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RESEARCH ARTICLE

An examination of the origin and evolution of additional tandem repeats in the mitochondrial DNA control region of Japanese sika deer (*Cervus Nippon*)

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Abstract

Tandem repeat units are only detected in the left domain of the mitochondrial DNA control region in sika deer. Previous studies showed that Japanese sika deer have more tandem repeat units than its cousins from the Asian continent and Taiwan, which often have only three repeat units. To determine the origin and evolution of these additional repeat units in Japanese sika deer, we obtained the sequence of repeat units from an expanded dataset of the control region from all sika deer lineages. The functional constraint is inferred to act on the first repeat unit because this repeat has the least sequence divergence in comparison to the other units. Based on slipped-strand mispairing mechanisms, the illegitimate elongation model could account for the addition or deletion of these additional repeat units in the Japanese sika deer population. We also report that these additional repeat units could be occurring in the internal positions of tandem repeat regions, possibly via coupling with a homogenization mechanism within and among these lineages. Moreover, the increased number of repeat units in the Japanese sika deer population could reflect a balance between mutation and selection, as well as genetic drift.

Keywords

Cervus Nippon, control region, evolution, tandem repeats

History

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Introduction

The highly variable control region in mitochondrial DNA plays a key role in initiating replication of the whole circular molecule (Clayton, 1992). In mammals, the control region, which lies between the $tRNA^{phe}$ and $tRNA^{pro}$ genes, is divided into three domains: a central conserved domain and two flanked domains on the left and right, respectively. In addition to nucleotide base substitutions and small insertions and deletions, variation in number of repeat units in either of the two flanked domains can also be observed, which contribute to the variation in the length of control region. These repeat units can be identified according to the size of the units and their position within the control region (Hoelzel, 1993). This pervasive feature in the mitochondrial DNA control region has been observed in an ever-growing list of species, which includes bats (Wilkinson & Chapman, 1991), shrews (Stewart & Baker, 1994), cats (Lopez et al., 1996) and so on (Broughton & Dowling, 1997; Hoelzel et al., 1994; Lunt & Hyman, 1997; Purdue et al., 2006).

The analogous tandem repeat units have also been found in the left domain of control region of sika deer, an important livestock species for velvet antler production and are distributed throughout Northeast Asia, from the Ussuri region of Siberia to North Vietnam including the Korean peninsula, Mainland China, Taiwan and Japanese islands (Figure 1) (Cook et al., 1999; Randi et al., 2001). The size of repeat units varies from 37 to 40 bp and the number of units from three to seven in the tandem repeat region (Nagata et al., 1999; Randi et al., 2001). The number of repeat units can serve as an indicator for distinguishing different sika deer populations, i.e. the sika deer in Asian continent/Taiwan often have three repeat units, but the Japanese sika deer population have more than three repeat units. Information from the available fossil records and molecular data indicate that sika deer could have colonized the Japanese islands from the Asian continent in the late Pleistocene (Ba et al., 2013; Kawamura, 1991). The evolution of additional repeat units in the Japanese sika deer population could have occurred independently since the late Pleistocene (Nagata et al., 1999; Randi et al., 2001).

A number of molecular mechanisms have been proposed to account for this variation in repeat units and, among these mechanisms, the most popular one is the slipped-strand mispairing (Broughton & Dowling, 1994; Buroker et al., 1990; Mundy & Helbig, 2004). However, the details of how these additional repeat units were generated in the Japanese sika deer population are poorly understood. Likewise, only limited research has been conducted to determine why these additional repeat units occur in the Japanese sika deer population, but not in the others. In the present study, we obtained the repeat units from an expanded dataset of the mitochondrial control region sequences from public domain databases. Our primary objective was to explore the mode of origin and evolution of these additional repeat units in the Japanese sika deer population by estimating the variation in the pattern of repeat units.

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Materials and methods

All control region sequences of sika deer were obtained from NCBI GeneBank ((2013). 05) by using the following key words: ''Cervus nippon'', ''control region'' and ''D-loop''. Based on the definition of tandem repeat region in sika deer (Randi et al., 2001), the incomplete control region sequences (i.e. only partially cover the whole tandem repeat region) obtained from the database were discarded, because the exact physical positions of the repeat units in the incomplete tandem repeat region were uncertain. The repeat units were obtained manually from the resultant high-quality control sequences.

As the origin and evolution of repeat units in sika deer could have occurred independently (Nagata et al., 1999; Randi et al., 2001), we considered each repeat unit as an independent operationally taxonomic unit and examined the degrees of divergence between them. In order to understand the evolution process of the additional repeat units in the Japanese sika deer population, we classified the population into five lineages based on the number of repeat units contained in the tandem repeat region (from three to seven repeat units). The repeat units were numbered from the 3′ end to the 5′ end based on the physical positions of H-strand of mitochondrial DNA (Figure 1).

All repeat units were aligned using ClustalX v1.83 software (Larkin et al., 2007). The pair-wise genetic divergences of each repeat unit within the five lineages were calculated based on Tajima-Nei model (Tamura & Nei, 1993) using the complete deletion option from MEGA v5.0 software (Tamura et al., 2011). Variance of genetic divergences was also estimated using 1000 bootstrap replicates. All haplotypes of repeat units were produced using the DnaSP v5 software (Librado & Rozas, 2009). In order to exclude potential sequencing errors in the repeat units obtained from NCBI, the haplotypes that had a frequency higher than 1 were used to construct a median-joining network using Network v4.6 software (Bandelt et al., 1999) with default options and all sites equally weighted. The most stable secondary hairpin structures of repeat units and the corresponding minimal folding energies were determined using RNA structure v3.7 software (Mathews et al., 1999).

Results and discussion

A total of 243 high-quality control region sequences were obtained. The number of repeat units in each sequence varies from three to seven. In total, 1023 repeat units were produced from these sequences.

If substitution rates are equal along each repeat unit and functional constraints are absent, then each repeat unit should diverge at equal rates. However, the pair-wise genetic divergences of each repeat unit show greater divergence varying from 0.00% to 13.64% among the five lineages. Within each lineage, the first repeat unit appears to be more conserved than the rest of repeat units (Figure 2A). This suggests that physical positions of repeat units influence the rate of sequence divergence. The functional constraints seem to act on the first repeat unit, and the constraints on internal repeat units are relaxed. The phenomenon of reduced variation on the edges of tandem repeat region was termed the ''edge-effect'' (Rand, 1993), which was also evident at the 3′ end of DNA in sika deer. The first repeat units may be the most important functional units in sika deer. Through further comparison in the pair-wise divergences between the two neighbor repeat units in each lineage, the ratio of the last two repeat units at the 5′ end (number four, five, six or seven depending on the size of the tandem array) is significantly higher than those of the other comparisons (Figure 2B). This result implies that the addition or deletion of additional repeat units is unlikely to occur at the 5′

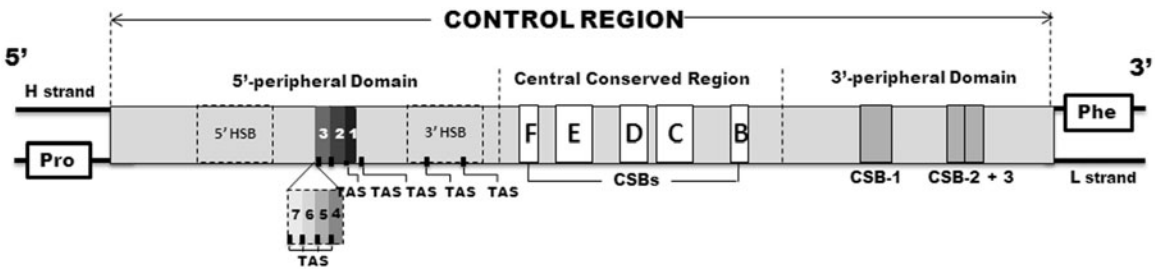

Figure 1. General structure of the mitochondrial control region (D-loop) in sika deer. The 3′-peripheral domain contains conserved sequence blocks (CSBs). Letters F to B denote conserved sequence in the central conserved region [from Douzery & Randi (1997)]. The 5′ HSB and 3′ HSB represent 5′ and 3′ hypervariable sequence blocks, respectively [from Randi et al. (2001)]. The 5′-peripheral domain contains tandem array represented by seven solid rectangles with gray levels. Each repeat sequence varies from 37 to 40 bp in length and contains a termination association sequence (TAS) element [from Randi et al. (2001)]. Japanese sika deer population has four to seven tandem repeat units.

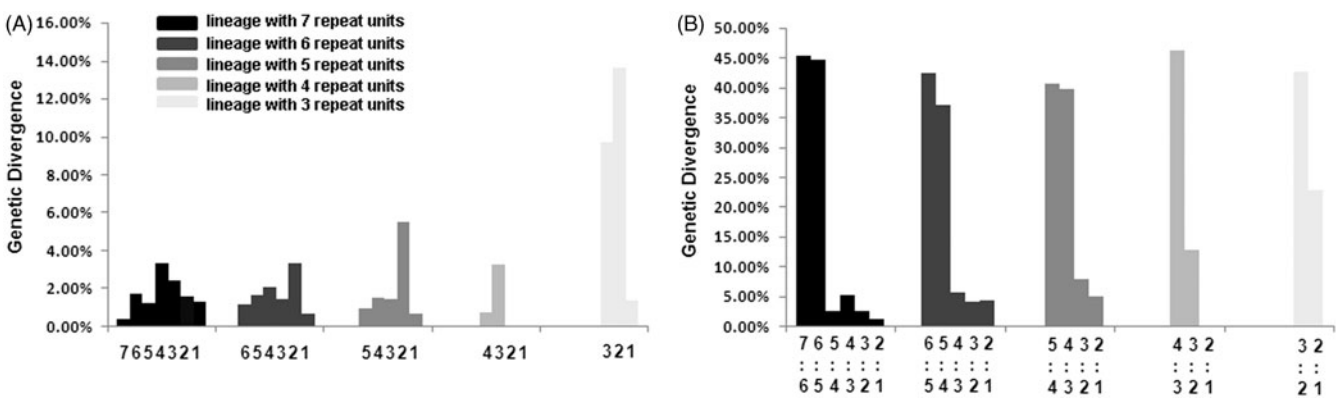

Figure 2. Estimation of pair-wise genetic divergences based on the five sika deer lineages, which contain three to seven repeat units. (A) Within lineages. (B) Between adjacent repeat units within lineages.

end of a tandem repeat region. This finding is plausible given that the evolution of additional repeat units in the Japanese sika deer population could have occurred independently since the divergence between species in the late Pleistocene (Nagata et al., 1999; Randi et al., 2001).

In total, 52 haplotypes were generated from the 1023 repeat units based on the 41 polymorphic sites (Table 1). Of these haplotypes, 30 had frequency more than 1 and were used to construct a median-joining network. Based on that network, three main clusters were identified (Figure 3A). Cluster A consisted of all repeat units except for the last two at the 5′ end; whereas Clusters B and C comprised the last two units only at the 5′ end, respectively. Within Cluster A, the two haplotypes with the highest frequency, H_16 ($n = 216$) and H_35 ($n = 141$), were derived from the first units of all five lineages, and account for 66.48% in total. The star-shaped node surrounding these two haplotypes implies that the first units should be the most ancestral units, from which the other units are derived, and the haplotypes H_16 and H_35 are likely the two most ancestral haplotypes. Within the haplotype network, the phenomenon that the two haplotypes H_16 and H_35 in Cluster A being flunked by the two independent Cluster B and Cluster C suggests that the last two units at the 5′ end could be derived from the first units. We also observed that not only haplotype H_50, located between haplotype H_33 in Cluster B and haplotype H_16 in Cluster A, were present at a low frequency; but also mv3, mv4 and mv5, located between mv2 in Cluster C and haplotype H_35 in Cluster A, were absent from the dataset. This suggests that the duplication

Table 1. List of 52 haplotypes from 1023 repeat units.

Haplotype	Lineage with 3 repeat units			Lineage with 4 repeat units				Lineage with 5 repeat units					Lineage with 6 repeat units						Lineage with 7 repeat units								
Repeat unit position	3	2	1	4	3	2	1	5	4	3	2	1	6	5	4	3	2	1	7	6	5	4	3	2	1	Total	Cluster in Figure 3
Hap_1								25					43						15							83	B
Hap_2																			1							1	–
Hap_3													11													11	B
Hap_4													1													1	–
Hap_5																				1						1	–
Hap_6									3					12						11						26	C
Hap_7									21					40						4						65	C
Hap_8										9					33						1					43	A
Hap_9								1																		1	–
Hap_10								2																		2	B
Hap_11										19					17	2					14	10				62	A
Hap_12																					1					1	–
Hap_13									3					1												4	C
Hap_14														1												1	–
Hap_15														1												1	–
Hap_16			36								10	25				45	16	49				3	4	13	15	216	A
Hap_17				1																						1	–
Hap_18	59			8																						67	B
Hap_19															1											1	–
Hap_20												1				1	2	3				2		1		10	A
Hap_21																						1				1	–
Hap_22															4											4	A
Hap_23									1																	1	–
Hap_24			1								1					3	30							1		36	A
Hap_25	31																									31	B
Hap_26	1																									1	–
Hap_27	18																									18	B
Hap_28	1																									1	–
Hap_29	14																									14	B
Hap_30	6																									6	B
Hap_31	2																									2	B
Hap_32	1																									1	–
Hap_33	2																									2	B
Hap_34																		1					2			3	A
Hap_35			97			9	9				5					4	6						10	1		141	A
Hap_36		26			1																					27	C
Hap_37					1																					1	–
Hap_38		15			3																					18	C
Hap_39		19			4																					23	C
Hap_40											11															11	A
Hap_41																	1									1	–
Hap_42											1															1	–
Hap_43		2																								2	C
Hap_44		33																								33	C
Hap_45		1																								1	–
Hap_46		1																								1	–
Hap_47		7																								7	C
Hap_48		31																								31	C
Hap_49																									1	1	–
Hap_50												2						1								3	A
Hap_51			1																							1	–
Hap_52																		1								1	–
Total	135	135	135	9	9	9	9	28	28	28	28	28	55	55	55	55	55	55	16	16	16	16	16	16	16	1023	

of the last two units at the 5′ end would have undergone a stochastic population process, e.g. a bottleneck effect, when they were derived from the first repeats. Furthermore, the last two units could be orthologous among all these five lineages.

Various slipped-strand mispairing mechanisms have been proposed to explain the addition or deletion of tandem repeat units (Broughton & Dowling, 1994; Buroker et al., 1990; Mundy & Helbig, 2004). A common feature of these mechanisms is the potential to form single-stranded hairpin structures in the tandem repeat units which may promote mispairing. Such a slipped-strand mispairing may have caused the addition or deletion of tandem repeat units. Therefore, we investigated the potential of forming hairpin structures in the tandem repeat region of sika deer. The average free energies of the repeat units in Cluster A (-4.03 ± 1.03 kcal/mol) was lower than those in both Cluster B (-2.40 ± 1.01 kcal/mol) and Cluster C (-2.39 ± 0.94 kcal/mol). A Mann–Whitney *U*-test indicates that there were significant differences in free energy between the repeat units within Cluster A and those within Cluster B and Cluster C, respectively ($p < 0.0001$). In contrast, no significant difference was detected between Cluster B and Cluster C ($p = 0.873$). It is obvious to note that the repeat units within Cluster A would have a greater tendency to form hairpin structures relative to those within Cluster B and Cluster C. Once two or more repeat units are present, mutations leading to extend the number and variation in repeat units may occur at a significantly higher rate (Arnason & Rand, 1992; Broughton & Dowling, 1994; Brown et al., 1992). This is not surprising as we also observed multiple adjacent repeat units from our data (data not shown) that have capability to create internal folds, which generates more stable hairpin structures with an increasing number of repeat units. It might be expected that formation of the specific hairpin structures of the additional repeat units may facilitate the addition or deletion of these repeat units in the Japanese sika deer population.

Based on the mechanism of slipped-strand mispairing, Buroker et al. (1990) proposed that an illegitimate elongation model could explain the origin and evolution of repeat units because of the existence of a three-stranded structure, known as a D-loop. Among these three strands, one strand of the nascent double helix can presumably slip easily and fold into the proposed secondary structure. The addition or deletion of repeat units could occur depending on a misalignment after a strand slip. Generally, this model predicts that the minimum number of units would be three and the internal repeat units should be perfectly conserved (Buroker et al., 1990). In sika deer, three or more repeat units have been observed, and the additional units in the internal

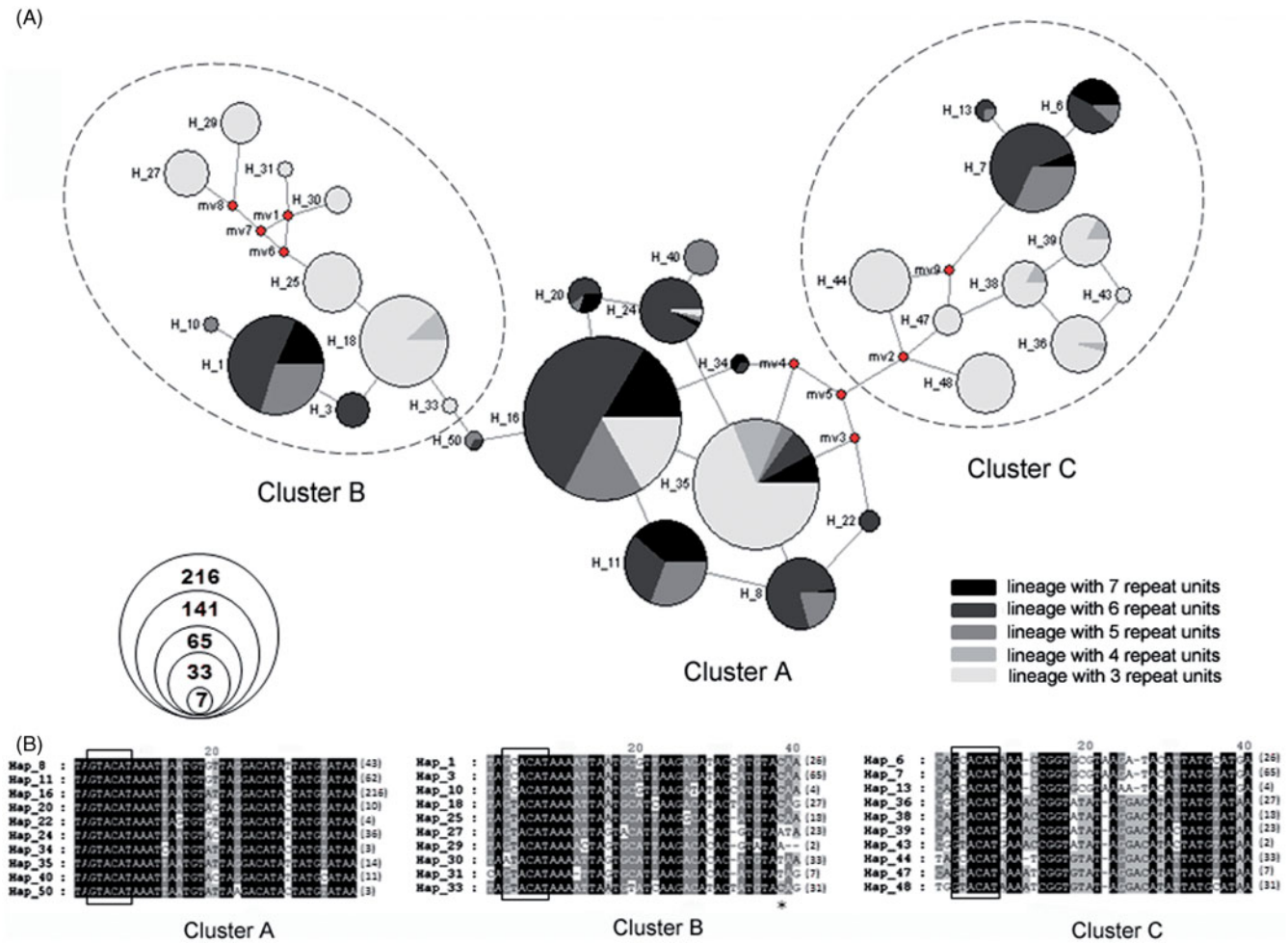

Figure 3. (A) Haplotype network comprising 30 common haplotypes (frequence >1) produced from 1001 repeat units, which locate in an expanded control region of sika deer. Three clusters are revealed in the network. Cluster A consists of the repeat units except the last two on the 5′ end, whereas Cluster B and C only comprise the last two repeat units on the 5′ end, respectively. Five gray levels correspond to five lineages that contain three to seven repeat units. Size of circles is proportional to the number of repeat units sharing a given haplotype, and each haplotype pie shows the percentage of repeat units that derive from different lineages. (B) Alignment of the haplotypes within three clusters, including invariable bases and gaps. TAS elements are framed. Asterisk indicates a single nucleotide substitution of transversion. The numbers in brackets indicate the frequence of haplotypes.

positions of the tandem repeat region are also highly conserved. It seems that the illegitimate elongation model is able to explain the origin and evolution of additional repeat units in the Japanese sika deer population.

The illegitimate elongation model can be adopted to explain the evolution of the tandem repeat units only when the termination-associated sequences (TAS) in each repeat unit are present (Buroker et al., 1990). These TAS sequences are extremely important in forming stable hairpin structures for the binding of a regulatory termination protein during the replication of mitochondrial DNA. Likewise, the apparently conserved elements (Figure 3B) in each repeat unit are similar to the sequences identified as TAS elements (Douzery & Randi, 1997; Randi et al., 2001; Wilkinson & Chapman, 1991), and particularly those that are present in the repeat units within Cluster A. Multiple conserved TAS elements in the Japanese sika deer population could provide redundant binding that would extend the tandem repeat region when binding is hampered by a point mutation (Buroker et al., 1990).

Analysis of the pattern of mutations in repeat units has revealed a phenomenon that is called concerted evolution (Broughton & Dowling, 1994; Fumagalli et al., 1996; Wilkinson & Chapman, 1991). The concerted evolution process may be homogenizing the repeat units by reiterated cycles of addition or deletion of repeat units (Tatarenkov & Avise, 2007). Within Cluster A in the haplotype network, the majority of haplotypes (80%, 8/10) are shared among the first and internal repeat units within the five lineages. However, within both Cluster B and C, smaller apparent subdivisions were observed between the repeat units. The mean sequence divergence calculated between repeat units within Cluster A is $1.70\% \pm 0.82\%$, which is much lower than those within Cluster B ($2.88\% \pm 0.87\%$) and Cluster C ($4.24\% \pm 1.46\%$). No small insertions or deletions were observed in the alignment of repeat units within Cluster A, except for a few within Clusters B and C, and all the base substitutions are transitional except for one being a transversion in haplotypes H_27 and H_29 (Figure 3B). These results suggest that there is remarkably less sequence variation among the repeat units in Cluster A, which contrasts with the divergence among the repeat units in Cluster B and Cluster C. This observation is consistent with the predictions of concerted evolution of repeat units occurring in other species (Broughton & Dowling, 1994; Fumagalli et al., 1996; Wilkinson & Chapman, 1991). Thus, we suggest that the additional repeat units within the internal positions of the tandem repeat region in the Japanese sika deer population could have undergone a concerted evolution processes within the lineages.

It is likely that the number of repeat units occurred in the left domain of the control region in mammals is the result of a balance between mutation and selection (Wilkinson et al., 1997). The Japanese sika deer population with the additional repeat units in the control region sequence is apparently more susceptible to the mutational events than other sika deer populations in the Asian continent and Taiwan. However, the number of repeat units is never higher than seven in the Japanese sika deer population, which may have a selective advantage around an optimal value, although exactly how this selection has taken place is unclear. Smaller mitochondrial genomes may have replication fitness advantages at the cellular level (Rand, 1993). Alternatively, the mutational events that produce the additional repeat units may be due to stochastic population processes, e.g. genetic drift. As the biogeographic boundaries emerged, such as the straits separating Japan from the Asian continent, which confined sika deer populations to each island, genetic drift would have resulted in the fixation of the additional repeat units in the colonized sika deer population in Japanese islands. This kind of spatial pattern of occurrence for the additional repeat units has also been observed in the white-tailed deer population native to the southeastern United States (Purdue et al., 2006).

Overall, the additional repeat units in the Japanese sika deer population could have occurred in the internal positions of the tandem repeat region, possibly coupling with a homogenization mechanism within the lineages. The mechanism underlying the generation of these additional repeat units can be explained by the illegitimate elongation model. Moreover, the increased number of repeat units in the Japanese sika deer population could reflect a balance between mutation and selection, as well as genetic drift.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper. We would also like to thank National 863 Program of China (No. 2011AA100603), and Science and Technology Commission, Jilin (No. 201262514) for providing funds for this research.

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