Paternal Analysis of Captive Red Kangaroos

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飼育下アカカンガルーの父子判定

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ABSTRACT. To determine the sire of captive red kangaroos (*Macropus rufus*), we conducted paternal analysis using 4 kinds of microsatellite DNA loci. We usually determine paternal lineage of captive animals on the basis of mating behavior or the strong dominant status of adult males. However, the sire of 7 offspring could not be determined as such behavior and status were not observed. Therefore, we used molecular DNA-based paternal analysis to determine their sires with positive likelihood. Our result indicates that molecular paternal analysis can be useful when a sire cannot be determined by the dominant status of adult males or observation of mating behavior.

Key words : captive population, microsatellite DNA, paternal lineage, red kangaroo

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INTRODUCTION

The red kangaroo (*Macropus rufus*) is one of the most abundant kangaroo species in Australia (e.g., [1]). It is known to be gregarious [2], and its mating system is polygamous [3]. In addition, the species has a unique reproductive system—embryonic diapause [4]—wherein a blastocyst remains quiescent and does not resume development until the previous offspring are about 200 days old and have left the pouch [4, 5].

The red kangaroo has been bred in 21 zoos in Japan, including Yokohama Zoological Gardens [6], where it is housed gregariously to reflect its natural lifestyle. Because all adult kangaroos are free to engage in physical contact with each other, there are no restrictions for establishing mating pairs. Although all adult males have the chance to mate with an oestrus female, only the dominant male, who is usually the largest male, commonly mates with the oestrus female. On the other hand, non-dominant males seem to have little chance of mating because the dominant male perpetually guards the oestrus female. On the basis of such observations, we assign the male who is observed to mate with a dam to the offspring of that dam. In situations when mating behavior cannot be recorded, it is assumed that the offspring is sired

by the dominant male. Both methods for assigning a sire are based on ordinal paternal analysis. The sire of some kangaroos, however, cannot be assigned on the basis of ordinal analysis because we cannot always observe mating behavior. Furthermore, succession of the dominant male was thought to occur when the offspring were conceived, although we did not know exactly when such succession occurred. Therefore, we could not assign the sire simply by knowing which male was the dominant male at a particular time. Since making a precise pedigree chart is important for managing a captive population, we need to clarify the paternal lineage of the captive kangaroos [7].

Molecular paternal analysis based on genotyping of microsatellite DNA loci is a popular technique even if no information on parental lineage is available (e.g., [8]). Although microsatellite DNA sequences have already been reported for several marsupial species, no such DNA for the red kangaroo has been reported to date [9].

In this study, to clarify the sire of offspring whose sire was unclear, we conducted molecular paternal analysis using a microsatellite DNA primer developed for other marsupial species.

MATERIALS AND METHODS

1) Kangaroos

We studied 15 kangaroos at the Yokohama Zoological Gardens (Kanagawa, Japan) (Table 1). Seven were born in captivity to resident adults. The sires of the offspring could not be assigned on the basis of mating behavior alone because no such behavior was observed. Of the 8 residents, 6 were females and dams to the offspring, while the other 2 were candidate sires. At one time, male No. 18 was the dominant male, but that role was ceded to male No. 27. Five of the seven offspring were thought to be fertilized during the succession of dominance from male No. 18 to No. 27. The youngest 2 offspring were thought to be fertilized by male No. 27 because of his current dominant status in the population. The sire of each offspring, estimated by our ordinal method, is shown in Table 2. The date of birth of each offspring was defined as the date 180 days before leaving the pouch.

2) DNA analysis

We extracted DNA from the blood of the kangaroos using a QIA DNeasy blood and tissue kit (Qiagen). We then used 4 kinds of polymerase chain reaction (PCR) primer sets designed for microsatellite DNA loci of other marsupial species (Me17, Pa593, G15-4, G26-4) [10-12]. For PCR, DNA primers and PCR cycles were performed as described previously [10-12].

PCR primer DNA was amplified in a 25 μ l reaction solution consisting of 2.5 μ l of 10 $\times$ buffer, 2 μ l of 2.5 mM dNTP and 0.75 U of Ex Taq polymerase (TaKaRa), dissolved in distilled water. The PCR products for one of two candidate male (No18) were cloned using a TA cloning kit (BioDynamics Laboratory Inc.). For sequencing, we used T7 sequence primers with an ABI PRIM 310 genetic analyzer (Applied BioSystems). For fragment analysis, each primer was labeled with 6-FAM, Hex and NED. Polymorphisms were analyzed using an ABI PRIM 310 genetic analyzer and GENESCAN version 2 Software (Applied BioSystems).

3) Paternal analysis

For paternal analysis, we used Cervus version 3.03 Software [13]. Before parental analysis, we estimated the frequency of null alleles in each locus using software in order to exclude loci with null alleles. Next, we conducted paternal analysis with the software. When we used the software to genotype several microsatellite DNA loci of the offspring, their dams and candidate sires, we obtained likelihood ratios for the candidate sires (LOD) and subsequently assigned the sire with a 95% level of confidence for LOD. A positive LOD score denotes a high probability that the candidate is the sire, whereas a LOD score of zero denotes a low probability. On the other hand, a negative LOD could occur if a candidate has one or more mismatched loci with the offspring.

Table 1 Subject characteristics, PCR product size of each of the 4 microsatellite DNA loci. Numbers in each locus indicate the size of each PCR product.

Kangaroo			Locus							
Individual Number	Characters	Known dam	Me17		Pa593		G15-4		G26-4	
No18	Candidate sire	—	146	156	117	121	226	226	186	202
No27		—	112	148	109	115	216	216	208	224
No13	Dam	—	114	154	103	119	216	234	214	218
No37		—	112	146	103	109	216	234	206	218
No57		—	154	156	113	121	226	226	202	214
No69		—	112	112	113	115	216	226	186	224
No86		—	156	156	117	121	226	226	186	202
No90		—	146	146	103	113	222	234	216	218
No79	Offspring	No13	146	154	103	117	216	226	202	218
No83		No37	146	156	103	117	226	234	202	218
No88		No69	112	112	113	115	216	226	186	208
No91		No57	148	156	109	121	216	226	202	224
No94		No57	148	154	115	121	216	226	202	224
No101		No86	148	156	109	121	216	226	186	208
No108		No90	146	148	103	109	216	234	218	224

Table 2 Assigned sires of the offspring on the basis of molecular analysis and LOD score

Offspring	Birthday	Molecular assigned sire	LOD score	Sire assigned by ordinal analysis
No79	2006/03/24	No18	3.62	No18 or No27
No83	2006/10/01	No18	3.63	No18 or No27
No88	2007/06/29	No27	3.34	No18 or No27
No91	2007/07/12	No27	4.23	No18 or No27
No94	2008/02/21	No27	4.46	No18 or No27
No101	2008/09/24	No27	4.75	No27
No108	2009/01/31	No27	4.24	No27

RESULTS

We amplified the DNA from all individuals using 4 primer sets and then verified the DNA sequences of the 4 loci. As a result, a TC repeat sequence was observed for G26-4, whereas a AC repeat was observed for Me17. And a GT repeat was observed for Pa593. The remaining locus included 3 kinds of repeat sequence: a TC repeat, AC repeat and TGTC repeat. Therefore, we were able to amplify the microsatellite DNA loci of the red kangaroo. Results of polymorphism analysis are shown in Table 1. The G15-4 locus included 4 alleles. The other 3 loci showed greater polymorphism. Me17 included 6 alleles whereas Pa593 included 7 alleles. And the remaining a loci included 8 alleles. Since all microsatellite loci were polymorphic, we used all 4 for paternal analysis. Before paternal analysis, however, we estimated the frequency of null alleles of each of the 4 microsatellite DNA loci using Cervus software. According to the manufacturer's instruction, a locus with a null allele ratio of >0.05 should be excluded from paternal analysis. In our case, the estimated frequency of null alleles in all 4 loci was <0.05 (-0.0937 to 0.0468). Therefore, we conducted paternal analysis without taking null alleles into consideration. Results of paternal analysis are shown in Table 2. Both males were assigned as candidate sires to all 7 offspring with a 95% confidence for LOD. The LOD score for a sire-offspring pair, given a known dam, was positive for all 7 pairs. For 5 offspring whose sires were unclear, male No. 18 was assigned as the sire of the 2 older offspring while male No. 27 was assigned as sire of the remaining 3 offspring (Table 2).

DISCUSSION

Molecular paternal analysis using microsatellite DNA polymorphism has been regarded as useful, although development of new PCR primers for microsatellite DNA loci for determining species has usually been necessary (e.g., [7]). However, a microsatellite DNA primer for one species can

also work for a closely related species [7]. In this study, we amplified 4 kinds of microsatellite DNA loci using a PCR primer set designed for other marsupial species, in which the loci shows relatively high polymorphism. Similarly, the same loci were polymorphic in the red kangaroo. This finding suggests that variable microsatellite DNA loci might be preserved among marsupial species.

For paternal analysis, we listed both adult males as candidate sires for the 7 offspring. Before conducting molecular analysis, 2 of the 7 offspring had already been assigned on the basis of observed dominant status, which was our ordinal method. The results of molecular paternal analysis closely matched the results of our ordinal method, indicating that our ordinal method is valid for captive paternal analysis. The sire of the remaining 5 offspring, whose sires could not be assigned by our ordinal method, could be assigned by using a positive LOD score. Therefore, molecular paternal analysis is useful when data of both mating behavior and dominant status are inadequate for ordinal paternal analysis. However, according to paternal analysis of the captive tammar wallaby (*Macropus eugeni*), the dominant male is the first to mate and sire half of the offspring, with a number of subdominant males also siring in captive populations [14]. We also observed copulation by non-dominant males, although such copulation is rare. Therefore, in order to clarify the paternal lineage in our captive population by molecular paternal analysis more precisely, we need to use not only the dominant male, but also non-dominant males.

Finally, microsatellite DNA polymorphisms are useful for not only parentage analysis, but also the study of genetic diversity [7]. Moreover, the study of genetic diversity in respect to captive animals is thought to be important for maintaining captive populations [7]. Therefore, in order to maintain a captive population of red kangaroo, we need to conduct a genetic study of the population using microsatellite DNA.

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要 約

よこはま動物園には、繁殖行動などにより父親推定が困難なアカカンガルーが7頭飼育されている。それらの父親候補は歴代の優位雄2頭のいずれかである。これら7頭の父親を明らかにするために、マイクロサテライトDNA多型に基づく父子鑑定を行った。その結果、7頭の父親を推定することができた。このことから、マイクロサテライトDNAを用いた父子鑑定は、繁殖行動などのデータが不足している場合には、飼育下カンガルーの父系推定法として有効であることが示唆された。

キーワード：飼育下個体群、マイクロサテライトDNA、父子関係、アカカンガルー

REFERENCES

1. Department of Environment and Conservation NSW. 2006. New South Wales Commercial Kangaroo Harvest Management Plan 2007-2011. Department of Environment and Conservation (NSW), Sydney.
2. Kirkpatrick TH. 1967. The red kangaroo in Queensland. *Qd Agric J* (93) 484-486.
3. Croft DB. 1981. Behaviour of red kangaroos, *Macropus rufus*, in north-western NSW, Australia. *Aust Mammal* 4: 5-58.
4. Sharman GB. 1963. Delayed implantation in marsupials. In Delayed implantation (Enders AC ed.). Univ. of Chicago Press
5. Clark MJ. 1968. Termination of embryonic diapause in the red kangaroo, *Megaleia rufa*, by injection of progesterone or oestrogen. *J Reprod Fert* 15: 347-355
6. Japanese association of zoos and aquariums. 2009. <http://www.jaza.jp/>
7. Frankham R, Ballou JD, Briscoe DA. 2002. Introduction to conservation genetics. Cambridge university press
8. Jones AG, Ardren WR. 2003. Methods of parentage analysis in natural populations. *Mol Ecol* 12: 2511-2523.
9. Coulson G, Eldridge M. 2010. Macropods. CISRO publishing
10. Spencer PBS, Odorico MD, Jones SJ, MarSh H, Miner DJ. 1995. Highly variable microsatellites in isolated colonies of the rock wallaby (*Petrogale assimilis*). *Mol Ecol* 4: 423-525.
11. Taylor AC, Cooper DW. 1998. A set of unlinked microsatellites from the Tammar wallaby (*Macropus eugenii*). *Mol Ecol* 7: 925-926.
12. Zenger KR, Cooper DW. 2001. A set of highly polymorphic microsatellite markers developed for the eastern grey kangaroo (*Macropus giganteus*). *Mol Ecol Notes* 1: 98-100.
13. Kalinowski ST, Taper ML, Marshall TC. 2007. Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. *Mol Ecol* 16: 1099-1106.
14. Hynes EF, Rudd CR, Temple-Smith PD, Sofronidis G, Paris D, Shaw G, Renfree MB. 2005. Mating sequence, dominance and paternity success in captive male tammar wallabies. *Reproduction* 130: 123-130.