

## Amplification of Microsatellites of Japanese Raccoon Dogs (*Nyctereutes procyonoides viverrinus*) with Primers for the Dog ZUBECA4 Locus

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### Abstract

The Japanese raccoon dog short tandem repeats (STRs, or microsatellites) were investigated using the primer set for the ZUBECA4 locus, one of the dog STR primers. The properties of Japanese raccoon dog PCR products were examined by electrophoresis and sequence analyses. Six alleles were detected by the electrophoresis analyses in nine Japanese raccoon dogs. The sequence analyses on the six alleles revealed that variants are based on the difference in the total number of repeats of GAAA and GCAA. The repeating units of Japanese raccoon dogs were simpler than those of dog alleles. The six alleles were designated as 12-16 and 18. Allele 15 and 18 were divided into 15<sup>12</sup>, 15<sup>14</sup>, 15<sup>15</sup>, 18<sup>13</sup> and 18<sup>14</sup> sub-alleles by the times GAAA was repeated. Their frequencies were estimated to be: allele 12=0.10, allele 13=0.17, allele 14=0.17, allele 15<sup>12</sup>=0.22, allele 15<sup>14</sup>=0.06, allele 15<sup>15</sup>=0.06, allele 16=0.06, allele 18<sup>13</sup>=0.06 and allele 18<sup>14</sup>=0.10. Heterozygosity was calculated to be 0.79 in STR alleles and 0.86 in sub-alleles. Further, new primers for Japanese raccoon dogs were developed based on the sequences of the Japanese raccoon dog alleles. This primer set was successfully amplified as a small-sized amplicon, which is more suitable for practical uses than dog primers. These findings indicated that the ZUBECA4 locus is a quite useful STR for species identification, individual identification, pedigree study and population study on Japanese raccoon dogs.

**Key words:** forensic genetics, DNA polymorphism, STR, microsatellite, dog ZUBECA4 primers, Japanese raccoon dog

### Introduction

Short tandem repeats (STRs), which are known as microsatellites, are potentially the most useful forensic DNA marker<sup>1</sup>. The fundamental information about STR loci, such as mutant alleles, internal alleles and three peak alleles, has been well documented<sup>2</sup>. The necessity of comparative studies of repeating units between animal species for judging the stability of tandem repeats has been pointed out<sup>3-5</sup>. In addition, in forensic practice, it is sometimes necessary to determine a sample's origin, i.e., human and animal. The specimens of Japanese raccoon dogs (*Nyctereutes procyonoides viverrinus*) are also subjects of the discrimination. Information on Japanese raccoon dog STRs is very limited. Therefore, we intended to investigate STRs of Japanese raccoon dogs as tools for species identification and the identification of individuals.

As we have previously reported that human STR primers effectively amplify Japanese macaque DNA<sup>6-9</sup>, we undertook a preliminary examination of 15 human core STR loci for the PCR amplification of DNA from Japanese raccoon dogs. All primers failed to amplify the Japanese raccoon dog DNA. We considered that the cause of failure is the differences between the nucleotide sequences of humans and Japanese raccoon dogs. The Japanese raccoon dog belongs to the family Canidae and there are many accumulated data for dog STRs<sup>10-14</sup>. Preliminary study showed that dog primers successfully amplify Japanese raccoon dog DNA and ZUBECA4 locus was the most polymorphic in raccoon dog loci amplified with dog primers. We describe herein not only the properties of Japanese raccoon dog PCR products amplified with dog ZUBECA4 primers but also the first primers prepared for the Japanese raccoon dog ZUBECA4 locus.

## Materials and methods

Nine Japanese raccoon dogs (RD1-RD9) were used in the present study. RD1, RD7 and RD9 were female and the rest were male. The roadkill raccoon dogs were collected in Kagoshima city by the prefectural road caretakers and municipal garbage men. The causes of death were due to traffic accident judging from the degree of their injuries and the situation of discovery. This study was carried out along the guideline of Kagoshima University commission for experiments using laboratory animals. Genomic DNA was extracted from the muscle tissues by organic methods<sup>16)</sup>. The sample of dog DNA as control (CD1) was extracted from cheek swabs of an adult female dog (Labrador retriever, 7 years) by organic methods<sup>16)</sup>. Primers for dog ZUBECA4 locus were prepared using information reported by Eichmann et al.<sup>12)</sup>. The reaction solution (total volume 50  $\mu$ L) for PCR amplification was prepared to have a final concentration of 1.5 mM MgCl<sub>2</sub>, 200  $\mu$ M for each dNTP, 1.25  $\mu$ M for each primer, 1.25 U AmpliTaq Gold<sup>®</sup> DNA polymerase and 200 ng genomic DNA. Amplifications were carried out using a PE-320 thermal cycling block (ASTECC, Fukuoka, Japan) as follows: 96°C for 11 min; 10 cycles at 98°C for 10 sec, 60°C for 90 sec, 72°C for 30 sec; 25 cycles at 90°C for 10 sec, 60°C for 90 sec, 72°C for 30 sec; and 60°C for 30 min. Electrophoresis analyses of the PCR products were performed using polyacrylamide gels with a BRL<sup>®</sup> model SA32 (Life Technologies, Rockville, MD, USA), followed by silver staining<sup>17)</sup>. The PCR products were directly sequenced using ABI Prism<sup>®</sup> BigDye<sup>™</sup> Terminator v3.1 Cycle Sequencing Ready Reaction Kits and an ABI Prism<sup>®</sup> 310 genetic analyzer according to the manufacturer's protocol (Applied Biosystems, Foster City, CA, USA). Allele frequencies and heterozygosity for STR locus were calculated using the formula reported by Nei and Roychoudhury<sup>18)</sup>.

## Results and discussion

Electrophoretograms of the PCR products obtained from the Japanese raccoon dogs (RD1-RD9) and a dog sample as control (CD1) using the ZUBECA4 primers are shown in Fig. 1. All Japanese raccoon dog DNA samples were successfully amplified with ZUBECA4 primers. Single or double bands were clearly detected from all PCR products. All of these bands migrated faster than the dog

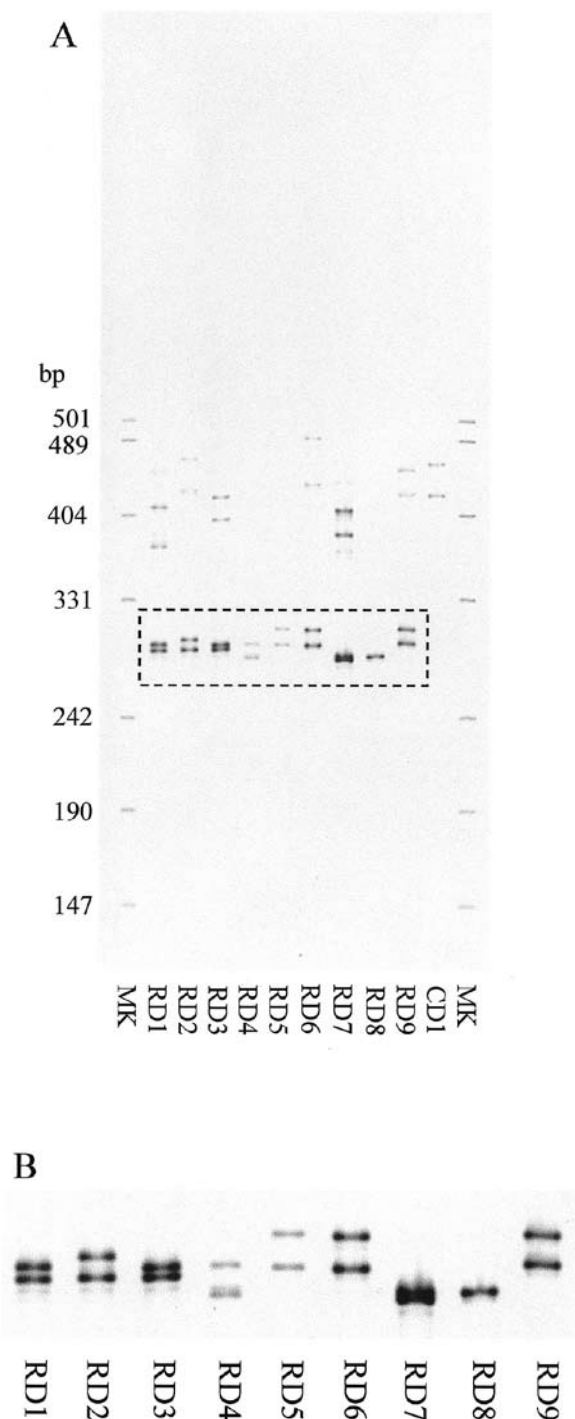


Fig. 1. Electrophoretograms of PCR products amplified with dog ZUBECA4 primers by electric focusing using unmodified polyacrylamide gels. A: overview of the focused gel, B: an enlargement of the region enclosed by a dashed line in Fig.1A. RD1-RD9: Japanese raccoon dogs. CD1: a dog. MK: DNA marker. Cathode at top.

bands, that is the Japanese raccoon dog PCR products were focused around 300 bp, and the dog PCR product to around 450 bp. Therefore, discrimination between the DNA of dogs and that of Japanese raccoon dogs is possible by electrophoresis analyses. Six different bands were observed. Each band from the nine individuals was separated and its nucleotide sequences analyzed.

The sequence data for the Japanese raccoon dog PCR products and the reference dog are shown in Fig. 2. The sequence studies revealed that the variants of nucleotide sequences in Japanese raccoon dogs arise from differences in the numbers of GAAA and GCAA tandem repeats: the numbers of the GAAA repeating unit ranged from 11 to 15 and those of the GCAA repeating unit from 0 to 5. The sequences in upper and lower flanking regions were similar to those of the dog DNA except for a C-G transversion in the upper flanking region and the insertion of GAATT into the front of repeat region. In the tandem repeat regions, GAAA repeats were observed in both Japanese raccoon dogs and dogs. These findings suggested that the dog ZUBECA4 primers amplify the same locus in Japanese raccoon dogs. The dog DNA has several nucleotides inserted around GAAA repeats<sup>10, 12</sup>. The repeating units of the Japanese raccoon dog alleles were very simple in contrast with those of the dog alleles. Therefore, we consider that the Japanese raccoon dog

ZUBECA4 locus is more favorable than the dog locus for practical uses. The six alleles of the Japanese raccoon dog ZUBECA4 locus were designated as 12-16 and 18 according to the total number of GAAA and GCAA repeats. Allele 17 was absent in this study. Allele 15 was subdivided into (GAAA)<sub>12</sub>(GCAA)<sub>3</sub>, (GAAA)<sub>14</sub>(GCAA)<sub>1</sub>, and (GAAA)<sub>15</sub> and allele 18 was subdivided into (GAAA)<sub>13</sub>(GCAA)<sub>5</sub> and (GAAA)<sub>14</sub>(GCAA)<sub>4</sub> based on the structure of their repeating units. Their alleles were tentatively designated as 15<sup>12</sup>, 15<sup>14</sup>, 15<sup>15</sup>, 18<sup>13</sup> and 18<sup>14</sup>, with the superscripts indicating the number of times GAAA is repeated. The ZUBECA4 types and subtypes in nine Japanese raccoon dogs are summarized in Table 1. The frequencies of STR alleles and their subdivided alleles were estimated to be 12=0.10, 13=0.17, 14=0.17, 15<sup>12</sup>=0.22, 15<sup>14</sup>=0.06, 15<sup>15</sup>=0.06, 16=0.06, 18<sup>13</sup>=0.06 and 18<sup>14</sup>=0.10. Heterozygosities on the Japanese raccoon dog ZUBECA4 locus were calculated 0.79 in STR alleles and 0.86 in their subtype alleles. These findings show that the Japanese raccoon dog ZUBECA4 locus has enough polymorphism to enable the identification of individuals.

Further, the first primer set for the Japanese raccoon dog ZUBECA4 locus was prepared. The amplicon of new primers was 150 bases smaller than that of a dog primer. The binding sites of these primers are shown in Fig. 2. Electrophoretograms of the PCR products obtained from

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RD: GAGGGCAGGAGTCATAAAAATCATTTTAAAGCCTAGCTATACTATATTGTTCTGTGTGTTG
      .....
CD: GAGGGCAGGAGTCATAAAAATCATTTTAAAGCCTAGCTATACTATATTGTTCTGTGTGTTG

RD: ATTTTATGTTCTGCACAATAAAACTTTATTGAATT
      .....
CD: ATTTTATGTTCTGCACAATAAAACTTTATT

RD: (GAAA)11(GCAA)1 : Allele 12
      (GAAA)11(GCAA)2 : Allele 13
      (GAAA)12(GCAA)2 : Allele 14
      (GAAA)12(GCAA)3 : Allele 1512
      (GAAA)14(GCAA)1 : Allele 1514
      (GAAA)15 : Allele 1515
      (GAAA)13(GCAA)3 : Allele 16
      (GAAA)13(GCAA)5 : Allele 1813
      (GAAA)14(GCAA)4 : Allele 1814

CD: (GAAA)3GAGAA(GAAA)8GAA(GAAA)2GAAGAAAAA(GAAA)3AAAAA(GAAA)3
      AGAAAAA(GAAA)4AA(GAAA)20AAGAAGAAAAAGAAA(GCTA)3

RD: GCTTAGCTGTTTGGGAAAGAGGCCAGTGATGTCAAAGTTAGAATGCACTTATCAAGTAGA
      .....
CD: GCTTAGCTGTTTGGGAAAGAGGCCAGTGATGTCAAAGTTAGAATGCACTTATCAAGTAGA

RD: CAGTATTTAATGTATTGGGAGTAAATAAGATCAGAGAAAAATCCTTCACAAGATTGTTT
      .....
CD: CAGTATTTAATGTATTGGGAGTAAATAAGATCAGAGAAAAATCCTTCACAAGATTGTTT

RD: GTCCCTGGGC
      .....
CD: GTCCCTGGGC
    
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Fig. 2. The nucleotide sequences of Japanese raccoon dog and dog PCR products amplified with dog ZUBECA4 primers. RD: Japanese raccoon dogs. CD: a dog. A solid underline indicates the binding sites of ZUBECA4 primers. A broken underline indicates the binding site of the ZUBECA4 primers prepared, for the first time, for Japanese raccoon dogs.

Japanese raccoon dog and dog samples using the new ZUBECA4 primer sets for Japanese raccoon dogs are shown in Fig. 3. The ladder marker for Japanese raccoon dog ZUBECA4 alleles was prepared with a template mixed with RD 2, RD 6 and RD 7 DNA. The ZUBECA4 types of nine Japanese raccoon dogs were easily typed by the use of the ladder marker. Since short amplicons are useful for working with old specimens that contain degraded DNA<sup>19)</sup>, it is suggested that the new ZUBECA4 primers for Japanese raccoon dogs are more suitable for the practical ZUBECA4 typing.

In conclusion, our findings indicated that the ZUBECA4 locus is a quite useful STR for discrimination between samples from Japanese raccoon dogs and samples from other animals in the field of forensic sciences. The multiplex typing of STR loci including the highly polymorphic ZUBECA4 would be also useful for the individual identification of Japanese raccoon dogs. In addition, since data on allele configuration in various animals contribute to assessing the stability of STR loci<sup>3-5)</sup>,

Table. 1. ZUBECA4 types and subtypes of nine Japanese raccoon dogs and their heterozygosities

Japanese raccoon dog	ZUBECA4	
	Types	Subtypes
RD1	14/15	14/15 <sup>15</sup>
RD2	14/16	14/16
RD3	14/15	14/15 <sup>12</sup>
RD4	12/15	12/15 <sup>12</sup>
RD5	15/18	15 <sup>12</sup> /18 <sup>14</sup>
RD6	15/18	15 <sup>12</sup> /18 <sup>13</sup>
RD7	12/13	12/13
RD8	13	13
RD9	15/18	15 <sup>14</sup> /18 <sup>14</sup>
Heterozygosity	0.79	0.86

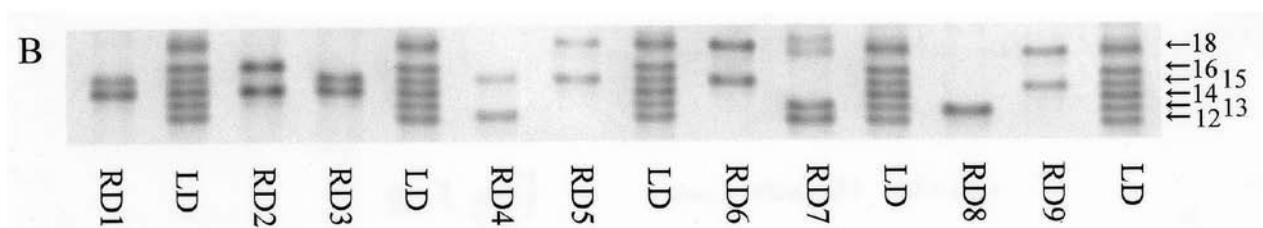
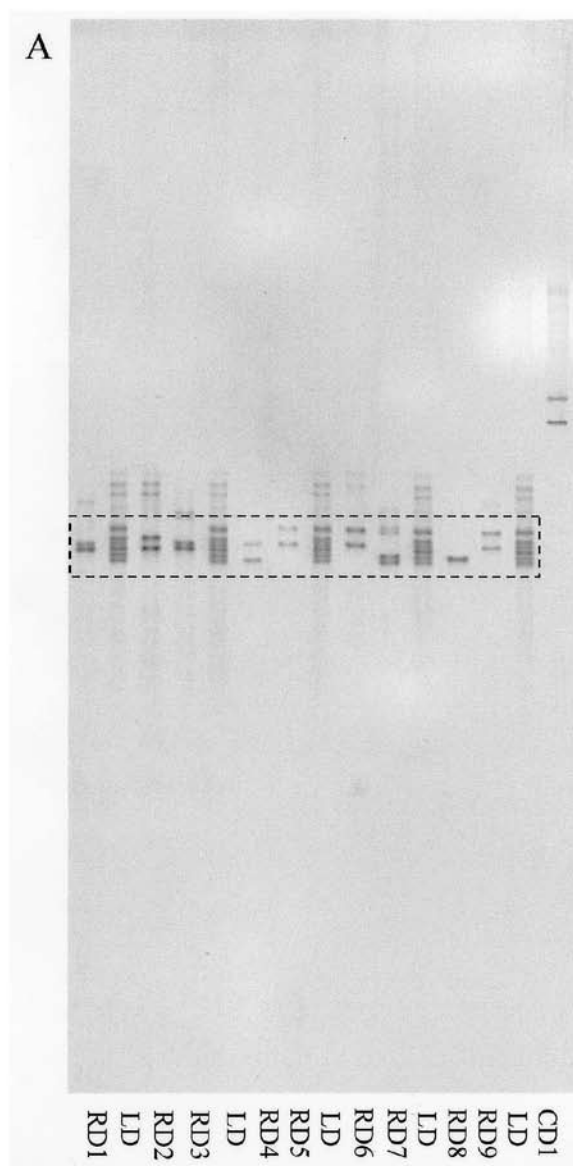


Fig. 3. Electrophoretograms of PCR products amplified with the prepared ZUBECA4 primers for Japanese raccoon dogs by electric focusing with unmodified polyacrylamide gels. A: overview of a focused gel. B: an enlargement of the region enclosed by a dashed line in Fig. 3A. RD1-RD9: Japanese raccoon dogs. LD: ladder markers. Cathode at top.

the present findings would be useful in a comparative study of canine animals. The ZUBECA4 locus can also inform pedigree study and population study on Japanese raccoon dogs because the information of Japanese raccoon dog STRs is currently very limited<sup>20)</sup>.

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## References

1. Butler JM. Genetics and genomics of core short tandem repeat loci used in human identity testing. *J Forensic Sci* 2006; 51: 253–265.
2. Butler JM. Forensic DNA typing. Biology, technology, and genetics of STR markers. 2nd ed. Amsterdam: Elsevier, 2005: 123–144.
3. Gusmao L, Gonzalez-Neira A, Alves C, Lareu M, Casta S, Amorim A, et al. Chimpanzee homologous of human Y specific STRs A comparative study and a proposal for nomenclature. *Forensic Sci Int* 2002; 126: 129–136.
4. Gomes I, Prinz M, Pereira R, Bieschke E, Mayr WR, Amorim A, et al. X-chromosome STR sequence variation, repeat structure, and nomenclature in humans and chimpanzees. *Int J Legal Med* 2009; 123: 143–149.
5. Gomes I, Pereira R, Mayr WR, Amorim A, Carracedo A, Gusmao L. Evaluation of DXS9902, DXS7132, DXS6809, DXS7133, and DXS7423 in humans and chimpanzees: sequence variation, repeat structure, and nomenclature. *Int J Legal Med* 2009; 123: 403–412.
6. Ago K, Ago M, Nakagawa S, Ogata M. Evaluation of the species specificity for six human short tandem repeat loci CAF1PO, TPOX, TH01, F13A01, FESFPS and vWA, in the Japanese macaque. *Leg Med* 2004; 6: 102–108.
7. Ago K, Ago M, Nakagawa S, Ogata M. Gender-specific amplification of Japanese macaque genes using primers for the DYS391 and DYS438 loci. *Leg Med* 2005; 7: 190–193.
8. Ago K, Ago M, Nakagawa S, Ogata M. Highly polymorphic STR marker amplified with human DYS389 primers in Japanese macaques (*Macaca fuscata*). *Am J Primatol* 2006; 68: 1025–1031.
9. Ago K, Ago M, Nakagawa S, Ogata M. Do A-stretches inhibit the emergence of new variants in STR loci? *Leg Med* 2009; 11: S446–S448.
10. Dolf G, Schläpfer J, Switonski M, Stranzinger G, Gaillard C, Schelling C. The highly polymorphic canine microsatellite ZuBeCa4 is localized on canine chromosome 3q15-q18. *Anim Genet* 1998; 29: 403–404.
11. Padar Z, Egyed B, Kontadakis K, Furedi S, Woller J, Zoldag L, et al. Canine STR analyses in forensic practice. Observation of a possible mutation in a dog hair. *Int J Legal Med* 2002; 116: 286–288.
12. Eichmann C, Berger B, Parson W. A proposed nomenclature for 15 dog-specific polymorphic STR loci for forensic purposes. *Int J Legal Med* 2004; 118: 249–266.
13. Eichmann C, Berger B, Reinhold M, Lutz M, Parson W. Canine-specific STR typing of saliva traces on dog bite wounds. *Int J Legal Med* 2004; 118: 337–342.
14. Eichmann C, Berger B, Steinlechner M, Parson W. Estimating the probability of identity in a random dog population using 15 highly polymorphic canine STR markers. *Forensic Sci Int* 2005; 151: 37–44.
15. Kanthaswamy S, Tom BK, Mattila AM, Johnston E, Dayton M, Kinaga J, et al. Canine population data generated from a multiplex STR kit for use in forensic casework. *J Forensic Sci* 2009; 54: 829–840.
16. Extraction of DNA. In: Budowle B, Smith J, Moretti T, DiZinno J, editors, DNA typing protocols: Molecular biology and forensic analysis, Natick, MA: Eaton Publishing, 2000; 13–43.
17. Bassam, BJ, Caetano-Anolles G., Gresshoff PM. Fast and sensitive silver staining of DNA in polyacrylamide gels. *Anal Biochem* 1991; 196: 80–83.
18. Nei M, Roychoudhury AK. Sampling variances of heterozygosity and genetic distance. *Genetics* 1974; 76: 379–390.
19. Hill CR, Kline MC, Cable MD, Butler JM. Characterization of 26 MiniSTR Loci for Improved Analysis of Degraded DNA Samples. *J Forensic Sci* 2008; 53: 73–80.
20. Matsuki R, Takeuchi T, Abe S, Nashimoto M. Microsatellite loci for raccoon dog. *DNA Polymorphism* 2006; 14: 188–192. (in Japanese)

## イヌZUBECA4プライマーによって増幅される ニホンタヌキマイクロサテライト座について

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### 和文要約

イヌZUBECA4プライマーにより増幅されたニホンタヌキPCR産物について, その性状を, ポリアクリルアミドゲル電気泳動分析ならびにダイレクトシーケンスによる塩基配列解析によって検討した. 電気泳動分析により, 9頭のニホンタヌキから分子量の異なる6種類のフラグメントが観察された. 塩基配列解析により, これらの変異はイヌと比べると単純なGAAAとGCAAの反復数の違いによることが明らかとなった. それぞれのアリルは繰り返し数の違いにより12-16, 18と名付けた. また, アリル15と18は, GAAAとGCAAそれぞれの繰り返し数に違いがある構造多型がみられ, GAAAの繰り返し数に基づいて $15^{12}$ ,  $15^{14}$ ,  $15^{15}$ ,  $18^{13}$ ,  $18^{14}$ とサブアリルに細分類できた. 各アリルの頻度は, 12:0.10, 13:0.17, 14:0.17,  $15^{13}$ :0.22,  $15^{14}$ :0.06,  $15^{15}$ :0.06, 16:0.06,  $18^{13}$ :0.06,  $18^{14}$ :0.10と推定された. ヘテロ接合度は, STRアリルの場合で0.79, 構造多型を含むサブアリルの場合では0.86と算出された. また, 今回明らかにしたニホンタヌキの塩基配列に基づき, アリルサイズが小さくなる実用的なニホンタヌキプライマーを作製することができた. これらの結果から, ニホンタヌキZUBECA4が, 種同定, 個体識別, 系統研究, 集団研究に利用可能であることが示された.