

Estimation of breeding population size using DNA-based pedigree reconstruction in brown bears

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Abstract

Robust estimates of demographic parameters are critical for effective wildlife conservation and management, but are difficult to obtain for elusive species. We estimated the breeding and adult population sizes, as well as the minimum population size, in a high-density brown bear population on the Shiretoko Peninsula, in Hokkaido, Japan, using DNA-based pedigree reconstruction. A total of 1,288 individuals, collected in and around the Shiretoko Peninsula between 1998 and 2020, were genotyped at 21 microsatellite loci. Among them, 499 individuals were identified by intensive genetic sampling conducted in two consecutive years (2019 and 2020) mainly by noninvasive methods (e.g., hair and fecal DNA). Among them, both parents were assigned for 330 bears, and either maternity or paternity was assigned to 47 and 76 individuals, respectively. The subsequent pedigree reconstruction indicated a range of breeding and adult ([?]4 years old) population sizes: 128–173 for female breeders and 66–91 male breeders, and 155–200 for female adults and 84–109 male adults. The minimum population size was estimated to be 449 (252 females and 197 males) in 2019. Long-term continuous genetic sampling prior to a short-term intensive survey would enable parentage to be identified in a population with a high probability, thus enabling reliable estimates of breeding population size for elusive species.

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Abstract

Robust estimates of demographic parameters are critical for effective wildlife conservation and management, but are difficult to obtain for elusive species. We estimated the breeding and adult population sizes, as well as the minimum population size, in a high-density brown bear population on the Shiretoko Peninsula, in Hokkaido, Japan, using DNA-based pedigree reconstruction. A total of 1,288 individuals, collected in and around the Shiretoko Peninsula between 1998 and 2020, were genotyped at 21 microsatellite loci. Among them, 499 individuals were identified by intensive genetic sampling conducted in two consecutive years (2019 and 2020) mainly by noninvasive methods (e.g., hair and fecal DNA). Among them, both parents were assigned for 330 bears, and either maternity or paternity was assigned to 47 and 76 individuals, respectively. The subsequent pedigree reconstruction indicated a range of breeding and adult ([?]4 years old) population sizes: 128–173 for female breeders and 66–91 male breeders, and 155–200 for female adults and 84–109 male adults. The minimum population size was estimated to be 449 (252 females and 197 males) in 2019. Long-term continuous genetic sampling prior to a short-term intensive survey would enable parentage to be identified in a population with a high probability, thus enabling reliable estimates of breeding population size for elusive species.

Keywords: Brown bear, breeding population size, pedigree reconstruction, population estimate, microsatellite analysis,

Ursus arctos

Introduction

The identification of demographic parameters is fundamental for understanding behavioral ecology (Roy, *et al.*, 2012; Stoen, *et al.*, 2006) and is essential for the effective management and conservation (Katzner, *et al.*, 2011) of wild animals. This includes quantity-related factors, such as population size/density and the number of reproductively active individuals, and quality-related factors, such as sex ratios, age structures, survival/mortality rates, reproductive rate, and population growth rate. Reliable estimates of these parameters are of particular importance for endangered animals or populations, but are usually difficult to obtain. This is particularly true for rare or elusive species, including large carnivores, most of which have declining population trends (Wolf and Ripple, 2018). In addition to habitat loss and fragmentation by deforestation (Zemanova, *et al.*, 2017), human-caused mortality, including culling for management purposes and hunting have become a serious threat to populations (Collins and Kays, 2011). On the other hand, an increase in the population of large carnivores presents a potential threat to human populations and livestock (Hristienko and McDonald, 2007). Therefore, population monitoring of wild carnivores inhabiting areas close to human populations is indispensable for the development of wildlife management and conservation policies, such as a determining harvest quotas (Kohira, *et al.*, 2009).

In the last two decades, DNA-based statistical models have been developed and used to estimate population sizes and trends. Most are based on noninvasive sampling methods. In large carnivore studies this includes the collection of hair (Rounsaville, *et al.*, 2022; Woods, *et al.*, 1999), feces (Kindberg, *et al.*, 2011; Kohn, *et al.*, 1999), and their combination (Ciucci, *et al.*, 2015). Hair and fecal samples allow DNA-based individual identification without capturing and handling the animals, which is of great advantage in terms

of cost-effectiveness (Kindberg, *et al.* , 2011), and animal welfare (Cattet, *et al.* , 2008). Several estimators have been developed for population size estimation based on noninvasive genetic data, including capture-mark-recapture (CMR) methods (Seber, 1986), rarefaction analysis (Kohn *et al.*, 1999), and, more recently, spatially explicit capture-recapture (SECR) methods (Efford, 2004). These methods have been applied to several large carnivore species, including brown bears (*Ursus arctos*) (Kindberg, *et al.* , 2011; Morehouse and Boyce, 2016), wolves (*Canis lupus*) (Caniglia, *et al.* , 2012), coyotes (*Canis latrans*) (Kohn, *et al.* , 1999; Morin, Kelly and Waits, 2016), and mountain lions (*Puma concolor*) (Russell, *et al.* , 2012). These methods use an individual's genotype as a molecular tag (Schwartz, Luikart and Waples, 2007). Genotypes can be a unique and permanent mark, which is superior to classic CMR approaches that use physical tags, such as ear-tags and leg bands. However, genotypic data are more than just tags; they contain further information, such as parent-offspring relationships and population structures, which sometimes improve the accuracy of estimates of population sizes and trends (Pearse, *et al.* , 2001).

As an alternative method for estimating demographic parameters, a DNA-based pedigree reconstruction approach has been developed (Creel and Rosenblatt, 2013). This approach has been widely used to estimate the number of breeding individuals in a population (Israel and May, 2010; Koch, *et al.* , 2008; Pearse, *et al.* , 2001; Quinn, Alden and Sacks, 2019), as well as to investigate many aspects of animal behavior, including population structure (Calboli, *et al.* , 2008; Hudy, *et al.* , 2010), breeding ecology (Levine, *et al.* , 2019; Shimozuru, *et al.* , 2019), and dispersal (Arora, *et al.* , 2012). Because population estimations based on statistical models do not provide age-related information, breeding population size estimates can offer more practical information regarding the reproductive potential of a population. One of the advantages of this method is that it enables the presence of breeders that were not directly sampled to be inferred if their offspring have been sampled, although it remains uncertain whether they were dead or alive at the time of sampling. Therefore, this method is particularly useful for estimating the number of breeding individuals under the circumstances where the inferred breeders can be determined to be alive or dead. For example, in a previous study in painted turtles (*Chrysemys picta*), Pearse *et al.* (2001) targeted hatchlings as offspring in a candidate parentage analysis, in addition to their mothers attending the nest, which enabled them to determine the number of male breeders that existed at the copulating period. In most mammals it is not possible to selectively sample newborns. In addition, it is almost impossible to obtain information on age by noninvasive genetic sampling, which makes it more difficult to know whether the breeders inferred by pedigree reconstruction are dead or alive. Such uncertainty over the survival/mortality of the breeders raises the ceiling of the maximum estimates and thereby impairs its accuracy. This holds particularly true for large carnivores that are relatively long-lived, for which multiple-generations can exist in a population, and mortality is difficult to detect. Therefore, studies of breeding populations based on the pedigree reconstruction approach are challenging and remain rare in large carnivore populations (Creel and Rosenblatt, 2013; Spitzer, *et al.* , 2016).

In this study, we estimated the breeding and adult population size, as well as the minimum population size, in a brown bear (Figure 1) population in the Shiretoko Peninsula, Japan, based on a pedigree reconstruction approach. The Shiretoko Peninsula is located in eastern Hokkaido, Japan (Figure 2). An area extending from the middle to the tip of the peninsula has been designated a UNESCO World Natural Heritage Site, as well as a national park, where the habitat of the brown bear is protected. However, human-bear conflict, including agricultural crop damage and intrusion into human residential areas, has become a serious problem on the peninsula. As many as 20–70 bears have been killed annually over the past decade (total 373 bears in 2011–2020), mainly for management purposes. This small peninsula consists of coastal area and precipitous mountains, and most of the area has limited accessibility, which makes it difficult to conduct a population estimation survey based on a systematic genetic sampling targeting all areas of the peninsula. As an alternative, a harvest-based method, based on the mortality records of brown bears, has estimated a population size as 559, although the wide confidence intervals (± 440) give little credibility to the estimates (Ministry of the Environment Government of Japan, 2017). The precise estimation of the population and/or breeding population is required for the appropriate management and conservation of brown bears. On the peninsula, information on genotypes, sex, and ages of dead bears (due to management culls, hunting, accidents, or

natural causes) has been accumulated for the past three decades. Due to the strong relationship between park managers and hunters on the peninsula, poaching or hunting without a report are very unlikely to have occurred over the past two decades. In addition, opportunistic noninvasive genetic sampling (hairs and feces) has been performed in some areas (Shirane, *et al.*, 2018), and continuous bear monitoring surveys (including DNA sampling) have been conducted for a decade or more in the Rusha area (Figure 2; Shimozuru, *et al.*, 2017). The accumulated information, if combined with large-scale genetic sampling, may be able to identify reliable demographic parameters, although other methods (e.g., the CMR method, a rarefaction analysis and the SECR method) are difficult due to geographical limitations. In the current study, we applied a pedigree reconstruction approach to this small but highly populated bear habitat. The population size of breeders and adults, and the minimum population size, were estimated based on large-scale genetic sampling events conducted in two consecutive years.

Methods

Ethical approval

All procedures involved in sample collection from live animals were conducted in accordance with the Guidelines for Animal Care and Use, Hokkaido University, and were approved by the Animal Care and Use Committee of the Graduate School of Veterinary Medicine, Hokkaido University (Permit Number: 1106, 1151, 1152, 15009, 17005, 18-0083, and 19-0047).

Study area and sampling

This study was conducted on the Shiretoko Peninsula (43°50′–44°20′ N, 144°45′–145°20′ E; Figure. 2), eastern Hokkaido, Japan. An area from the middle to the tip of the peninsula has been on the UNESCO World Natural Heritage List since 2005. It is valued for the unique ecosystem formed by the interrelationship of its marine and terrestrial environments. Genetic samples were collected in and around the peninsula using multiple methods that detailed in previous studies (Shimozuru, *et al.*, 2020; Shimozuru, *et al.*, 2019; Shimozuru, *et al.*, 2020; Shimozuru, *et al.*, 2017; Shirane, *et al.*, 2018; Shirane, *et al.*, 2019). In this study, the area consisting of Shari, Rausu, and Shibetsu towns was defined as being inside the peninsula (approximately 1,760 km²), with additional samples from Kiyosato and Nakashibetsu towns also included in the analysis (Figure 2). Most samples (blood and tissue) were from bears that were dead due to nuisance control (mostly outside the national park) or hunting (limited to the October to January period outside the national park), or that were captured for research purposes. For bears captured or killed between 1998 and 2020, age was estimated by counting the dental cementum annuli (Craighead, Craighead and Mccutchen, 1970; Tochigi, *et al.*, 2018). In addition, we also obtained hair collected by hair-traps in several locations during 2010–2020, skin tissues collected by biopsy dart sampling during 2011–2020, and fecal samples collected during 2009–2020.

During 2019–2020, we conducted intensive, noninvasive genetic sampling for hair and feces. For hair, 63 and 67 tree-rub traps (For details, see Sato, *et al.*, 2020; Shimozuru, *et al.*, 2020) were placed throughout the peninsula in 2019 and 2020, respectively, except for areas where it was difficult to gain access (Figure 2). In the tree-rub trap, the trunk was partially smeared with wood preservative (Creosote R; Yoshida refinery, Tokyo, Japan) to lure bears (Sato, *et al.*, 2020), and barbed wire was wrapped around the trunk between 30 and 230 cm above the ground. From late May to October, we visited each trap at approximately 2-week intervals (a total of 10 and 11 collections in 2019 and 2020, respectively), and collected hairs from individual barbs, which then were stored separately in envelopes. Samples were dried and kept at –30°C until DNA extraction. Each tree-rub was monitored by an automatic camera (HykeCam SP108-J; Hyke Inc., Asahikawa, Japan). The recording time and intervals were set to 25 and 5 s, respectively. All videos were checked to estimate the number of bears that potentially rubbed against the tree, and their sex/age status was determined visually if possible. Through a combination of genetic analysis and video data, breeding status was clarified in some females, e.g., by the accompanying presence of cubs or yearlings. For fecal samples, we collected bear scats with ages of 0–4 days as estimated by field collectors. They were stored in Inhibitex buffer (Qiagen Inc., Tokyo, Japan) and kept at –30°C until DNA extraction. Bear scats were collected every time field collectors

found them during bear patrols in and around popular tourist areas and farmland, driving on forest roads, and during exploratory investigations in the forest. To collect DNA samples from the areas without tree-rub traps, field collectors periodically (1–2 times a month from June to September) made explorations on foot into those areas, e.g., high-elevation areas and the tip of the peninsula.

Extraction of DNA and genotyping

The extraction of DNA, microsatellite genotyping, and a mitochondrial DNA haplotype analysis were conducted using the procedure described in previous studies (Shimozuru, *et al.*, 2019; Shirane, *et al.*, 2018). Briefly, DNA was extracted using the DNeasy Blood & Tissue Mini Kit (Qiagen Inc., Tokyo, Japan) for blood and tissue samples, the DNA Extractor FM Kit (Wako, Osaka, Japan) or Isohair Easy (Nippon Gene, Inc., Tokyo, Japan) for hair samples, and the QIAamp DNA Stool Mini Kit (Qiagen Inc.) for feces samples. Twenty-one microsatellite markers and one sex marker, amelogenin (Yamamoto, *et al.*, 2002), were analyzed by a multiplex PCR assay (Shimozuru, *et al.*, 2020; Shimozuru, *et al.*, 2019; Shimozuru, *et al.*, 2020). Allele size was determined using an ABI PRISM 310 genetic analyzer or SeqStudio Genetic Analyzer (Thermo Fisher Scientific K.K., Tokyo, Japan). In addition, eight Y-linked microsatellite alleles (Y318.1, Y318.2, Y318.4, Y318.6, Y318.9, Y369.1, Y369.4, and 15020.1; Hirata, *et al.*, 2017) were determined by a multiplex PCR assay, using the same primer sets as those used in previous studies (Bidon, *et al.*, 2014; Hirata, *et al.*, 2017). The mitochondrial and Y chromosome haplotype information were used to select candidate mothers for offspring, and candidate fathers for male offspring, respectively.

Pedigree reconstruction

Parentage analysis was performed using a likelihood-based approach with the CERVUS software (version 3.0.7) (Kalinowski, Taper and Marshall, 2007), followed by the COLONY software (version 2.0.6.4) (Jones and Wang, 2010; Wang, 2004). Mitochondrial and Y chromosomal haplotype data were used for the selection of potential mother–offspring relationships and potential father–son relationships, respectively. Analyses were run systematically in accordance with a previous study (Shimozuru, *et al.*, 2019). First, all individuals, sampled during 1998–2020, were analyzed with CERVUS, which selected the most likely parent among the existing candidates. The same simulation parameters were set as in a previous study (Shimozuru, *et al.*, 2019). In the first step of the CERVUS analysis, we assigned a parent pair. The confidence level was set at 80%, and no mismatching was allowed in a parent–offspring combination (i.e., mother–father–offspring trio). One mismatch was allowed in a parent–offspring combination obtained at a [?] $\geq 95\%$ confidence level when the same mother and father were selected as the most likely parents ([?]1 mismatch per pair) in maternity and paternity assignment analyses, respectively. If a parent pair could not be assigned due to a low (<80%) confidence level or the presence of [?]1 mismatching loci, we assigned maternity or paternity as a second step. The confidence level was set at 80%, and no mismatching was allowed in a mother/father–offspring combination. Furthermore, bears that were not assigned a mother and/or father in CERVUS were included in a parentage analysis using COLONY. The COLONY software generates hypothetical parents in a sib-ship reconstruction with the assumption that both females and males are promiscuous (Steyaert, *et al.*, 2012), which enables the assignment of parentage to individuals whose parent(s) were not present in the parent candidate data set. To reduce the possibility of multiple generations in the candidate offspring leading to a false parentage assignment, only bears that were sampled during 2019–2020 were included as candidate offspring in COLONY analyses.

Breeding/adult population estimates

In this study, breeders and adults were defined as bears that produced [?]1 offspring between 1998 and 2020, and bears that had the potential ability to breed, respectively. For females, adults included both breeders and bears [?]4 years old (the youngest age of the first mating in this population, reported by Shimozuru, *et al.* (2017)). For males, the youngest age of the first mating in this population was 6 years of age (Shimozuru, *et al.*, 2020), whereas males potentially reach sexual maturation at 3.5 years of age in the Scandinavian population (Zedrosser, *et al.*, 2007). In the current analysis, bears [?]4 years old and sexually experienced males (indicated by a parentage analysis) were included in the adult population, which allowed us to compare

the breeding/adult population size between sexes. In this study, we estimated the breeding/adult population size as of 2019, the first year of the intensive genetic sampling period. This was because 2019 was expected to be the year when the highest number of breeders/adults would be identified as alive. For example, females identified with cubs for the first time in 2020 could be counted as breeders in 2019. Likewise, all females and males identified in 2019 and/or 2020 were confirmed as breeders if their offspring were sampled during 1998–2020. In addition to breeding experience, some bears were confirmed to be ≥ 4 years old based on their appearance when identified in an ongoing bear monitoring survey that has been continuously conducted in recent decades in the area between Rusha and Utoro (Shimozuru, *et al.*, 2020; Shimozuru, *et al.*, 2017). Some bears were confirmed to be ≥ 4 years based on the year of first genetic identification, or on the year when their parent was dead (e.g., if the father was dead in 2014, his offspring could potentially be born in 2015, suggesting they were ≥ 4 years old as of 2019).

First, we calculated the minimum number of breeders, including the existing bears and hypothetical parents. The former included bears identified in 2019 and/or 2020 that were confirmed as sexually experienced based on a parentage analysis. The later included hypothetical parents generated by the COLONY software, which were estimated to produce cubs during 2018–2020. For example, if a 1-year-old bear, killed in 2019, was not assigned a mother from the list of candidate mothers, it was reasonable to assume that his/her mother, although not genetically identified, gave birth in 2018 and was alive until the timing of mother–offspring separation in 2019. Similarly, if a cub-of-the-year, sampled in 2020, was not assigned a father from the list of candidate fathers, it could be assumed that his/her father was alive and mated with the mother in 2019.

Second, we estimated the maximum breeding population size by a pedigree reconstruction approach, based on the simple assumption that the number of breeders would not exceed the total number of parents that produced bears identified in 2019–2020. We estimated the number of breeders that produced bears identified during 2019–2020, but whose mother and/or father (genetically identified during 1998–2018) were not sampled during 2019–2020, or whose mother and/or father were missing from the list of existing candidates sampled during 1998–2020. One of the problems of this approach is that the assumption that each missing parent constitutes a new individual would most likely cause an overestimation (Spitzer, *et al.*, 2016). In this study, COLONY allowed hypothetical parents to produce multiple offspring, which reduced the likelihood of overestimation due to this issue. Another problem is that it is difficult to know how many of the parents that were identified as alive until 2018, but not sampled during 2019–2020, were still alive as of 2019. To account for mortality among those individuals, we calculated the period between the year of the last identification and 2019, and multiplied it by the survival rate to estimate his/her survival probability. Because the adult survival rate was not investigated in this population, we applied the median value of the survival rates (0.94 for females and 0.89 for males) among the other brown bear populations (0.89–0.96 for females, 0.62–0.94 for males; reviewed in Schwartz 2003). For example, a mother identified as alive in 2017 was counted as 0.88 of an individual (i.e., 0.94×0.94). In addition, we assumed that adult bears who had not been sampled for the last 5 years (2016–2020) in the area where successive surveys were conducted were no longer alive. Continuous genetic samples from hair-traps, biopsy darts, and fecal collection have been conducted in the area between Rusha and Utoro (Figure 2; Shimozuru, *et al.*, 2020; Shimozuru, *et al.*, 2017), enabling us to assume that those individuals were less likely to be alive.

A similar but more serious concern, reported by Creel and Rosenblatt (2013), was that there is no way to ascertain how many of the hypothetical parents are actually alive. To avoid overestimation, we made several assumptions. First, females ≥ 30 years old and males ≥ 28 years old were not counted as breeding individuals. This assumption was based on previous studies regarding reproductive senescence in brown bears (Schwartz, *et al.*, 2003; Van Daele, *et al.*, 2001; Zedrosser, *et al.*, 2007). Upon pedigree reconstruction, the age of each hypothetical parent was estimated based on the age of the oldest offspring and generation intervals. The generation interval between mother and offspring was set at 7.3 years based on our bear monitoring survey in the Rusha area. We calculated the first age when females gave birth to cubs that survived the first year (for eight females = 5–9 years, average of 7.25; Shimozuru, *et al.*, 2017), and used it as the minimum interval between generations. This value was more realistic than their primiparity age (for 15 females = 5–6 years, average of 5.3; Shimozuru, *et al.*, 2017), which was more likely to induce

overestimation in the current analysis. The generation intervals between father and offspring, i.e., the first age when males sired cubs that survived the first year, was not well investigated in this population. Males become sexually mature at 3.5 years old (Zedrosser, *et al.*, 2007), but it is rare to gain a reproductive opportunity until physical maturation at around 9–11 years of age (Moriwaki, *et al.*, 2018; Shimozuru, *et al.*, 2020; Shirane, *et al.*, 2020). Therefore, we set the same value (7.3 years) as for females, based on the assumption that the generation intervals between father and offspring were not less than those between mother and offspring. The second assumption was that more than four matrilineal generations do not exist at the same time, which was also based on our bear monitoring survey conducted in the Rusha area. In this area, four generations (offspring, mother, grandmother, and great-grandmother) exist at the same time, but a great-great-grandmother has never been identified (Shimozuru, *et al.*, 2017). Similarly, our previous pedigree reconstruction conducted in the same population revealed that more than three paternal generations (offspring-father-grandfather) do not exist at the same time (Shimozuru, *et al.*, 2019). Hypothetical mothers and fathers that correspond to great-great-grandmother and great-grandfather, respectively, were assumed to be dead, and were not counted as breeding individuals in the current analysis. Finally, we calculated the minimum and maximum number of adults, by adding the number of bears confirmed to be [?]4 years old in 2019 (based on the criteria described above) to the minimum and maximum number of breeders.

Minimum population estimates

The minimum population size as of 2019 included bears identified in 2019, including bears that died in 2019; bears not identified in 2019–2020, but whose presence as of 2019 was inferred by pedigree reconstruction; and bears [?]1 year old, identified not in 2019 but in 2020. The second category included existing bears (i.e., bears identified only before 2019) and hypothetical bears generated by the COLONY software, as described above. In the third category, the age or minimum age of bears was determined based on the year of first genetic identification (i.e., bears identified before 2019 were included), the year when their parent was dead, or on the video data obtained at the time of genetic identification. The combination of a DNA-based parentage analysis and video data taken at the hair-trap site sometimes enabled us to determine the age of young bears (i.e., 0–1 years old) that accompanied their mother. Some bears were confirmed to be [?]1 year old when their DNA was collected, and their body sizes were able to be assessed by the video data. Significant differences in body sizes between 0- and 1-year-old bears enabled us to determine if bears were [?]1 year old, even if information regarding their age was unavailable. This assessment was done only when we could identify the bear in the video clip with 100% confidence. Therefore, bears that had the possibility of being cub-of-the-year were not included in the minimum population size as of 2019.

Results

The number of samples analyzed and the number of bears identified by an intensive survey during 2019–2020 are shown in Table 1. The distribution of feces that was successfully analyzed is shown in Appendix A. From the 2-year intensive genetic survey in 2019–2020, 499 unique bears (281 females and 218 males) were identified. Among them, 172 bears (96 females and 76 males) had been genetically identified by 2018. Finally, with the samples collected between 1998 and 2020, we genotyped 1,288 bears (616 females and 672 males), including 1,221 bears from the Shiretoko Peninsula (i.e., Shari, Rausu, and Shibetsu towns), and 67 bears from areas adjacent to the peninsula (i.e., Kiyosato and Nakashibetsu towns). Approximately 58% of the sampled bears (748 bears) were confirmed to be dead, due to management culls, hunting, accidents, or natural causes. All bears were genotyped at all of the loci. We found seven haplotypes in the mitochondrial analysis, which was similar to the results of previous studies on the same population (Shirane, *et al.*, 2018): HB-02 ($N = 37$), HB-10 ($N = 139$), HB-11 ($N = 703$), HB-12 ($N = 66$), HB-13 ($N = 122$), HB-new1 ($N = 107$), and HB-new2 ($N = 1$); and one heteroplasmic pattern: HB-10/11 ($N = 113$). For the Y chromosomal haplotype analysis, we found four haplotypes (BR1.02, BR1.04, BR1.05, and BR1.06) that were reported in a previous study (Hirata, *et al.*, 2017). In addition, based on two markers, UarY369.4 and 15020.1, which were excluded in the above study due to the pseudoheterozygous genotypes identified in bears sampled outside Hokkaido, the haplotypes BR1.04 and BR1.05 were classified into two and three sub-haplotypes, respectively. Finally, we found six haplotypes, BR1.02 ($N = 32$), BR1.04a ($N = 1$), BR1.04b ($N = 339$),

BR1_05a ($N = 57$), BR1_05b ($N = 91$), BR1_05c ($N = 1$), and BR1_06 ($N = 149$). Two samples were not available for Y chromosomal haplotypes due to an unstable amplification.

Table 2 summarizes the results of the parentage analysis with CERVUS. Among the 499 unique bears identified in 2019–2020, 7 males with the HB-02 mitochondrial haplotype were considered to be immigrated males from outside the peninsula, because no females with HB-02 were identified on the peninsula (Shirane, *et al.*, 2018). Therefore, those males were excluded from the candidate bears that were possibly born inside the peninsula. Both parents were assigned for over two-thirds of the remaining 492 bears, and less than 8% of the bears were unassigned to one parent. Among the 499 bears, including the seven immigrant males, 125 females and 65 males were confirmed to be breeders, due to the existence of [?]1 offspring between 1998 and 2020. In addition, 27 females and 18 males were identified as [?]4 years old as of 2019, based on the year of first visual/genetic identification (12 females and 6 males), the year of death of their parent (15 females and 8 males), or an age estimation at death by counting the cementum annuli of the teeth (4 dead males in 2019–2020), although they did not have any breeding record. Among the 499 bears, no bears were assigned as daughters/sons, or mothers/fathers of bears sampled outside the peninsula (i.e., Kiyosato and Nakashibetsu towns). Taken together, among the 499 bears identified in 2019–2020, 152 females and 83 males were confirmed to be adults (i.e., bears with reproductive experience or [?]4 years old) as of 2019.

Table 3 summarizes estimations of the breeding population by including past-identified breeders (previously existed, but not identified in 2019–2020) and hypothetical parents, based on a pedigree reconstruction by the combination of CERVUS and COLONY analyses. Among the bears identified between 1998 and 2018 but not in 2019–2020, 16 females and 10 males (identified between 2008 and 2018) were assigned as parents of bears identified in 2019–2020. Among them, one female was assigned as a mother of a bear that was born in 2018 and was dead in 2020. She was included in the minimum breeding population because it was reasonable to assume that she survived until the time of separation with the offspring in 2019. On the other hand, four females were estimated to be [?]30 years old based on the reconstructed pedigree, and one female (unidentified since 2012) and three males (unidentified since 2008–2015) were assumed to be dead due to their long-term non-identification in the Rusha area where continuous genetic monitoring had been conducted. By excluding these bears, 8.8 and 5.4 bears were included in the maximum breeding population as of 2019. Subsequently, COLONY generated 51 hypothetical mothers and 37 hypothetical fathers as potential parents of the bears (identified in 2019–2020) that remained unassigned to both or either of the parents in the CERVUS analysis. Among them, two females and one male were included in the minimum breeding population because they were assigned as parents of bears born in 2019 (two hypothetical females) and in 2020 (one hypothetical male). Among the remaining hypothetical parents, 13 females and 16 males were excluded due to the estimated age (two females and three males were estimated to be [?]30 and [?]28 years old, respectively), and due to the limitation of maximum maternal/paternal generations (9 females and 13 males were considered to be great-great-grandmothers and great-grandfathers, respectively). In addition, two females were assumed to be dead because they were mothers of resident adult females in the Rusha area, but were not observed in the past 12 years. Finally, the minimum/maximum adult populations of females and males were estimated to be 155–200 and 84–109, respectively.

The minimum bear population in 2019 in the Shiretoko Peninsula is shown in Table 4. It was found that a total of 449 (252 females and 197 males) existed as of 2019 in the Shiretoko Peninsula. Changes in the cumulative number of unique bears counted as the minimum population in 2019 are shown in Figure 3. Bears identified visually (one female) or inferred by pedigree reconstruction (one existing female, two hypothetical females, and one hypothetical male) were excluded from this analysis. Three females were counted as adults, not at the timing of first genetic identification, but when they were proven to be an adult (e.g., at a time when they were confirmed to be present with offspring).

Discussion

We applied a pedigree reconstruction approach to estimate the breeding and adult population size of brown bears on the Shiretoko Peninsula, Japan. Large-scale, intensive genetic sampling enabled a high rate of parentage assignment, which allowed us to raise the minimum size of the breeder/adult populations. The

adults ([?]4 years old as of 2019) accounted for 47.1% of the total unique bears identified in 2019–2020, which was comparable to the percentage of adults (43.0%; [?]5 years old, defined in Craighead, Sumner and Mitchell, 1995) in Yellowstone bears monitored at Trout Creek, 1960–1968. This suggests that the current method is effective enough to detect breeders/adults among bears without information on age. The estimated breeding/adult population size, although that was the minimum value, was higher than among other brown bear populations in the world, suggesting that this population, which inhabits a small area, has a very high reproductive potential (Schwartz, Miller and Haroldson, 2003). The breeding/adult population size is a very useful indicator for determining population dynamics and set harvest/hunting quotas (Swenson, *et al.*, 1994), which is essential for the development of wildlife management and conservation policies. Kohira *et al.* (2009) estimated the population growth rate to be >1 under the conditions where [?]81 adult females [?]5 years old (among 150 females in total) existed in the Shiretoko Peninsula, excluding Shibetsu Town (which accounts for 31% of the total forest cover in the current study area), with eight adult female mortalities/year (7.2 adult [?]5 years old] female mortalities/year in the same area during 2011–2020). Our results suggest that the current harvest rates are below the sustainable level; however, careful attention is still required because some of the parameters used in Kohira *et al.* (2009) were extrapolated from data obtained from other brown bear populations.

To estimate the maximum breeding population, we made an assumption that the breeding population would not exceed the total number of parents that produced bears identified in 2019–2020. This assumption was unreliable if the sampling efforts were insufficient or if the sampling area was too limited. In these circumstances, the maximum population size would be severely underestimated. Although most of the hair-trap sites were placed in coastal areas for ease of access, the combination of hair-trapping and scat collection enabled intensive genetic sampling in the current study, which was supported by the accumulative curve of unique bears shown in Figure 3. Brown bears in this population range from high elevations (e.g., to eat alpine stone pine cones in summer) to coastal areas (e.g., to eat salmon in autumn) depending on seasonal changes in food availability (Shirane, *et al.*, 2021), suggesting that most of the bears on the peninsula had the potential to be sampled. In addition, one of the advantages of the current method is that it was possible to infer the presence of the parents without sampling if their offspring were sampled. Male bears disperse from their birthplace at around three years of age (Shirane, *et al.*, 2019), which allowed mothers living in the area with a low sampling probability to be detected by the pedigree reconstruction. Therefore, it was unlikely that the true breeding population exceeded the current estimation, but there is a need to give careful attention to the possibility of overestimation. One of the disadvantages of this method is that with an increase in the number of bears whose parent(s) are unknown, the number of hypothetical parents increases, which raises the ceiling of the estimate. This concern was partially mitigated by the use of COLONY software, which allowed each hypothetical parent to be assigned to multiple bears based on the promiscuous mating ecology of bears (Steyaert, *et al.*, 2012). However, because it is not always possible to know whether they are alive or dead, this leads to an overestimation, particularly in short-term surveys, as discussed in Creel and Rosenblatt (2013) and Spitzer *et al.* (2016), in which a population estimation was conducted based on a similar method. In the present study, more than two-thirds and over 90% of the bears were assigned for both parents and either parent, respectively. This rate of parentage assignment is high compared to other studies targeting brown bears (Norman and Spong, 2015; Sawaya, Kalinowski and Clevenger, 2014; Spitzer, *et al.*, 2016) and other bear species (Zeyl, *et al.*, 2009), which allowed us to reduce the generation of hypothetical parents in this study.

This “alive or dead problem” holds true not only for hypothetical parents but also for existing ones. Although the parentage assignment rate was high, the lack of information regarding their survival also leads to overestimations. In this study, among the 295 existing parents (170 females and 125 males) assigned as the parents of the 492 unique bears identified in the 2-year period, 222 bears (113 females and 109 males) had already been identified by 2018, of which 196 (97 females and 99 males) were confirmed to be dead. This enabled us to reduce the number of breeders without information on their survival, which in turn reduced the difference between the minimum and maximum breeding populations. This was mainly achieved by the accumulation of over 20 years of genetic information preceding large-scale sampling events. Furthermore,

information on age for dead bears (obtained mainly by an analysis of their teeth) and the date of first identification for living bears were very useful to assign the minimum age, which helped improve the accuracy of estimates of the minimum population size as of 2019. We suggest that the current method based on pedigree reconstruction offers less advantage in terms of estimating breeder/adult population sizes based on genetic data obtained by limited sampling events, but works well for populations where continuous genetic surveys, particularly targeting harvested bears, have been conducted in advance.

To assume the mortality of hypothetical parents and bears identified only before 2019, we defined three criteria, i.e., a maximum number of generations, maximum age as a breeder, and long-term absence of observation records in the areas with high survey activity. This enabled us to exclude 33% (37/114) of those bears from the maximum population size. The adoption of these criteria was a realistic approach on the basis of previous studies; however, it may be too conservative. For example, the minimum ages of some parents were estimated based on the age of the oldest daughter/son in the offspring list, but it was unlikely that the daughter/son was the first offspring that they raised successfully. In fact, among bears included in the maximum breeding number ($N = 49$ and 28 , for females and males, respectively), the minimum age for 16 females and 11 males was estimated to be 20 years of age or older, but their real ages may have exceeded the threshold criteria as a breeder. In addition, opportunistic hair-trapping and scat collection has been conducted throughout the peninsula over the last decade; thus, those older bears should have had a higher possibility of being sampled. Therefore, it is reasonable to think that the maximum breeding size still included a certain number of bears that were already dead. This suggests that the true breeding population size was closer to the minimum than maximum number, which is supported by the accumulative curve of unique adult bears that almost reached a plateau at the end of the 2-year period.

The sex ratio of breeders was more than two-fold (2.04) biased in favor of females, which is unusual compared to other brown bear populations (e.g., 1.20–1.30 in Swedish population; Spitzer, *et al.*, 2016). It is generally accepted that there are no sex biases at birth in brown bears (Schwartz, *et al.*, 2003), and this result therefore raises some issues. It was most likely due to sex differences in reproductive opportunities; male reproduction is competitive (Steyaert, *et al.*, 2012), and breeding opportunities tend to be biased toward physically mature males, which reduces the possibility for young males with limited breeding experience to be assigned as a father in a parentage analysis. This is consistent with a previous report showing that the frequency of breeding was low in 5- to 9-year old males but high in 10- to 14-year old bears in the Rusha area of the Shiretoko Peninsula (Shimozuru, *et al.*, 2020). However, if the bias were solely due to this reason, the number of males assigned as [?]4 years old based on a pedigree reconstruction should have been larger than that of females, which was not true (27 females vs. 18 males). In addition, the number of bears of unknown age was not very different (49 females vs. 47 males) in the minimum population. Furthermore, the number of bears whose father was unknown (47) was fewer than that of bears whose mother was unknown (76), which reduced the possibility that males had a lower probability of sampling than females did in the current field survey. This suggests that the female-biased breeding population (128 vs. 66) or adult ([?]4 year) population (155 vs. 84) was not strongly influenced by procedural matters in the current analysis. The adult sex ratio has been shown to vary in other brown bear populations, but is more or less biased to females (Schwartz, *et al.*, 2003), similarly to this population. This was partially supported by the male-biased probability of human-caused death in this population, particularly for 2- to 3-year-old bears when males initiate natal dispersal (Kohira, *et al.*, 2009; Shimozuru, *et al.*, 2020). In addition, the high mortality rate in males due to natural causes, e.g., starvation due to the high energy demand during development in males (predicted by Mattson and Reid, 1991) or intraspecific killing (Schwartz, *et al.*, 2003), may have accelerated this tendency, although the sex differences in the natural survival rate are still unknown in this population.

The minimum population size (449 individuals as of 2019) in the study area (total area of three towns: 1,760 km²; total forest cover in the area: 1,378 km²) indicated that the Shiretoko Peninsula has one of the highest brown bear populations area in the world. The minimum density (25.5–32.6 bears/100 km²) was much higher than the estimated brown bear density in the interior populations of Europe (e.g., Swedish population: 0.8–1.2 bears/100km²; Bellemain, *et al.*, 2005) and North America (0.4–8.0 bears/100km²; Haroldson, *et al.*, 2021; Schwartz, *et al.*, 2003), and also higher than or comparable to the coastal populations in Alaska

(18.4–40.0 bears/100km²; Schwartz, *et al.*, 2003), where a high-nutrient diet (e.g., salmon) is available in the hyperphagia period. In this study, genetic sampling conducted in two consecutive years (2019–2020) allowed us to increase the minimum population by 28% compared to the number obtained solely in the first year (2019). This was partially achieved by the minimum age assignment for bears identified for the first time in 2020, based on pedigree reconstruction and also on body size assessment in cases where video data were allowed to specify the donor bear. This suggests that a simple count of the detected genotypes, a very classic method, can still provide practicable data through a combination of long-term, continuous genetic monitoring for dead/alive bears and a subsequent multi-year large-scale sampling event. We still need to ascertain how close the minimum value is to the true population size through the use of more sophisticated statistical methods, e.g., SECR approaches. However, population estimates using statistical models sometimes have wide confidence limits (Lukacs and Burnham, 2005). Therefore, a precise estimation of the minimum population size sometimes provides more applicable and conservative information for wildlife management and conservation, and can be a useful indicator to select the best-fit model (Solberg, *et al.*, 2006), thereby helping to refine population estimates.

In conclusion, our study suggests that pedigree reconstruction is a very useful tool for estimating breeding/adult populations and minimum population size in elusive wildlife species. This approach is also applicable to wildlife populations under circumstances where population estimation using statistical models, e.g., the SECR approach, is difficult for various reasons, e.g., geographical limitations and the behavioral characteristics of study animals. It should be emphasized that not only the sampling intensity for large-scale sampling events but also the preceding accumulation of information on the genotypes and ages of dead individuals are essential to maximize the utility of this approach. The current study indicates how important an accurate knowledge of animal mortality (due to management culls, hunting, accidents, poaching, and natural deaths) and secured recovery of samples are for monitoring populations of wildlife. A large-scale, intensive genetic survey is very costly, and therefore it is not often conducted. In preparation for the opportunity of such surveys, continuous genetic monitoring efforts are needed to maximize the amount and quality of the information regarding demographic parameters.

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Tables

Table 1. Number of samples and bears identified during 2019–2020.

Samples
Year
No. samples analyzed
No. successful analysis
No. unique bears
female/male
*Other methods; biopsy-darting, blood stains and saliva.
#Dead; management kills, hunting and natural death.
+The number included one and three visually-identified, not genetically-identified individuals in 2019 and 2020, respectively

Table 2. Key characteristics of the parentage analysis by CERVUS showing the number of breeders and adults identified in 2019–2020.

Bears identified in 2019–2020
Females
Males
Triads
Dyads
With "no parent"
Female breeders ^b
Male breeders ^b
Ratio of dams : sires
Females [?]4 y, no breeding record
Males [?]4 y, no breeding record
No. breeders or [?]4 years old (Females/Males)
^a Seven males, originated out of the peninsula, were included, but excluded as potential offspring in the parentage analysis.
^b Individuals with at least one offspring between 1998 and 2020.
^c 4 males, originated outside the peninsula, were included.
^d Percentage among same sex.

Table 3. Population size of breeders and adults estimated by a pedigree reconstruction.

	Female	Male
No. breeders (existed)	16	10
Estimated as dead	5	3
Counted as minimum number	1	0
Counted as maximum number	8.8	5.4
No. hypothetical parents	51	37
Estimated as dead	13	16
Counted as minimum number	2	1
Counted as maximum number	36	20
Minimum No. of parents	3	1
Maximum No. of parents	45	25
Breeding population size	128–173	66–91
Adult ([?]4 y) population size	155–200	84–109

Table 4. Minimum population size in the Shiretoko Peninsula, Hokkaido, Japan, as of 2019.

Age
4
2–3
1
0
Unknown
Subtotal
Total
Number in the parenthesis indicates the number of bears died in the given year.
¶Three females and one male that were not identified in 2019–2020 but inferred by pedigree reconstruction were included.
*One and §three visually-identified bears were included.
#One bear (age unknown) and one cub that died due to natural causes were included in 2019 and 2020, respectively.
+Fifty bears born and four bears possibly born in 2020 were excluded.

Figure Legends

Figure 1. Brown bears copulating in the Rusha area of the Shiretoko Peninsula, Hokkaido, Japan (Photo: Masami Yamanaka).

Figure 2. Map of the Shiretoko Peninsula, eastern Hokkaido, Japan. The dotted yellow line indicates the Shiretoko National Park. This map was created using QGIS version 3.4.7-Madeira (*QGIS.org*, 2022. QGIS Geographic Information System. QGIS Association. <http://www.qgis.org>) and edited by the author. The topographic features are based on Digital Topographic Map 1:25000 published by Geospatial Information Authority of Japan (available from <https://fgd.gsi.go.jp/download/mapGis.php?tab=dem>, accessed 18-May-2019). Administrative divisions were created by the National Land Numerical Information published by Ministry of Land, Infrastructure, Transport, and Tourism of Japan (available from <https://nlftp.mlit.go.jp/ksj/index.html>, accessed 21-Jul-2021). National park boundaries were created using GIS data for national parks published by Biodiversity Center, Ministry of the Environment (available from <http://gis.biodic.go.jp/webgis/sc-026.html?kind=nps>, 20-Jul-2021). The vegetation was created using the 1:25,000 GIS-based vegetation map "Hokkaido" published by Biodiversity Center of Japan, Ministry of

the Environment, Japan (available from <http://gis.biodic.go.jp/webgis/sc-025.html?kind=vg67>, 19-Jul-2021).

Figure 3. Changes in the cumulative number of unique bears counted as the minimum population in 2019. Bears identified visually or inferred by pedigree reconstruction were excluded from this analysis. Three females were counted as adults, not at the timing of first genetic identification, but when they were proven to be an adult (e.g., at a time when they were confirmed to be present with offspring).

Data Accessibility

The data (microsatellite genotypes, mitochondrial haplotypes, and Y-chromosomal haplotypes) will be made available in Dryad.

Competing Interests

The authors declare no competing interests.

Author contributions

Michito Shimozuru : Conceptualization (lead); Data curation (lead); Formal analysis (lead); Funding Acquisition (lead); Investigation (equal); Methodology (lead); Project administration (lead); Visualization (equal); Writing – Original Draft Preparation (lead). **Mina Jimbo** : Data curation (equal); Investigation (equal); Visualization (equal); Writing – Review & Editing (equal). **Keisuke Adachi** : Data curation (equal); Formal analysis (supporting); Investigation (equal); Writing – Review & Editing (equal). **Kei kawamura** : Data curation (equal); Investigation (equal); Writing – Review & Editing (equal). **Yuri Shirane** : Data curation (equal); Investigation (equal); Writing – Review & Editing (equal). **Yoshihiro Umemura** : Data curation (equal); Investigation (equal); Resources (equal); Writing – Review & Editing (equal). **Tsuyoshi Ishinazaka** : Data curation (equal); Investigation (equal); Project administration (equal); Resources (equal); Writing – Review & Editing (equal). **Masanao Nakanishi** : Data curation (equal); Investigation (equal); Resources (equal); Writing – Review & Editing (equal). **Mayu Kiyonari** : Data curation (equal); Investigation (equal); Resources (equal); Writing – Review & Editing (equal). **Masami Yamanaka** : Data curation (equal); Funding Acquisition (lead); Investigation (equal); Resources (equal); Writing – Review & Editing (equal). **Yukihiro Amagai** : Data curation (equal); Investigation (equal); Resources (equal); Writing – Review & Editing (equal). **Ayaho Ijuin** : Data curation (equal); Investigation (equal); Resources (equal); Writing – Review & Editing (equal). **Tomoki Sakiyama** : Data curation (equal); Investigation (equal); Resources (equal); Writing – Review & Editing (equal). **Shinsuke Kasai** : Data curation (equal); Investigation (equal); Resources (equal); Writing – Review & Editing (equal). **Takane Nose** : Data curation (equal); Investigation (equal); Resources (equal); Writing – Review & Editing (equal). **Masataka Shirayanagi** : Data curation (equal); Investigation (equal); Resources (equal); Writing – Review & Editing (equal). **Hifumi Tsuruga** : Data curation (equal); Investigation (equal); Resources (equal); Writing – Review & Editing (equal). **Tsutomu Mano** : Data curation (equal); Investigation (equal); Resources (equal); Writing – Review & Editing (equal). **Toshio Tsubota** : Supervision (equal); Writing – Review & Editing (equal). **Keita Fukasawa** : Supervision (equal); Writing – Review & Editing (equal). **Hiroyuki Uno** : Funding Acquisition (lead); Project administration (equal); Writing – Review & Editing (equal).

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