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Inbreeding status and implications for Amur tigers

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Abstract

Inbreeding more likely occurs in small, isolated and endangered populations, and may influence the sustainable survival of a population. As the Amur tiger *Panthera tigris altaica* population in China experienced a severe decline in the 1990s, the recovering population may be prone to inbreeding and its potential impacts on population health. However, the inbreeding status has not been evaluated and relationships with health remain poorly understood in wild animals. Based on the genetic samples collected from the main Amur tiger habitats in China, this study analyzed the population inbreeding level, major histocompatibility complex polymorphism, parasitic infections and gut microbial structures and functions, and then explored the influence of inbreeding on these traits. Our results indicated that more than 50% of individual relationships were in cousin or half sibs, and 22.73% of individuals had moderate or high inbreeding coefficients. There was a significant positive correlation between the inbreeding level of an individual and the *Toxocara cati* parasitic load. Gut microbiota community structure and function were also impacted by inbreeding intensity. In conclusion, results indicate that the Amur tiger population in China has reached a moderate level of inbreeding and that there are direct interactions between inbreeding intensity and parasitic load and gut microbiota. This study thus provides an early warning on the Amur tiger population health and should prompt the construction of national and international ecological corridors and/or the re-introduction of new individuals to relieve the evident inbreeding pressure.

Introduction

Inbreeding, the result of mating among close relatives, frequently occurs in small and isolated animal populations, and has a negative influence on most species, ultimately reducing the fitness of offspring and the sustainable survival of the population (Lande, 1988; Kokko & Ots, 2010; Mattey, Strutt & Smiseth, 2013; Weiser *et al*., 2015). Previous research has suggested that inbreeding could result in a decrease in heterozygosity and increase the expression of deleterious recessive alleles (Fox, 2005). Inbreeding also has severe consequences on fitness-related traits. For instance, it commonly has a negative impact on immune function, leads to a decrease in resistance to gastrointestinal parasitic infection, and possibly reshapes the gut microbial communities (Reid, Arcese & Keller, 2003; Cassinello, Gomendio & Roldan, 2010; Yuan *et al*., 2015; Wang *et al*., 2020).

A thorough knowledge base shows that when a population reaches a certain level of inbreeding, the number of major histocompatibility complex (MHC) unique genotypes and the number of alleles will decline (Richardson & Westerdahl, 2003; Siddle *et al*., 2010; Ellison *et al*., 2012). For example, Morris *et al*. (2014) using MHC-linked microsatellite markers found that the MHC diversity of outbred Australian cats was significantly higher than that of purebred Burmese cats with high inbreeding coefficient, which may also be conducive to disease resistance. MHC genes code for proteins that can recognize foreign peptides which are delivered to

T cells, and hence MHC genes can initiate an adaptive immune response against invading pathogens (Peng *et al.*, 2020). Furthermore, MHC polymorphism, found in most vertebrates, plays a pivotal role in increasing individual fitness (Piertney & Oliver, 2006; Sepil, Lachish & Sheldon, 2013).

Parasites live on or inside organisms, have complex and dynamic relationships with their mammalian hosts, and the resources they acquire bring potent stress on the host (Wenzel & Piertney, 2015). The effect of parasite infection intensity on host fitness and health status is still a hotspot area of research, with effects including body weight loss, decreased egg mass production, increased risk of infectious disease and manipulation of host's behavior (Brown, 1999; Jarvi *et al.*, 2004; Kutz *et al.*, 2004; Blair & Webster, 2010; Bakker & Traniello, 2017). Inbreeding is one of the multiple factors which plays an essential role in host vulnerability and parasite infection levels (Overall *et al.*, 2005). A recent study on female dolphins *Stenella coeruleoalba* detected that the load of lung nematodes (*Skrjabinalius guevarai*) in the host was significantly negatively correlated with the individual's heterozygosity, implying the influence of inbreeding on parasite infection (Gkafas *et al.*, 2020).

Gut microbiome composition is also suggested to be influenced by kinship, and this influence could work through two ways: vertical transmission from mother to embryo and horizontal transmission by association with close relatives (Lombardo, 2008; Yuan *et al.*, 2015; Amato *et al.*, 2017). The effect of inbreeding on gut microbiota should be an important part of inbreeding research, especially considering the microbiota's critical significance in immune system function, nutrient acquisition, regulating metabolic disease and beneficial phenotypic plasticity (Clemente *et al.*, 2012; Alberdi *et al.*, 2016; Brahe, Arne & Larsen, 2016; Ning *et al.*, 2020). In the case of gopher tortoises *Gopherus polyphemus*, the diversity of gut microbiota has a significantly negative correlation with the inbreeding coefficient, and the similarity of the microbial community increases between relatives (Yuan *et al.*, 2015). However, studies are still few and far between on the effects of inbreeding on gut microbiota, parasite infection and MHC polymorphism in many animal groups, including birds, fish and large carnivores.

In 2010, the International Tiger Forum held in St. Petersburg, Russia reached an agreement among all 13 tiger range countries to double the number of wild tigers by the year 2022. In the case of the Amur tiger *Panthera tigris altaica* in north-east Asia, the number of individuals fell to less than 50 in Russia in the 1940s and approximately 10 in China in the 1980s (He, Yu & Shi, 1997; Russello *et al.*, 2004). Since the St. Petersburg agreement in 2010, China and Russia, which have always valued Amur tiger conservation and are the main range countries of this subspecies, have further stepped up their efforts and significantly restored tiger habitats and populations (Soh *et al.*, 2014). In China, the wild Amur tiger population increased from a total of 12–16 individuals in 2000 to 26 individuals in one of the primary core areas assessed between 2012 and 2014 (Wang *et al.*, 2016*b*). Although the number of Amur tigers and the size of suitable habitat is recovering rapidly in China, severe isolation of habitat patches prevents genetic communication between populations and low levels of genetic diversity have been reported in both China and Russia (Henry *et al.*, 2009; Sorokin *et al.*, 2016; Ning *et al.*, 2019). Considering that the current global population of this subspecies has evolved from only a handful of individuals, the issue of Amur tiger inbreeding and its potential effects on health and hence continued recovery in the wild is an essential area of research and understanding. Nevertheless, this area is poorly understood, especially in China (Henry *et al.*, 2009; Dou *et al.*, 2016; Wang *et al.*, 2016*a*).

In this research, based on genetic samples of wild Amur tigers in China, we used molecular genetic markers to reveal the intensity of inbreeding and analyze its effects on fitness-related traits, including MHC polymorphism, gut parasitic infections and gut microbial structures and functions. Specifically, we aimed to answer the following questions: (1) How many Amur tiger individuals (total, and proportion) have reached the level of moderate ($0.125 \leq f < 0.25$) or high inbreeding ($f \geq 0.25$)? (2) What is the diversity of MHC polymorphism in wild Amur tigers? (3) What is the degree of the intensity of parasite infection in wild Amur tigers? (4) Does inbreeding intensity impact immunity-related genes, infection status (of common gastrointestinal parasites) and/or composition and function of intestinal microbial communities in the Amur tiger?

Materials and methods

Study area and sample collection

Based on previous survey information, the distribution of Amur tigers encompasses an area totaling 83 360 km^2 in north-east China, ranging from 126.517°E to 134.582°E, and from 42.534°N to 48.712°N. Laoyeling, Zhangguangcailing, Wandashan and Lesser Khingan Mountain are all partially included in this range. The climate is characterized as temperate continental monsoonal and experiences long and cold winters. Deciduous forest and mixed coniferous and broad-leaved forest are the main forest types, composed mainly of oak *Quercus mongolica* Fisch. ex Ledeb., birch *Betula platyphylla* Suk., basswood *Tilia tuan* Szyszyl., korean pine *Pinus koraiensis* Sieb. et Zucc., larch *Larix gmelinii* Rupr and other locally common tree species. Roe deer *Capreolus pygargus* and wild boar *Sus scrofa* are the most common ungulate species and the main prey for Amur tigers (Gu *et al.*, 2018). Sika deer *Cervus nippon*, red deer *Cervus elaphus* and musk deer *Moschus moschiferus* also occur in some areas within this range. Sympatric large predators include Amur leopard *Panthera pardus orientalis*, Eurasian lynx *Lynx lynx*, brown bear *Ursus arctos* and black bear *Ursus thibetanus*.

Since 2013, a comprehensive fecal sample collection survey has been conducted in the entire known Amur tiger distribution area in China, including transect surveys, footprint tracking and forest patrols. The transect surveys were conducted in winter when the ground was covered with snow, increasing probability of track and spoor detection (and

ultimately fecal sample collection) and temperatures were typically below −20°C, hence preserving genetic quality of samples. The transects were designed with a stratified sampling method, and the survey intensity was no <15 km/100 km^2 in each forest type. Reports arising from forest patrols and local residents were also followed up with footprint tracking (in winter) to increase the probability of obtaining a fecal sample, while also encouraging local forest workers to collect suspected Amur tiger fecal samples during patrols. Each fecal sample was paired with a record card noting location information, and all samples were kept in a freezer at −80°C until experiments were performed.

DNA analysis

Total DNA was extracted from frozen fecal samples by using a QIAamp DNA Fecal Mini Kit (Qiagen, Valencia, CA, USA), strictly following the operation manual. Then, species and individual identification analysis was conducted. We amplified a 271-bp fragment of cytochrome b gene in the mitochondrial DNA for species identification by following the methods of Sugimoto *et al.* (2006). The 18 short tandem repeat and polymorphic microsatellite markers (FCA5, FCA32, FCA43, FCA44, FCA69, FCA77, FCA90, FCA94, FCA105, FCA161, FCA176, FCA211, FCA220, FCA290, FCA293, FCA304, FCA310, FCA391) that were genotyped across all tiger samples were selected to identify individuals (Zou *et al.*, 2015). We used the Excel Microsatellite Toolkit v3.1.1 (available at http://ms.biomed.cas.cz/MSTools/) to calculate the number of alleles, polymorphism information content (PIC) and observed (H_o) and expected heterozygosity (H_E) for each locus. We calculated the multi-loci potential for correct identification of individuals in GIMLET (Valière, 2002). We also tested Hardy–Weinberg equilibrium (HWE) and identified the null allele by using Genepop and Microchecker (Raymond & Rousset, 1995; Oosterhout *et al.*, 2004).

To calculate the population relatedness coefficients (r), we evaluated the applicability of seven relatedness estimators in the program COANCESTRY (Wang, 2015). Based on the observed microsatellite allele frequencies, we simulated 2000 pairs of three common relatives (half sibs, cousins and unrelated) and then compared with the true value relatedness of each simulated relative to calculate the total variance of every method. Therefore, the kinship estimation method with the lowest sum of variance was selected as the most reliable estimation method for this population. We calculated the likelihood of obtaining the observed relatedness distribution from any mixture of half sibs, cousins and unrelated simulated distributions. Then, the triadic likelihood estimator (TrioML) (Wang, 2007) was applied to estimate the inbreeding coefficient (f) of each Amur tiger based on microsatellite data. According to the conventional cut-off standard, 'moderate inbreeding' is defined as when $0.125 \leq f < 0.25$ and 'high inbreeding' defined as when $f \geq 0.25$ (Keller & Arcese, 1998; Marshall *et al.*, 2002; Hu *et al.*, 2017). To rigorously confirm whether our 18 microsatellite loci could represent the heterozygosity of whole-genome microsatellite, EASYPOP software was used to simulate the genotyping of 22 individuals with 100 microsatellite loci under a mutation rate of 10^{-3}. We then used the same method to calculate the inbreeding coefficient and compared the difference between the simulated and observed results (Balloux, 2001).

In order to evaluate the allelic variation of MHC class II DRB gene and to amplify the homologous DRB gene sequence in tiger DNA, we amplified the target fragment with primers 61a (5′-CCGCTGCACTGTGAAGCT-3′) and 219a (5′-CCACACAGCACGTTTCC/TT-3′) (Luo *et al.*, 2004; Wei *et al.*, 2010) and added multiple unique 8-base pair (bp) DNA tags as the barcodes in front of the primers to distinguish the samples. To analyze the MHC DRB sequences of nucleotide mutation sites, we mixed the purified polymerase chain reaction (PCR) fragment together and used the enzyme-linked method for library construction, and then applied next-generation sequencing (NGS) technology based on the illumina Hiseq ×10 platform with the sequencing kit of HiSeq X™ Ten Reagent Kit v2.5 (Illumina Inc., San Diego, CA, USA). Raw sequences were first analyzed by using AmpliMERGE to combine the paired-end reads in each run to obtain a complete amplification product (Sebastian *et al.*, 2016). Then, we performed AmpliCHECK to assess the variation/artifact frequency and make a preliminary exploration of our dataset. After sequence de-multiplexing, clustering and artifact filtering by using AmpliSAS, finally we identified the artifact and true alleles. NGS data analysis tools in this section are available (and described) at http://evobiolab.biol.amu.edu.pl/amplisat/.

Toxocara cati is the most prevalent gastrointestinal roundworm found in cats (Hart, 2006). After identifying tiger individuals from fecal samples, we selected one fresh sample from each individual to measure the infection intensity and take the average egg number from three different parts of the single sample. We used a saturated solution of sodium chloride as a floating medium to isolate *T. cati* and identified egg species by PCR with primer FM1 (5′-TTGAGGGGAAA TGGGTGAC-3′) and FM2 (5′-TGCTGGAGGCCATATCGT-3′) (Mikaeili *et al.*, 2017; Peng *et al.*, 2020). The detailed PCR conditions were as described in Peng *et al.* (2020), and the amplicons were Sanger-sequenced after examining with the 1% agarose gel. A modified McMaster technique was then applied to determine the intensity of infection. Sixty eggs per gram (epg) was set as the lower threshold limit for *T. cati* infection intensity to evaluate whether the host was infected or not (Peng *et al.*, 2020).

For exploring the relationship between kinship and gut microbiota, we selected three samples from inbred individuals (f = 0.1748, 0.4118 and 0.4264) and three samples from noninbred individuals (f = 0.0256, 0.0519 and 0.0985) for metagenomic sequencing analysis by using NEXTflex Rapid DNA-Seq Kit DNA on the Illumina Hiseq platform, aiming to detect any differences in composition and function of gut microbiota in the two sets of samples. The FastQC program (v0.11.8) (Babraham Institute, Babraham, Cambridgeshire, UK) was used to check the quality of raw illumine reads and subsequently removed the adaptor sequences and trimmed the low quality reads by using Trimmomatic

(v0.38) (Andrews, 2014; Bolger, Lohse & Usadel, 2014). Finally, Metaphlan2 was used to calculate the relative abundance of each sample in the taxonomic profile and HUMAnN2 counted the pathway abundance with the UniRef90 databases (Truong *et al.*, 2015).

Statistical analysis

We tried model lm and glm, glm.nb and glmer (MASS package, R Core Team, 2013) to test the relationship between the individual inbreeding coefficient and the number of *T. cati* eggs per individual. Models were evaluated based on normal residuals, standardized residuals and Cook's distance. We used Mann–Whitney *U* test to identify the difference in individual MHC diversity between the inbred group and the noninbred group. We performed the above analyses in R. Linear discriminant analysis (LDA) effect size (LEfSe) was carried out to test the abundance differences of gut microbiota taxa with the criteria of LDA score >2 and $P < 0.05$ (Kruksal–Wallis test) through the online interface at http://huttenhower.sph.harvard.edu/lefse/, and STAMP software was used to identify the pathway abundance differences.

Results

In this research, 662 transects with a total length of approximately 3300 km and 36 footprint chains with a total length of 158 km were completed. Through these surveys, 163 fecal samples were collected, of which 150 were successfully determined to be of Amur tiger based on the sequencing of mtDNA fragments. After amplifying 18 microsatellite loci, a total of 30 Amur tiger individuals were identified. However, only 22 individuals (16 males, 4 females and 2 of unknown sex) obtained MHC and parasite results of sufficient quality in order to perform further analysis (Fig. 1). The genetic diversity of 18 microsatellite loci in 22 individuals is presented in Supporting Information Table S1, and the mean H_E and H_O heterozygosity were 0.561 and 0.569, respectively. Cumulative PI (biased) and PI (sibs) values of 18 polymorphic microsatellite loci in 22 Amur tiger individuals were $2.261e^{-11}$ and $1.427e^{-5}$, which could be accurately identified different individuals in a small population (Supporting Information Figure S1). The results based on Genepop and Micro-checker showed that there was no significant deviation from HWE in any locus after Bonferroni correction and no

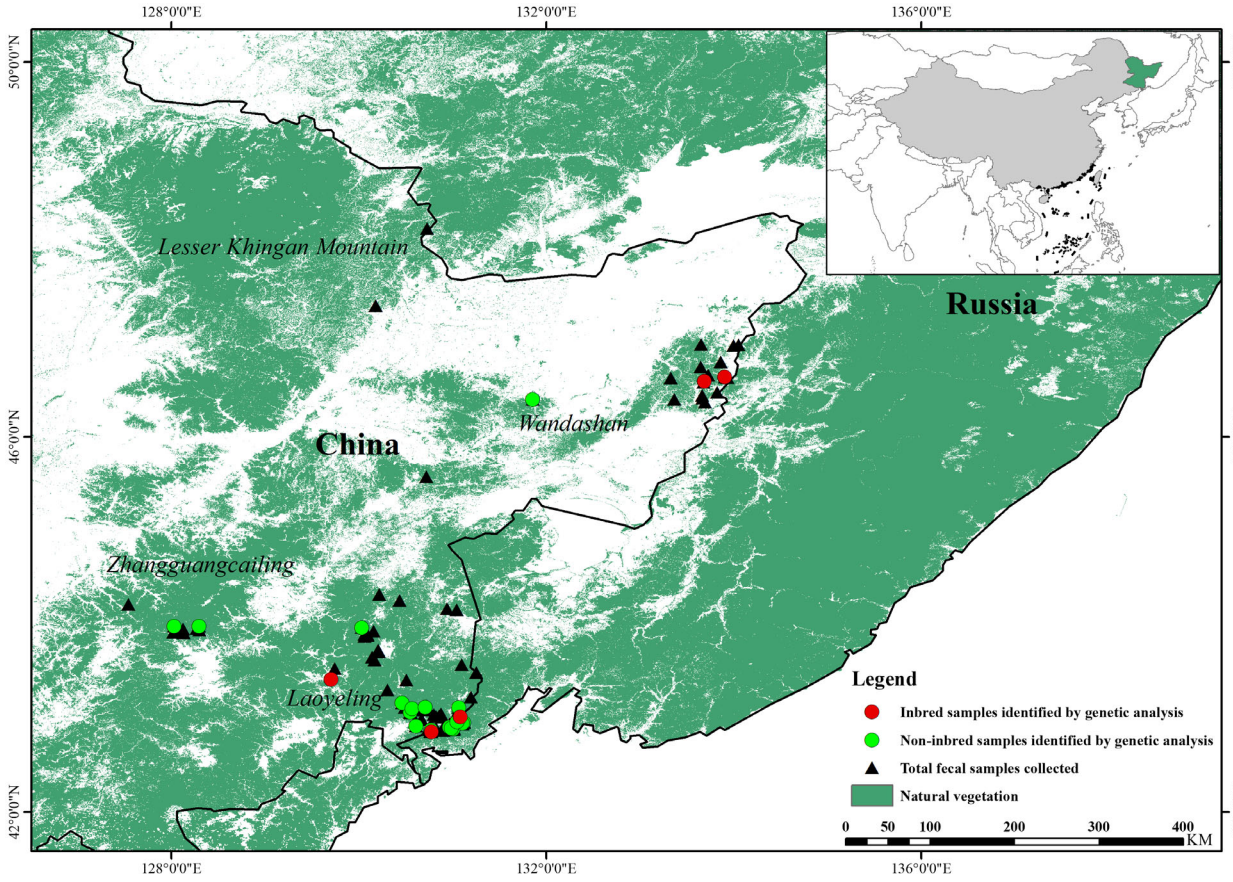

Figure 1 Geographical distribution of the Amur tiger fecal samples collected in north-east China (n = 150; black). Final analyses were of 47 samples representing 22 individuals; 17 individuals were identified as non-inbred (green) and five were identified as inbred (red).

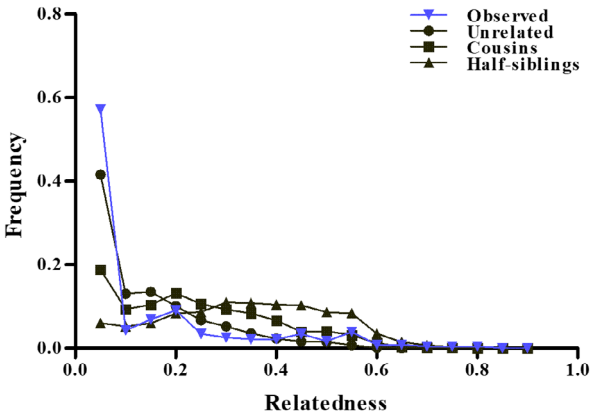

Figure 2 Observed relatedness distribution of Amur tiger (blue triangles) and expected distribution for half sibs (black triangles; true $r = 0.25$), cousins (black squares, true $r = 0.125$) and unrelated (black circles, true $r = 0$).

evidence for null alleles or large allele dropout or stuttering on 18 loci.

According to the simulation results we chose DyadML as the method for estimating kinship between Amur tigers, because this method had the smallest variation compared to the true value. The likelihood analysis showed that the observed distribution was achieved when 45.31% was drawn from the unrelated distribution, 24.31% from the cousin distribution and 30.37% from the half sibs distribution (Fig. 2). The difference in the inbreeding coefficient between our data and the simulated population using 100 microsatellite loci was $P = 0.086$, indicating that our panel of 18 microsatellite loci was a good proxy for the alternative whole-genome microsatellite heterozygosity analyses. According to our inbreeding results, 5 of the 22 individuals were identified as either moderately or highly inbred with specific inbreeding values of 0.1399, 0.1748, 0.2089, 0.4118 and 0.4264. The frequency of tigers infected with *T. cati* was 68.18% and the estimated individual infection intensity ranged from 10 to 3080 epg in 22 Amur tiger individuals. After screening the model, finally we used a linear model which revealed a significant positive correlation between coefficient of five inbred individuals and infection intensity ($r = 0.742$; $P = 0.038$; slope = 4177.6; SE = 1181.5; intercept = −637.8; sample size = 5), that is, inbred individuals with a significantly higher inbreeding coefficient were at higher risk of *T. cati* infection.

We obtained 7 702 515 high-quality sequences from 22 samples (average, 350 114; range, 36 314–996 653 reads per sample). A total of seven unique sequences were homologous to the MHC II DRB exon 2, and the number of alleles ranged from 2 to 6 in 22 individual Amur tigers (Supporting Information Figure S2). There was no evidence of a significant difference in MHC diversity between 5 inbred and 17 noninbred individuals, analyzed by Mann–Whitney *U* test ($P = 0.872$).

The gut microbiota composition of wild Amur tigers was dominated by phyla *Firmicutes* (61.65% in inbred and 86.42% in noninbred group) and *Proteobacteria* (38.27% in inbred and 8.33% in noninbred group), followed by *Bacteroidetes* (0.084% in inbred and 5.09% in noninbred group) (Fig. 3a). LEfSe revealed the existence of three taxa distinctly different between inbred and noninbred populations, namely *Collinsella unclassified*, *Escherichia coli*, *Escherichia* and *Enterobacteriaceae* (LDA score >2, Kruskal–Wallis test $P < 0.05$) (Fig. 3b). Additionally, except for *Collinsella*

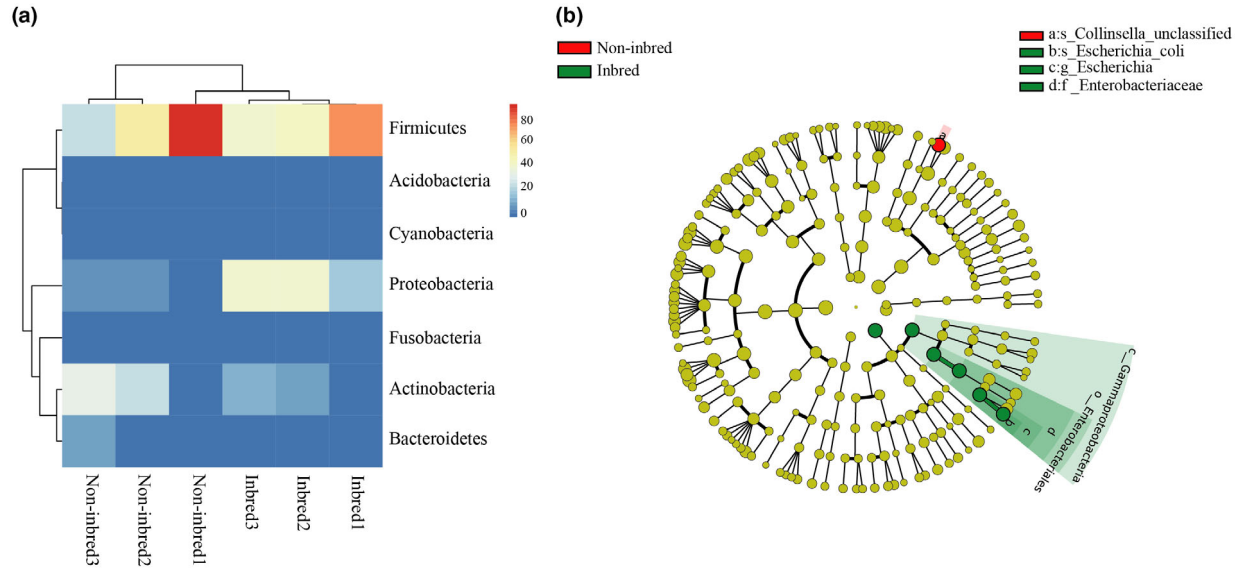

Figure 3 Analysis on the different gut microbiota of inbred and noninbred Amur tigers. (a) The heat map displaying the relative abundance at the phylum level based on the complete cluster method. (b) The cladograms generated by LEfSe analysis showing the significant divergence of seven taxa between inbred and noninbred group. LEfSe, linear discriminant analysis effect size.

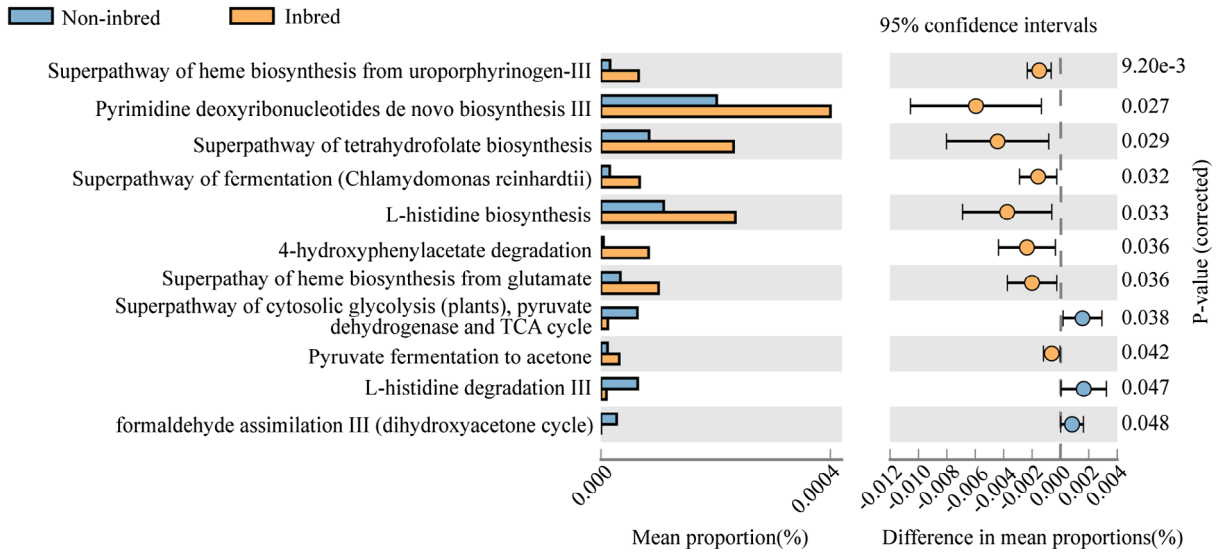

Figure 4 Significant differences in pathways between inbred and noninbred groups of Amur tigers computed by STAMP statistical software using Welch *t* test and the threshold *P* values of 0.05.

unclassified, the other three bacterial taxa were all more abundant in the inbred group than in the noninbred group.

In addition, we determined that the relative abundance of 11 bacterial functional pathways were significantly different between the inbred and noninbred group (Fig. 4). Those pathways associated with biosynthesis include L-histidine, pyrimidine deoxyribonucleotides de novo, tetrahydrofolate and heme, while pathways associated with degradation/utilization/assimilation include 4-hydroxyphenylacetate, formaldehyde assimilation and L-histidine. The other three pathways belong to generation of precursor metabolites and energy. The pathways associated with L-histidine biosynthesis were more observed in the inbred group, while pathways associated with L-histidine degradation were more abundant in the noninbred group. There were two heme biosynthesis-related pathways which were both more abundant in the inbred group.

Discussion

The Amur tiger experienced a severe population decline in the mid-20th century and was isolated into two separate populations of south-west Primorye and Sikhote-Alin, making effective population communication difficult (Umbers *et al.*, 2016). While Laoyeling Mountain in China is adjacent to south-west Primorye, dispersal beyond this was also severely restricted (Ning *et al.*, 2019). Previous research has shown low and worrying levels of genetic diversity of wild tigers both in China and Russia, so the research on inbreeding and its implications is necessary and urgent to not only better understand the genetic status of wild Amur tigers, but also to inform targeted conservation strategies (Russello *et al.*, 2004; Wang *et al.*, 2016*a*).

According to camera trap data collected from the Laoyeling landscape between 2013 and 2014, the sex ratio of Amur tigers in China was almost 1:1 (Wang *et al.*, 2018). In our research however, most Amur tiger fecal samples were from males, probably because the home range of female tigers is mostly close to the Sino-Russian border (unpubl. data) and our data collection here was far more extensive across a larger area.

The mean Amur tiger relatedness value of 0.124 found in this study was very close to cousin relationship. The average inbreeding value of wild Amur tigers in this research was 0.0813, higher than many other wild populations, such as bighorn sheep *Ovis canadensis*, African dogs *Lycaon pictus* and pronghorn fawns *Antilocapra americana* (Rioux-Paquette, Festa-Bianchet & Coltman, 2010; Dunn *et al.*, 2011; Spiering *et al.*, 2011; Nielsen *et al.*, 2012). In addition, 3 of the 22 individuals were identified as moderately inbred and 2 individuals were highly inbred. Some individuals even have higher inbreeding coefficients than captive South China tigers which had an average coefficient of 0.3584 in 2016 (Yuan, Pei & Liu, 2020). Inbreeding, without effective population restoration measures, may erode all tiger populations except the 4–5 largest wild populations in the next 70 years (Miquelle *et al.*, 2015). This has been witnessed before in the tragedy in Sikhote-Alin Biosphere Zapovednik, where a population of 3–4 tiger individuals recolonized in 1996, reached a peak of 38 in 2005, then rapidly dropped to less than 10 in 2012, probably due to disease, demonstrating the instability and vulnerability of small Amur tiger populations (Miquelle *et al.*, 2015).

Although wild animals generally have mechanisms to avoid inbreeding, previous research has shown that inbreeding of red wolves *Canis rufus* increased significantly over time, for reasons which include limited founders, existing as a single small population, and limited gene flow between individuals (Brzeski *et al.*, 2014). One of the most common consequences of inbreeding is immune deficiency (Reid

et al., 2003; Ellison *et al*., 2012). The cell-mediated immune response of song sparrows *Melospiza melodia*, for example, significantly decreased with the increase of the individual's inbreeding coefficient (Reid *et al*., 2003). The natural antibody level of inbred Galapagos hawks *Buteo galapagoensis* was generally low and had less variation than that of the outbred population (Whiteman *et al*., 2006). In our study, tiger individuals possessed more than two DRB alleles, which indicated that there were at least two MHC DRB loci. Further, a total of seven DRB alleles were identified in 22 Amur tigers, suggesting the molecular genetic variation of DRB sequence in wild Amur tigers was at a moderate level compared with other mammals, including other felids. Wei *et al*. (2010), for example, studied the diversity of MHC DRB genes in 11 felid species of five lineages, identifying 14 unique alleles in 47 tiger individuals and 6 alleles in 3 clouded leopards *Neofelis nebulosa* and 7 leopards *Panthera pardus*. However, only four alleles were detected after amplification of the same gene fragment in 16 Bengal tigers (Pokorny *et al*., 2010). Here, there was no significant difference in the MHC diversity between the inbred group and the noninbred group, which also indicates that wild Amur tigers in China are at a moderate level of inbreeding and have not yet experienced such expected impacts on immunity genes.

However, the correlation analysis between inbreeding and parasitic infection and gut microbiota showed different results. *T. cati* can cause diseases such as internal visceral or eye larva migrans (González *et al*., 2007). In this study, we found that a total of 15 Amur tigers (68.18%) have a parasite burden of more than 60 epg, consistent with the reported *T. cati* infection rate of Amur tigers in Russia of 63.4%, thus validating that *T. cati* is a prevalent parasite in the wild Amur tiger population (Moskvina, Yu & Begun, 2018). Just as the correlation analysis between inbreeding coefficient and the intensity of *T. cati* infection reveals here in Amur tigers, inbreeding can also increase the body's sensitivity to pathogens. Similar results have been found in other animals, such as increased sensitivity of sea lions *Zalophus californianus* to infectious disease as a result of inbreeding and the response time of inbred individuals to treatment was also known to be delayed (Acevedo-Whitehouse *et al*., 2003). In another case, highly homozygous ring-tailed lemur *Lemur catta* individuals appear to be more vulnerable to warbles and higher parasite burden than heterozygous individuals (Charpentier, Williams & Drea, 2008).

In our analyses of the effects of inbreeding on gut microbiota, our comparative group selection was based on the inbreeding coefficient while we recognize that other factors, such as sex, age range and season may have greater influence (Bergmann *et al*., 2015; Janiak *et al*., 2021). All samples were collected during winter and thus we expect limited variability between individuals due to timing of sample collection, but unfortunately, we were unable to control for the biological factors (sex and age) as the Amur tiger occurs at low densities in the wild, and thus, our sample size was limited.

Concerning gut microbiota in wild Amur tigers, our results demonstrated that the most prevalent phyla detected were *Firmicutes*, *Proteobacteria* and *Bacteroidetes*, consistent with previous studies on Amur leopard, captive Amur tiger, Namibian cheetahs *Acinonyx jubatus*, house mice and Antarctic seals (Linnenbrink *et al*., 2013; Nelson *et al*., 2013; Wasimuddin *et al*., 2017; He *et al*., 2018; Han *et al*., 2019). The abundance of several gut microbiota taxa was significantly different between the inbred and noninbred group. *Collinsella*, for example, which is positively associated with circulating insulin and altering intestinal permeability (Chen *et al*., 2016; Gomez-Arango *et al*., 2017), was found to be more prevalent in the noninbred group than in the inbred group. However, genus *Escherichia* (including *E. coli*), which is the most common found in the large intestine and also a typical cause of intestinal disease in humans and wildlife (Onyango *et al*., 2009; Katouli, 2010), was more abundant in the inbred group. In addition, the family of *Enterobacteriaceae*, which was characterized with high-pathogenicity (Schubert, Rakin & Heesemann, 2004), were more prevalent in the inbred group. Thus, we conclude that inbreeding leads to an increase in the abundance of pathogenic bacteria in the body.

In order to better understand the potential functional role of the gut microbiomes, our research used HUMAnN2 to detect the functional gene pathway. We found that some functional pathways were significantly different between the inbred and noninbred group based on the Welch *t* test. It was found that L-histidine degradation was high in the noninbred group, while L-histidine biosynthesis was prevalent in the inbred group. There is evidence to suggest that superfluous L-histidine could lead to taste abnormalities and suppress the host's appetite (Okusha *et al*., 2017). The second major finding concerning microbiome function was that heme biosynthesis, a highly conserved pathway (Franken *et al*., 2011), was significantly higher in the inbred group. Heme is necessary to maintain life under aerobic conditions (Paulley, Anderson & Roop, 2007; Larsen *et al*., 2012; Staroń *et al*., 2017); further research is needed to identify and characterize the mechanism.

Conclusion

The rationale to initiate this study was to understand the level of inbreeding and its impact on wild Amur tigers in China based on highly variable gene marker and metagenome analysis. Our results suggested that the population inbreeding level was moderate. Although current inbreeding has not (yet) led to a significant difference in MHC diversity, inbreeding coefficient was significantly correlated with the intensity of parasite infection. In addition, inbreeding also changes the structure and function of the gut microbiota, increasing the abundance of pathogenic bacteria and affecting the body's functions of biosynthesis, degradation and utilization. Future conservation efforts should aim to connect existing habitat patches in a timely manner to achieve effective genetic communication between populations within China and with those in Russia, release inbreeding pressure and increase the effective population size as a meta population which can cope with the constantly changing environment.

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Conflict of interest

The authors have no conflict of interest.

Author's contributions

G.J. designed the study; J.Q., J.G., Z.H., E.Y., Y.R., J.L., Z.L., S.Z., M.H., J.M. and G.J. collected the genetic samples from wild; Y.N. and Z.P. carried out the laboratory work; Y.N. and Z.L. analyzed the data; Y.N., N.J.R., J.Q. and G.J. wrote and revised the paper.

Data availability statement

All metagenomic and MHC sequence data are available on NCBI under project PRJNA633309 and PRJNA764009, respectively. Microsatellite genotypes and fecal egg counts can be found in Supporting Information Appendix S1. Microsatellite and fecal egg counts can be viewed in Supporting Information Appendix S1.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. The cumulative PI (biased) and PI (sibs) values of 18 polymorphic microsatellite loci in 22 Amur tiger individuals.

Figure S2. A total of 94 homologous alleles of MHC II DRB exon 2 were identified in 22 Amur tiger individuals.

Table S1. Summary statistics of 18 polymorphic microsatellite loci on 22 tiger individuals.

Appendix S1. Raw data of microsatellite and parasite.