ORIGINAL INVESTIGATION

Male reproductive success in plateau pikas (*Ochotona curzoniae*): A microsatellite analysis

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Abstract

In many social species, competition and cooperation between group members may lead to a large variance in reproductive success among individuals, especially for adult male. From April to August 2002, we studied the adult male reproductive success of plateau pikas in Haibei Alpine Meadow Ecosystem Research Station, Chinese Academy of Science, using microsatellite analysis of paternity, mark-recapture and behavioral observations. Our result indicated that the reproductive success of adult males had a large variance. Its average was 6 individuals and range was from 0 to 13 individuals. One-third of males sired 63.22% offspring. There was a hierarchy system in polygynandrous families. Although the reproductive success of dominant males was higher than that of subordinate males, subordinate males still play an important role in the reproductive success of the population.
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Keywords: Male reproductive success; Microsatellites; Paternity analysis; Plateau pika

Introduction

The plateau pika (*Ochotona curzoniae*), called "black-lipped pika", is a small (160 g), non-hibernating, native diurnal lagomorph that only inhabits alpine meadows above 3000 m on the Qinghai-Xizang (Tibetan) plateau, People's Republic of China (Smith et al. 1986; Smith and Wang 1991; Dobson et al. 2000). Plateau pika plays dual roles on the diversity of the Qinghai-Xizang. For decades the plateau pika has been considered as a pest because it degrades rangeland (Lang et al. 1997; Fan et al. 1999) and destroys the forage for domestic livestock, such as yak, sheep, horses, etc. However, the plateau pika is also considered as a keystone species for the biodiversity of the Qinghai-Xizang plateau (Smith and Foggin 1999; Lai and Smith 2003). For instance, the pikas' burrows may minimize soil erosion, enhance soil absorption, increase soil nutrient cycling, and create microhabitats by increasing plant species richness, as well as provide living place for small birds and reptile (Lai and Smith 2003). In addition, the plateau pika serves as the principal prey for nearly all of the plateau's predators (Wei and Zhou 1997).

The primary social unit of plateau pika is the family, inhabiting an interconnected series of burrows on continuous and generally flat meadows. Within a population, these families are composed of a variable number of breeding adult males, females and their young. Members in the same family are usually affiliative to each other, but are aggressive towards ones from other families. Both males and females fend off intruders from other families (Wang and Smith 1989;

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Wang and Dai 1990; Smith and Wang 1991). The mating system of plateau pika is generally considered as monogamous, polygynous or polygynandrous and there is a hierarchy system among adult males (Smith et al. 1986; Wang and Smith 1989; Wang and Dai 1990; Smith and Wang 1991; Wei et al. 2000), but all of these researches focused mainly on the observational data. This approach is limited to studying small mammal's mating activity, especially for the assignment of parentage (Westneat 2000; Coltman et al. 2002). Most copulating activities of plateau pika take place in burrows and a single female is usually copulated with several males during her receptive period. So, it is very difficult to identify the mating pattern and evaluate individual reproductive success.

A large number of studies have applied different DNA markers, such as mini- and microsatellites to examine the reproductive activities of animal populations (Birkhead and Møller 1998). However, currently no species-specific primers are available for plateau pika. This study was aimed at investigating male reproductive success in a single plateau pika population by integrating the results of microsatellite, mark-recapture and behavioral observations, and then to examine the hypothesis that there is no difference between the reproductive success of dominant males and that of subordinate males. We also analyzed the characteristics of the microsatellites based on 10 loci derived from the related species of plateau pika.

Materials and methods

Study area

From April to August in 2002, this study was conducted at the Haibei Alpine Meadow Ecosystem Research Station of the Chinese Academy of Sciences (HAMERS) in Qinghai Province, China. HAMERS is located at the northeast Qinghai-Tibet Plateau, a large valley in the Qilian Mountains ($37°29'$–$37°45'$N, $101°12'$–$101°33'$E). The climate is a continental monsoon type dominated by the southeast monsoon and high pressure from Siberia, with about $-1.7\,°C$ of the average temperature, ranging from -37.1 to $27.6\,°C$. The winter is long and severe and the summer is short and cool. Most rainfall occurs from May to September, and heavy snow packs in winter (Sun et al. 2005). The major vegetations include alpine meadow, alpine shrub and swamp meadow. Almost all plateau pika have lived in alpine meadow. It serves as the principal prey for nearly all predator species, such as foxes (*Vulpes vulpes* Linnaeus, *V. ferrilata* Hodgson), alpine weasel (*Mustela altaica* Pallas), Asia polecat (*M. eversmanni* Lesson), desert cat (*Felis bieti*

Milne-Edwards), upland buzzard (*Buteo hemilasius* Temminck and Schlegel), falcons (*Falco cherrug* J.E. Gray, *F. tinnunculus* Linnaeus, *F. peregrinus* Tunstall) and owls (*Bubo bubo* Linnaeus, *Athene noctus* Scopoli, *Asio otus* Linnaeus) (Wei and Zhou 1997; Lai and Smith 2003; Yang et al. 2007). Besides the plateau pika, other small animals in the area include root vole (*Microtus oeconomus* Pallas), gansu pika (*O. cansus* Lyon) and Himalayan marmot (*Marmota himalayana* Hodgson) (Yang et al. 2007).

The study site is located at about 5 km east of the HAMERS. Vegetation at study site was *Kobresia humilis* meadow community, with a variety of sedges, grasses and forbs that served as pika forage. The area of 1.0 ha was divided into one hundred $10 \times 10\,m^2$ grid cells using clods. Each grid cell was assigned a unique letter and number code. So, all captures and behavioral observations were referenced to their spatial location (Dobson et al. 1998, 2000).

Study species

Plateau pikas exhibited the male-biased natal dispersal that is common among mammalian species. Disperse occurs within a narrow window of time just before initiation of the mating season and juvenile pikas remained within their natal family range during the annual breeding season (Smith and Wang 1991; Dobson et al. 1998). After the mixing that results from dispersal, each newly formed family becomes a cohesive social unit and hierarchy among males was established (Smith and Wang 1991). In the reproductive period, from April to August, all adult females breed, producing 3–5 sequential litters of 3–6 young in 3-week intervals. Over-winter mortality is high, and few adults live to 2 years of age (Smith and Wang 1991; Dobson et al. 1998; Yin et al. 2004).

In early April, pikas (22 males and 32 females) were caught with string nooses, anchored to soil near the hole with chopsticks (Dobson et al. 1998, 2000). Captured pikas were identified, weighed and labeled with numbered, plastic ear-tags with a unique combination of colors to facilitate recognition of individuals in subsequent behavioral observations. Blood samples (100 μl) of the labeled pikas were collected from suborbital veins before being released at the point of capture. The blood samples were stored in DNA buffer (1.21% Tris, 3.72% EDTA and 1.0% SDS, pH = 8.0) and immediately frozen with liquid nitrogen. About 12 days after birth, newborn pikas began to move on the ground (Wang and Smith 1988). When lots of new young began to move on the ground, all young and adult pikas were trapped, marked and weighted again. New young were sampled if they were more than 40 g.

Measurement of behavior and determination of family composition

The recorded behaviors of plateau pikas include: (1) *moving*: moving from one locality to another on the meadow in a non-social context, (2) *feeding*: gathering or chewing vegetation, (3) *observing*: sitting on meadow with neck outstretched or standing with front feet off the ground, or raising head during feeding, (4) *Calling*: uttering long and short, (5) *affiliative behavior*: contacting, grooming, mouth or nose rubbing, nursing, mating, etc. among pikas, (6) Aggressive behavior: chasing, fighting, etc., among pikas (Smith et al. 1986; Yang et al. 2007).

The behaviors were observed with focal-animal sampling by observers sitting on or adjacent on the study area at the active peaks from 7:00 to 11:00 a.m. and from 4:00 to 7:00 p.m. after all pikas were being captured for 5 days. Binoculars were used to identify focal animal and observe its behaviors. The frequency of each behavior variable every 15-min focal-animal sampling interval was recorded and expressed as the number $15 \, min^{-1}$. Animals were repeatedly observed at different times of different days to ensure a complete representation of each age and sex in a population. During the 15-min observation sessions, the localities of focal animals on the x, y coordinate grid system at 1 min intervals were mapped.

We determined the family composition based on the behavioral and spatial information according to the procedures of Dobson et al. (1998). They included (1) affiliative and aggressive behavior (99% of all recorded affiliative behaviors occur within families and 96% of all aggressive behaviors involved males from different families, see Smith and Wang 1991), (2) the activity centers of individuals (the average distance between centers of activity of individuals within families was 5.7 ± 0.25 m, whereas distances to the center of activity of the nearest individuals of the same gender outside the family were 23.8 ± 0.68 m, see Dobson et al. 2000). If 2 or more than 2 adult males belong to the same family, the dominant male was determined by analyzing the occurring frequency of their aggressive behavior (the higher was the dominant male).

Molecular analysis

DNA was extracted from blood using a standard phenol/chloroform protocol (Sambrook et al. 1996). Twenty-four microsatellite loci isolated from European wild rabbit (*Oryctolagus cuniculus*) (Mougel et al. 1997; Surridge et al. 1997) and North American pika (*Ochotona princes*) (Peacock et al. 2002) were optimized for plateau pikas. The markers Sat5, Sat12, Sol33, OCP1, OCP2, OCP3, OCP4, OCP7, OCP8 and OCP9

were selected for PCR amplification. The 15 μl PCR reaction contains 100 ng (approximately) of genomic DNA, $1 \times$ reaction buffer, 0.2 μM of each primer, 0.2 mM dNTPs, 1 U of *Taq* DNA polymerase and $MgCl_2$ at different concentrations according to each locus. The conditions for amplification include 5 min initial denaturation at 95 °C, followed by 30 cycles of 30 s denaturation at 95 °C, 30 s annealing at 55 °C, and 45 s polymerization at 72 °C, and a final extension of 10 min at 72 °C. PCR products were checked on 8% polyacrylamide denaturing gels and analyzed by the silver staining method, and PBR322DNA/MSPI marker (622–123 bp) was used to calibrate allele length. The genotypes were determined by visual inspection. Allele sizes were determined by using Bandscan software. Samples that were not amplified or amplified weakly were reamplified up to 5 times. The ambiguous results were treated as missing data (3.45%, 6 of 174).

Paternity analysis

Mothers could be identified based on the field observations of the timing of reproduction and maternal–offspring behaviors (Smith and Wang 1991). Exclusion analysis could be used to exclude the adult males that have at least one microsatellite allele incompatible with the genotype of the cub (Queller et al. 1993). Such an approach was possible since each cub could also be matched to the known maternal profile. However, if 2 or more candidate adult males had no mismatches, the father should not be identified only by exclusion analysis. The program CERVUS 2.0 (Marshall et al. 1998) also can be used for paternity analysis. In CERVUS maximum likelihood approach was used to assign paternity based on the log-likelihood ratio scores (LOD score) between the most and the second-most likely candidates. However, CERVUS support criteria to allow genotyping errors and assign paternity despite mismatching loci that can lead to the false exclusion of true fathers (Vigilant et al. 2001). Only stringent criteria allowing no mismatches should be used to avoid false paternity assignments (Worthington Wilmer et al. 1999).

In this research, paternities were assessed using 2 methods. Exclusion analysis was first performed to exclude the adult males that have at least one microsatellite allele incompatible with the genotype of the cub. If only one candidate adult male in one family had no mismatches, this male was assigned as the father. If 2 or more candidate adult males in one family had no mismatches, each descendant is assigned to the most likely parent by program CERVUS. The theoretical predictions of correct assignments used allelic frequencies found in each family under study. The parameters set for CERVUS were at 2 confidence levels (relaxed: 80%; strict: 95%), including 10,000 cycles, 100% of

candidate parents sampled, 100% of loci typed and 1% for rate of typing error.

Sometimes, all males in one family had one or more mismatches. Then, all adult males in the study site were included as candidate fathers. Extra-pair paternity was determined combining direct and probability methods. If all adult males in the study site had one or more mismatches with the genotypes of the cubs, we generally considered the father to be unknown and unsampled.

To analyze the microsatellite polymorphism of plateau pikas, the program CERVUS calculates also the observed and expected heterozygosities, and the frequencies of possible null alleles for every locus from all typed individuals. Tests of Hardy–Weinberg equilibrium and of linkage disequilibrium were performed using GENEPOP 3.3 (Raymond and Rousset 1995) on all adults to avoid bias due to family structure and null alleles.

Result

Molecular analysis

Ten loci did not show high levels of polymorphism for paternity analyses, with an average of 5 alleles per locus. Significant departures from Hardy–Weinberg expectations were not detected and linkage disequilibrium between loci was also not detected. The per-locus expected heterozygosity ranged from 0.312 to 0.855, with a mean of 0.506, and the exclusion probabilities ranged from 0.210 to 0.735, with a high combined average exclusion probability of 0.92 (Table 1).

Family composition and paternity assignment

The members of 8 families (about 2.4 adult males and 2.9 adult females per family) were determined based on behavioral and spatial information. Two families were socially monogamous, and the other 6 families were polygynandrous. One family had 2 adult males and 4 adult females, and the remaining 5 families had 3 adult males and 3 or 4 adult females (Table 2). The paternities of 118 offspring (70 females and 48 males) were analyzed. 65.3% (77 of the 118) offspring were determined by exclusion analysis and 28.8% (34 of the 118) offspring were theoretically assigned by the program CERVUS 2.0 (Table 2). The paternities of 2 offspring were extra-pair paternity (1.7%). Presumably the father(s) of 7 of the 118 (5.9%) offspring either died or dispersed from the site before the commencement of the study.

Male reproductive success

There was a large variance in the reproductive success of males in these families, especially for the dominant and subordinate males. Its range was from 0 to 13 individuals with an average of 6 individuals per adult male. The reproductive success of dominant males was prominently higher than that of subordinate males (Table 2). However, some subordinate adult males still reproduced and sired 30.6% offspring (34 of the 111).

Behavior of the dominant and subordinate males

Table 3 shows the behavior of the dominant and subordinate males. A mixed linear model was used to test the behavioral differences between dominant and subordinate males considering the effect of male status, month, family and repeated sampling. The observing and aggressive behaviors were significantly different between the dominants and subordinates ($P < 0.05$). While feeding, calling and familiar behaviors were similar between them ($P > 0.05$) (Table 3).

Table 1. Statistics of 10 microsatellite loci

Locus	No. of alleles	Fragment size	H_o[a]	H_e[b]	Null freq.[c]	Exclusion probability
Sat5	2	307–317	0.338	0.312	−0.0008	0.213
Sat12	4	122–134	0.406	0.397	−0.0102	0.367
Sol33	2	207–214	0.576	0.563	0.0180	0.642
OCP1	2	308–310	0.306	0.327	−0.0422	0.210
OCP2	3	365–395	0.484	0.424	−0.0010	0.483
OCP3	4	302–319	0.436	0.468	−0.0212	0.497
OCP4	2	244–249	0.742	0.606	0.0111	0.361
OCP7	23	258–358	0.984	0.855	−0.0386	0.735
OCP8	3	236–250	0.758	0.651	−0.0287	0.580
OCP9	5	185–203	0.694	0.556	−0.0252	0.632

[a] H_o = observed heterozygosity.
[b] H_e = expected heterozygosity.
[c] Null freq. = estimated frequency of null alleles or nonamplifying alleles.

Discussion

Determining mating patterns and variance in reproductive success in mammals is complicated by the lack of paternal care. In some mammals, maternal care is a good indicator of motherhood. A female's reproductive

Table 2. Offspring amount of each adult male in 8 families of plateau pikas (*Ochotona curzoniae* Hodgson)

Family	Adult male	No. of overall offspring	Theoretically assign
F1[a]	L168[b]	10	20.00% (2/10)
	L11	6	0
	L88	0	0
F2	L106[b]	11	27.27% (3/11)
	L52	5	40.00% (2/5)
	L8	0	0
F3	L23[b]	7	0
	L59	3	66.67%(2/3)
	L6	3	100% (3/3)
F4	L27[b]	13	23.08% (3/13)
	L25	5	20.00% (1/5)
	L116[c]	4	50% (2/4)
F5	L16[b]	11	18.18% (2/11)
	L61	6	16.67% (1/6)
	L54	3	66.67%(2/3)
F6	L4[b]	10	50.00% (5/10)
	L51	5	80.00% (4/5)
F7	L19	6	33.33% (2/6)
F8	L82	3	0

[a]Families F1, F2, F3 had 3 adult males and 3 adult females; F4, F5 had 3 adult males and 4 adult females; F6 had 2 adult males and 4 adult females; F7, F8 had 1 adult male and 1 adult female.
[b]Dominant male.
[c]Of its offspring was extra-pair mating.

success could be determined by behavioral observations but a male's reproductive success is much more difficult to determine (Schwagmeyer et al. 1998), especially for small mammals. Also, when a female mates with multiple males, the paternities of young are difficult to determine. In the present research, by integrating the behavioral observations with the genetic data, we investigated the mating activities of plateau pika. Our behavioral and genetic data indicated that different mating systems (monogamy and polygynandry) emerge in the plateau pika population. This pattern might result from the dispersal of juvenile males and the competition for mates. The natal dispersal of plateau pika was male-biased and females were most philopatric (Dobson et al. 1998). Philopatric females were much more likely to have close kin among them. If there are more than one female in a family, these females are easy to form reproductive alliances. For example, adult females took care of juveniles that are not their own offspring within the family (Smith and Wang 1991). The environment may be relatively uniform to plateau pikas, which might limit the capacity of males to control their mates; meanwhile, females in estrus were often observed to mate alternately and repeatedly with all males in their family (Smith and Wang 1991; Dobson et al. 1998). So, it is very difficult for every adult male to control more than one female. If there is only one adult female in one family, monogamy might be the mating system in this family. If there are 2 or more adult females in one family, polygynandry might be the mating system.

The plateau pikas show a stable breeding group during the reproductive season. Most successful mating occurs between adults of the same family and both males and females were effective at chasing off trespassing males and non-family males. So, the probability of extra-pair copulations might be very low (Wang and Dai 1990; Dobson et al. 1998). In our research, the proportion of extra-pair paternities was only 1.7%.

Our study showed that the reproductive success of dominant males was prominently higher than that of subordinate males (Table 2). So our results reject the hypothesis that there is no difference between the reproductive success both of dominant males and of subordinate males. Opposing forces (competition and cooperation) may lead to reproductive skew. In our

Table 3. Frequencies (number $15\,min^{-1}$) of the behaviors of the dominant and subordinate adult males

	Moving	Feeding	Observing	Calling	Affiliative behavior	Aggressive behavior
Dominants[a]	5.76 ± 1.05	4.32 ± 0.24	9.82 ± 1.59	0.15 ± 0.09	0.22 ± 0.11	1.56 ± 0.39
Subordinates[b]	4.10 ± 0.42	4.59 ± 0.30	6.56 ± 1.24	0.33 ± 0.09	0.27 ± 0.14	0.80 ± 0.24
F	0.041	0.461	18.293	0.669	0.432	23.132
P	0.843	0.493	0.001	0.731	0.507	0.000

[a]Dominants includes adult males: L168, L106, L23, L27, L16, L4.
[b]Subordinates includes adult male: L11, L88, L52, L8, L59, L6, L25, L116, L61, L54, L51.

research, adult males may guard the mating of females, especially for the dominant males that are highly aggressive to any males who approached the female they were guarding. The aggressive behaviors of the dominant and subordinate plateau pikas were very different. The aggressive behaviors of males may lead to a stable dominant family hierarchy, in which dominant males have priority of access to receptive females (Wang and Dai 1990). So, the reproductive success of dominant males was prominently higher than that of subordinate males. In many social species, competition and cooperation between group members may lead to a large variance in reproductive success among individuals, especially for adult males. The "Optimal Skew Hypothesis" and the "Limited Control Hypothesis" have emerged to explain the reproductive skew. "Optimal Skew Hypothesis" suggests that dominant individuals have complete control of the reproduction of subordinates (Vehrencamp 1983). However, "Limited Control Hypothesis" suggests that dominant individuals have incomplete control of subordinate reproduction. Subordinate individuals also have a few offspring (Clutton-Brock 1998). Our results showed that some subordinate adult males still had many offspring (Table 2), which indicate dominant male pikas did not fully control the reproduction of their subordinates. So our results agree with the viewpoint of "Limited Control Hypothesis".

Males provided abundant paternal care and emit alarm calls when they deter a predator. The observing behavior of mature males had a significant positive correlation with the survival of juveniles (Wang and Dai 1990; Yin et al. 2006). Adult females and dominant males may benefit from maintaining group cohesion with subordinates. To retain the subordinate male in the group, dominant males have to concede enough reproduction to them, which might be the main reason why 2 or more males decide to share mating. Similar findings have been reported for alpine marmot, Marmota marmota (Grimm et al. 2003). Meanwhile, adult females might influence male–male competition by mating with several males during their estrous periods. Some field studies indicate that females then can actively seek extra-pair copulations and there is considerable evidence that they do so for genetic benefits (Foerster et al. 2003; Cohas et al. 2006, 2007). It thus seems reasonable to conclude that although the dominant male in the study area contributed most to the gene pool, the subordinate males also play a role in the reproductive success of the population.

Acknowledgements

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