already been detected previously (Henry et al. 2009), likely linked to founder effect of the post-ice age colonization of the tiger's far eastern distribution range just about 10,000 years ago. This renders Siberian tigers very young compared to other large cats (Luo et al. 2004; Wilting et al., 2010). The study (Henry et al. 2009) however, did not detect a more recent population size contraction, possibly due to the very high rate of missing data across individuals and loci (53–63%).

To our knowledge, our results are the first to demonstrate a quite recent genetic bottleneck in Siberian tigers, a result that matches the well-documented severe demographic decline of the Siberian tiger population in the 1940s (Kaplanov 1948). We do not believe this to be coincidence because mode shift and heterozygosity excess are transitory phenomena that become erased over approximately 0.2–4 N_e generations (Luikart and Cornuet 1998).

Our results likewise underline the cautious approach researchers and conservationists should take when studying the unknown past of populations by means of microsatellite allele distribution analyses (Cristescu et al. 2010).

The findings presented in our study should be taken into account in future molecular studies of this population, because recently bottlenecked populations may have lost lineages that affect inferences of their demographic history from contemporary molecular data sets (Johnson et al. 2007).

The molecular bottleneck pattern presented in our study is of pivotal interest, because optimal Siberian tiger management and conservation programs require additional interventions such as

establishing corridors and buffer zones and/or implementing reintroduction programs (Tilson et al. 2001), which in turn require knowledge regarding the genetic background of the individuals included in such measures.

Acknowledgements

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