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Evolutionary Dynamics of Copy Number and Meiotic Recombination in Murine 5S rDNA: Possible Involvement of Natural Selection

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Running head: Evolution of mouse 5S rDNA

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Abstract

We investigated evolutionary trends of the 5S ribosomal RNA gene in the house mouse, *Mus musculus*. First, we assessed the 5S cluster and copy numbers in eight laboratory strains by pulsed-field gel electrophoresis. The copy numbers in seven lines were estimated to be around 130–170 copies per cluster, with 63 copies in the remaining strain, implying that the copy number can change drastically and has been maintained under certain evolutionary constraints at ~140 copies. Second, we addressed the frequency of meiotic recombination mediated by the 5S cluster by performing a mating experiment with laboratory strains, and found that the 5S cluster did not accelerate recombination events. Third, we surveyed recombination events of the 5S-containing chromosome region in wild mice from the Japanese Islands, where the two subspecies lineages, *M. m. castaneus* and *M. m. musculus*, are historically mingled, and found that the influence of the 5S cluster on meiotic recombination was limited. Finally, we examined the nucleotide diversity of six genes in the neighboring regions of the 5S cluster and found reduced genetic diversity in the regions on both sides of the cluster, suggesting the involvement of either positive or background selection in the population-level sequence similarity of the 5S clusters. Therefore, the mouse 5S genes are considered to be evolving toward sequence similarity within a given cluster by certain intrachromosomal mechanisms and toward sharing of a specific 5S cluster within a population by certain selective processes.

Introduction

Ribosomal RNA genes (rDNA) are a specific class of multigene families engaged in generating products of a single function, and exhibit peculiar evolutionary patterns (Ohta 1980). In mammals, the two types of rDNA encode small (5S) and large (18S-5.8S-28S) rRNAs, and flanking spacer regions comprising units of 1–2 and 40 kb, respectively. The copies are tandemly repeated, head to tail, and generate clusters in the genome (Yu and Lemos 2016). While both types of rDNA exhibit substantial levels of copy number variation within and among species, the copy numbers are predicted to be maintained by natural selection at optimal dosages (Gibbons et al, 2015). The coding regions are highly conserved among vertebrate lineages, but the spacer sequences evolve rapidly (e.g. Suzuki et al. 1994a, b; 1996). Nevertheless, repeating units including the spacer regions display uniformity within and among rDNA clusters and even within natural populations, implying the existence of mechanisms that shape the sequence similarity (Nagylaki 1984; Nagylaki and Petes 1982).

The greater level of sequence uniformity between reiterated copies has two explanations. One explanation presumes homogenization events, which are expected to drive the homogenous state within genomes and populations by recurrent exchanges of genetic elements among the repeating units (Ganley and Kobayashi 2007; Stage and Eickbush 2007). In homogenization events, the unequal crossover between rDNA clusters and gene conversions, within and between clusters, is the most plausible mechanism that facilitates the exchange of genetic information among repeating units (Smith 1976). Another explanation assumes the repeated occurrence of gene duplication and deletion within clusters in organisms such as fungi (Rooney and Ward 2005), shells (Vierna et al. 2009; Vizoso et al. 2011), fish (Fujiwara et al. 2009; Pinhal et al. 2011), and yeasts (Ganley and Kobayashi 2011). Since this scenario only covers the evolutionary mode within a cluster, an additional system is needed to interpret sequence similarity at the population level.

Among mammalian species, for which evolutionary patterns of the 5S genes are less extensively studied (Sørensen and Frederiksen 1991; Stults et al. 2008), the house mouse (*Mus musculus*) is an ideal subject to better understand these evolutionary dynamics. The sequence of the repeating units of 5S rDNA has been identified in several inbred strains of mice (Suzuki et al. 1994a). Based on fluorescence in situ hybridization (FISH) analyses of mice (Matsuda et al. 1994) and rats (Suzuki et al. 1996), and genomic analysis of humans

(Gaubatz et al. 1976; Little and Braaten 1989; Stults et al. 2008), the 5S rDNA is postulated to be present as a single cluster within a haploid genome. The copy numbers of the 5S clusters were 16–210 according to genome-wide sequence data (Gibbons et al. 2015). The length of a single copy is ~ 1–2 kb (Suzuki et al. 1994a; 1996), compared with larger genes (18S-5.8S-28S rDNA), offering a chance to assess the copy number per haploid genome by pulsed-field gel electrophoresis, which allows measurement of >1,000 kb fragments (Stults et al. 2008).

Mus musculus is frequently used as a model organism; its complete genome sequence is available (e.g., Takada et al. 2013, 2015), and it carries a large number of inbred strains. Genetic surveys of natural populations of *M. musculus* have indicated the presence of three major subspecies groups: the west European subspecies group of *M. m. domesticus* (DOM), the Southeast Asian subspecies group of *M. m. castaneus* (CAS) and the northern Eurasian subspecies group of *M. m. musculus* (MUS). Genetic studies have predicted their possible homelands, ranging from the Middle East to the Indian subcontinent, and their extensive dispersal with prehistoric human movements along multiple routes resulted in several sites of secondary contact, as is seen in the Japanese Islands (e.g., Boursot et al. 1993; Kodama et al. 2015; Kuwayama et al. 2017; Nunome et al. 2010; Suzuki et al. 2013). Substantial levels of genetic diversity in the nuclear gene have been documented in *M. musculus* (Kodama et al. 2015; Nunome et al. 2010), which enable detection of signs of the acceleration of 5S cluster-mediated meiotic recombination and reduction of genetic variation (e.g., selective sweep), indicative of the involvement of natural selection, by monitoring of natural populations.

In this study, we conducted pulsed-field gel electrophoretic analysis of several inbred mouse strains to assess copy number variability. We next addressed the issue of meiotic recombination acceleration at the 5S cluster. We explored mouse crossing, using nearby microsatellite markers to monitor recombination. We also conducted a genetic survey of wild mice from the Japanese Islands, where two different subspecies groups have mingled (Nunome et al. 2010), to measure the relative rate of recombination between the 5S clusters that originated from CAS and MUS. Finally, we performed an experiment to capture the signature of natural selection at the 5S cluster, using specimens of wild-derived mice of the three subspecies groups (Kodama et al. 2015; Kuwayama et al. 2017; Nunome et al. 2010; Suzuki et al. 2013) and whole genome sequences (Takada et

al. 2013; 2015). Our current efforts would contribute to better understanding of the evolutionary dynamics of the 5S rDNA of *M. musculus*.

Materials and Methods

Materials

The laboratory-inbred strains AKR/J, BALB/cA, BALB/cBy, CBA/J, C3H/HeJ, C57BL/6J and DBA/2J obtained from the National Institute of Genetics, Mishima, Japan, and SM/J from Hamamatsu University School of Medicine, Japan, were used to assess the copy number of the 5S cluster (Table 1). The strains SM/J, MOA (Hamamatsu University School of Medicine; strain originated from wild mice captured in Anjo, Japan) and BALB/cUcsd (the National Institute of Genetics) were used in a genetic crossing experiment. We examined one inbred strain (MSM/Ms) and 51 wild-captured mice, of which 22 were from Japan and 30 from outside Japan. Most of these mice were genetically characterized previously using mitochondrial and nuclear gene sequences (Kodama et al. 2015; Kuwayama et al. 2017; Nunome et al. 2010; Suzuki et al. 2013). These samples were collected in China, India, Russia and other countries, on expeditions organized by Dr. K. Moriwaki during 1987–2003 (MG series, stored in the National Institute of Genetics, Japan and BRC series stored in RIKEN BioResource Center, Japan), and Drs. H. Ikeda and K. Tsuchiya during 1989–1992 (HI series, stored at Hokkaido University). We also used DNA samples stored at Hokkaido University (HS series).

Pulsed-field gel electrophoresis

Agarose blocks for pulsed-field gel electrophoresis were prepared according to a method described by Katakura et al. (1993). Freshly isolated thymus lymphocytes were warmed to 37°C at a concentration of 1×10^7 cells/ml in Hank's balanced salt solution (HBSS; Sigma, St. Louis, MO, USA), and mixed with an equal volume of 1% agarose with a low gelling temperature (Sea Plaque; FMC Bioproducts, Rockland, ME, USA) in 75 mM phosphate buffer, pH 8.0, containing 65 mM NaCl and 1% glucose. Forty microliters of the mixture containing 2×10^5 cells were solidified into $5 \times 5 \times 1.5$ -mm blocks. The agarose blocks were treated with 1% sarcosyl

(Sigma) and 2 mg/ml proteinase K (Sigma) in 0.5 M ethylenediaminetetraacetic acid (EDTA), pH 8.0, at 50°C for 2 days. Samples were washed with 1× TBE (90 mM Tris, 90 mM boric acid and 2 mM EDTA, pH 8.0) and stored in 0.5 M EDTA at 4°C until used. Total DNA embedded in the agarose gel was washed with 10 volumes of each restriction enzyme buffer at room temperature for 30 min and digested with each restriction enzyme at 37°C overnight; enzymes were chosen according to the sequences of the 5S rDNA repeating units (Suzuki et al. 1994a). We used a lambda DNA ladder (FMC Bioproducts) and lambda *Hind*III-digested DNA (TaKaRa, Kyoto, Japan) as size markers. Electrophoresis was performed in 1.5% agarose at 180 V in 0.5× TBE at 12°C with a pulse interval of 10 s for 23 h using a turn-table type pulsed-field gel electrophoresis apparatus (Cross Field Gel Electrophoresis; ATTO Corp., Tokyo, Japan). This apparatus was designed to rotate a 20-cm circular gel plate at an angle of 110° alternately with a defined pulse time. After electrophoresis, the gel was stained with 0.1 µg/ml ethidium bromide in 0.5× TBE for 1 h and photographed. Southern blot analysis was performed as described previously, using a ³²P-labeled probe for the fragment containing the 5S rRNA gene (Suzuki et al. 1994a).

We calculated 5S rDNA copy numbers based on the resultant fragments from the pulsed-field gel electrophoresis and the known lengths of cloned 5S repeating units (Suzuki et al. 1994a). The lengths of the spacers for BALB/cCrSlc, which varied between repeating units due to indels and two cloned 5S units (the total spacer plus 37-bp coding regions), have been reported as 1,611 (accession number: D14832) and 1,631 (D14833) bp. We therefore tentatively set the total repeating unit length as 1,700 bp, considering the length of the remaining coding region (84 bp).

Detection of recombination by breeding experiments and wild population survey

Recombination events associated with the 5S cluster were assessed using inbred strains from the subspecies groups DOM (BALB/cUcsd, SM/J) and MUS (MOA). A total of 101 mice, consisting of 40 [(BALB/c X MOA) F1 X BALB/c], 13 [(BALB/c X MOA) F1 X MOA] and 48 [(BALB/c X MOA) F1 X SM/J] animals, were analyzed to determine the recombination rate acceleration mediated by the 5S cluster at meiosis. Three simple sequence length polymorphism (SSLP) markers (MIT microsatellites) that localized near the 5S cluster

(D8MIT12, D8MIT13 and D8MIT42) were used for genotyping. Genotyping was performed by PCR, followed by electrophoresis in a 3% agarose gel (Nusieve agarose 3:1). Information about marker positions was obtained from the NCBI website (GRCm38.p8). The subspecies types of the 5S clusters were assessed by Southern blot analysis with the ³²P-labeled 5S probe mentioned above, based on the presence or absence of the 68-bp duplication, using the restriction enzyme *Pst*I, which cuts once in the 5S repeating unit (Suzuki et al. 1994a).

Genetic analyses of the 5S rDNA cluster and its flanking gene regions

We examined sequence variation in six gene regions flanking the 5S cluster in 52 wild mice, including 22 from Japan and 30 from outside Japan, using PCR primers (Supplementary Table S1). Four species—*M. caroli* (HS598), *M. terricolor* (HS1469), *M. macedonicus* (HS537) and *M. spretus* (inbred line, SEG)—were used as outgroups. We analyzed the gene regions *Afg3l1* (482 bp), *Dbn1d1* (555 bp), *Gas8* (549 bp), *Gm19600* (554 bp), *Rhou* (414 bp) and *6030466F02Rik* (518 bp). Most of the sequence data from the gene regions of *Afg3l1* and *Dbn1d1* were obtained from previous studies (Kodama et al. 2015; Nunome et al. 2010). We designed primers using the Ensemble Mouse Genome Database (<http://www.ensembl.org/>). PCR was performed using the AmpliTaq Gold 360 Master Mix Kit DNA (Thermo Fisher Scientific, Inc., Waltham, MA, USA), with the conditions presented in Table S1. PCR products were sequenced according to the manufacturer's instructions using a BigDye Terminator v3.1 Cycle Sequencing Kit and an ABI 3130 automated sequencer (Thermo Fisher Scientific, Inc.) with two PCR primers. Sequence alignment was performed using the MEGA5.2 program (Tamura et al. 2011). Sequences with more than two heterozygous sites were separated into two haplotypes according to PHASE version 2.1 software (Stephens et al. 2001; Stephens and Scheet 2005). Sequences determined in this study were deposited in the DDBJ/EMBL/GenBank databases under the accession numbers LC318299–LC318371.

A phylogenetic network was constructed using the neighbor-net method, implemented in Splitstree4 software, version 4.12.8 (Huson and Bryant 2006), to assign alleles to subspecies groups. Following the network analyses, we assigned alleles to subspecies groups (*c*: CAS, *d*: DOM, *m*: MUS) according to previously

described criteria (Kuwayama et al. 2017; Nunome et al. 2010). Some alleles could not be assigned due to sharing more than one subspecies group (*u*: unknown).

Haplotype patterns demonstrated the presence or absence of recombination (breakpoint) between haplotypes representing the MUS and CAS mouse subspecies groups, under the assumption that hybridization between the two subspecies groups occurred over a substantial period of time (*e.g.*, 700–800 generations; Kuwayama et al. 2017; Nunome et al. 2010; see Supplementary Material, Fig. S1). The relative rates of meiotic recombination were measured by counting the breakpoints and distances (kb) between gene markers.

The six nuclear gene sequences determined for mice from outside Japan were taken to represent the three major subspecies groups of *M. musculus*, namely MUS (*n* = 14), CAS (*n* = 10) and DOM (*n* = 6). Nucleotide diversities (π) were analyzed using Arlequin 3.5 (Excoffier and Lischer 2010). Selective neutrality of these nuclear gene loci was tested by Tajima's *D* test and Fu's *F_s* test with 10,000 coalescent simulations, as implemented in Arlequin 3.5.

The Hudson–Kreitman–Aguadé test (HKA; Hudson et al. 1987) was performed using DnaSP (Librado and Rozas 2009). We focused on the two subspecies groups MUS (five genes, excluding *Rhou* due to marked interruption by introgressed CAS alleles) and CAS (six genes). We divided the genes into two groups of neighboring (*Gas8*, *Gm19600*, *Rhou*) and distant (*Afg311*, *Dbndd1*, *6030466F02Rik*) genes and performed the test.

Genome sequences covering the six gene regions flanking the 5S cluster were obtained from the NIG_MoG2 Mouse Genome Database (<http://molossinus.lab.nig.ac.jp/mog2/>) (Takada et al. 2013, 2015). The π values were calculated in 10-kb intervals across both flanking regions using the MEGA5.2 program with the Tamura and Nei substitution model (Tamura et al. 2011).

Sequence analysis of the 5S cluster

We amplified fragments of the spacer regions in the repeating units within the 5S cluster, using primers (5S-F, 5S-R; Supplementary Material, Table S1) that recognized both ends of the coding region. We performed PCR and direct sequencing using the conditions shown in Table S1. In this study, we focused only on the 5' distal

part of the spacer region (174 bp) because the remaining downstream region of the spacer was prone to indel mutations and did not yield good sequences.

Results

5S rDNA cluster copy number

To assess the copy number of the 5S rDNA cluster, a pulsed-field gel electrophoretic analysis was performed on the eight inbred mouse strains (Table 1). We used the restriction enzyme *Bgl*III to cleave an entire 5S rDNA cluster because the restriction site was predicted to be absent in the repeating units according to a sequence analyses of cloned spacer regions of the mouse 5S rDNA (Suzuki et al. 1994a). Southern blot hybridization analysis confirmed that each of the eight mouse strains produced single bands (AKR/J, 250 kb; BALB/cBy, 115 kb; CBA/J, 225 kb; C3H/HeJ, 225 kb; C57BL/6J, 240 kb; DBA/2J, 240 kb; SM/J 243 kb), with the exception of a single strain, BALB/cA, which had two bands (250 kb and 290 kb; Fig. 1A). Considering that the *Bgl*III restriction site is present approximately once per 4,096 (4^6) bp, the proximate lengths of the 5S rDNA cluster for AKR/J, BALB/cA, BALB/cBy, CBA/J, C3H/HeJ, C57BL/6J, DBA/2J and SM/J were 242 (*i.e.*, $250 - 8 = 242$), 282 (242), 107, 217, 217, 232, 232 and 235 kb, respectively. Given that a single repeating unit is 1,700 bp (Suzuki et al. 1994a), the average copy number was calculated to be 137 repeats, ranging from 63 (BALB/cBy) to 165 (BALB/cA) (Table 1).

Because the copy number calculated for BALB/cBy was low, it is possible that new *Bgl*III sites emerged and substantially shortened the 5S. We performed a pulsed-field gel analysis of the BALB/cBy strain using four other restriction enzymes (*Eco*RI, *Bam*HI, *Hind*III and *Pvu*II), which were predicted to be absent in the 5S repeating units (Suzuki et al. 1994a), in addition to *Bgl*III (Experiment 2 in Table 1). Restriction fragments of similar lengths were observed among the five enzymes (Fig. 1B), with slight variation from 125 kb (*Eco*RI, *Bam*HI, *Hind*III) to 120 kb (*Pvu*II, *Bgl*III). This experiment provided robust evidence for copy numbers of 63–69 in the 5S cluster of the BALB/cBy strain (Table 1).

Assessing meiotic recombination in the 5S cluster

To assess the efficiency of the meiotic recombination associated with the 5S rDNA cluster, we first performed a cross-breeding experiment with progeny from F1 mice of the two subspecies groups DOM (BALB/c) and MUS (MOA), with BALB/c, MOA, or SM/J. We genotyped 101 individual progenies in the 5S rDNA cluster and the three flanking SSLP markers (Supplementary Material, Fig. S2). The numbers of recombination events between the SSLP markers D8MIT12 and D8MIT13, and D8MIT13 and D8MIT42, were 17 and 2, respectively, among 101 N2 mice examined (Supplementary Material, Fig. S2). These results provided genetic distances of 17 and 2 cM, respectively, which were comparable to those presented by Mouse Genome Informatics (MGI, <http://www.informatics.jax.org/>).

We examined the haplotype structure of the chromosome region comprising the 5S cluster and six flanking genes (Fig. 2A) in 52 wild-derived mice, including 22 from Japan (localities 1–22) and 30 from outside Japan, representing the territories of the three subspecies groups: MUS (localities 23–36), CAS (37–46) and DOM (47–52; Table 2). We constructed networks with the six gene sequences and specified subspecies-specific clusters to assign each allele to either one of the subspecies-specific groups (Supplementary Material, Fig. S3). This resulted in haplotype patterns with the six linked genes in Japanese mice (Table 2) and allowed us to infer that recombination break points resulted from hybridization events between the two subspecies groups of CAS and MUS in nature, which was thought to have started 700–800 generations ago in northern Japan (Kuwayama et al. 2017). We ignored the inter-subspecies breakpoints seen in the two genes of *Afg3l1* (allelic type *d1*) and *Dbn1* (*c2*), as the chimeric segments are thought to have been generated prior to the arrival of MUS on the Japanese Islands (Kodama et al. 2015; Nunome et al. 2010; see Supplementary Material, Fig. S1). In addition, three haplotypes possessing DOM alleles in all gene regions examined, suggestive of recent introgression from DOM mice (Kuwayama et al. 2017; Nunome et al. 2010), were not included in this analysis. The relative rates of meiotic recombination per length (in kb) were estimated based on the physical map of the genome database. The predicted number of recombination events between *Gas8* and *Gm19600* flanking the 5S cluster was two, and the predicted relative rate of recombination was calculated to be 0.008/kb, under the assumption that the size of the 5S rDNA cluster was 240 kb, based on the results of the present study (Table 1). The relative recombination was lower in the chromosome segment harboring the 5S cluster (interval c in Fig. 2A) than in

neighboring chromosome regions (0.049–4.841/kb).

We analyzed a portion of the spacer region (~200 bp) immediately downstream of the 5S rRNA-coding region in the 22 mice from Japan and 9 from outside Japan, using a direct sequencing method. Two variations were visible: a single nucleotide substitution from C to T at site 132 (132C>T; Supplementary Material, Fig. S4) and a duplication of a 68-bp fragment (sites 143–210). We roughly quantified the proportion of these mutations by measuring the peak heights of the chromatogram of each individual specimen (Supplementary Material, Fig. S4). The 132C>T variation was detected in northern Japanese mice, in which the 5S repeating units were suggested to have originated from CAS based on genotypes of the neighboring genes of *Gas8* and *Gm19600*. The 132C>T mutation was not found in the predicted CAS haplotypes of mice from southern Japan (localities 19, 21, 22; Table 2) and the five mice from outside Japan (localities 42–46). The 68-bp duplication mutation was present in the 5S repeating units in most mice from Japan and Korea.

Signs of selection in the 5S cluster

The neutrality tests of Tajima's and Fu's methods using the sequences from outside Japan did not provide evidence of positive selection in any of the six gene regions or any subspecies group (Supplementary Material, Table S2). We measured π , accounting for all sites across the chromosome region, in each of the three subspecies groups, and their combination ($n = 60$). The π values of *Gas8*, *Gm19600* and *Rhou* were lower than those of *Afg311*, *Dbndd1* and *6030466F02Rik* in each of the data sets (Fig. 2B). The trend was conspicuous in the combined data set; the three higher values were >1.3% and the lower values were <0.4% (Table S2). We further analyzed the π values across the chromosome region using genome sequences from the C57BL/6J and MSM/Ms mouse strains, which represent the two major subspecies groups, DOM and MUS, respectively. Comparison of the genome sequences showed similar trends in π across the chromosome region (Fig. 2C).

We performed the HTK test, with *M. terricolor* as an outgroup, using the gene sequences of the flanking regions of the 5S cluster. We focused on the two subspecies groups, MUS (five genes excluding *Rhou* due to marked interruption by introgression) and CAS (six genes), because the DOM sequence data were interrupted by the introgression of other subspecies fragments (Table 2). We divided the genes into two groups

with high (*Afg3l1*, *Dbndd1*, *6030466F02Rik*) and low (*Gas8*, *Gm19600*, *Rhou*) genetic variations. The HKA test showed a significant difference between the two gene groups in the MUS and CAS data sets ($P < 0.05$; Supplementary Material, Table S3).

Discussion

Copy number variability in the mouse 5S gene

To our knowledge, this study is the first examination of copy number variation in the 5S rDNA cluster in *M. musculus*. Pulsed-field gel electrophoresis of the eight inbred mouse strains and the mouse 5S rDNA probe allowed us to assess the copy numbers of the 5S clusters of the haploid genomes. The 5S rDNA clusters were estimated to have an average of 135 copies per haploid genome, ranging from 63 (BALB/cBy) to 165 (BALB/cA) copies in the inbred strains examined (Table 1). This copy number magnitude is consistent with the findings of previous studies in mammals. Pulsed-field gel electrophoresis analysis indicated that humans have 70–130 copies per haploid genome (Stults et al. 2008). According to analyses on genome-wide sequence data, the 5S copy numbers in mice and humans are estimated to be 32–224 and 16–210, respectively (Gibbons et al. 2015).

Our pulsed-field gel electrophoresis yielded single bands in the majority of the inbred strains examined. These results simply indicated that only one 5S cluster exists per haploid genome, which is consistent with previous *in situ* hybridization analyses in *M. musculus* (Matsuda et al. 1994), *Rattus norvegicus* (Suzuki et al. 1996) and primates, including humans (Henderson et al. 1976; Sørensen et al. 1991, but see Lomholt et al. 1996). Given this consideration, it is reasonable to think that the 5S cluster of mammals has been situated in the genome as a single cluster at a specific chromosome position since the time prior to the divergence between humans and mice, while the non-array type of 5S copies has been generated and persisted, being scattered in a genome in an evolutionary course (Richard et al. 2008).

BALB/cBy and BALB/cA, which have 63 and 165 (and 142) copies, respectively, of 5S rDNA repeating units, are sublines originating from the same ancestral stock of BALB/c. The difference between the BALB/c sublines implies that the copy number can change within a small number of generations. Notably,

BALB/cA showed two bands (Fig. 1A), suggesting the presence of two different 5S clusters, distinguished by variation in copy number. Possible reasons for this result are that BALB/cA has two different clusters in one haploid genome, or that it is a heterozygote of two chromosomes with different numbers of copies. Because the BALB/cA band intensities were relatively faint compared to single bands of the other mice, the latter explanation is more feasible. Thus, one could consider that the copy numbers can be changed somewhat drastically by a single event (Ganley and Kobayashi 2011). Accordingly, the copy numbers of the 5S cluster in humans has been reported to change by 20–30 copies per cluster in a single generation (Stults et al. 2008).

The copy numbers in the inbred strains, with the exception of BALB/cBy, were similar (~140 copies), despite their varying nuclear genome origins. Southern blot analysis of F1 hybrids between the two mouse subspecies groups indicates that the mouse strains representing DOM and MUS have similar 5S copy numbers (Supplementary Material, Fig. S2B). The magnitude of the copy number is in agreement with the findings of previous studies in *M. musculus* and other mammals, as mentioned above. These considerations indicate that whereas the copy number of the 5S cluster can change over a short period, the 5S copy number of mammalian species is somewhat constant at ~140. It is possible that the genes are evolving under certain constraints stabilizing the copy numbers of the 5S cluster in mammals (Gibbons et al. 2015; Stults et al. 2008), and perhaps in other organisms (*e.g.*, wheat; Kellogg and Appels 1995). Gibbons et al. (2015) proposed the hypothesis that the 5S copy number is partly determined by the requirement to balance the dose with the 45S rDNA array. In yeast, the copy number is regulated genetically by a mechanism that involves controlling the copy number and manipulating the amplification that responds to copy number reductions (Kobayashi 2011).

Less frequent meiotic unequal crossover by the 5S cluster

In this study, we addressed whether the 5S cluster is a hotspot of meiotic recombination, based on crosses among inbred strains and a survey of the wild population in the Japanese Islands. The former experiment indicated no apparent acceleration of meiotic recombination mediated by the 5S cluster (Fig. 2). In the latter experiment, we genotyped the six genes in the flanking regions of the cluster in natural populations where the two mouse subspecies groups, MUS and CAS, mingled for a substantial time (*e.g.*, 700–800 generations;

Kuwayama et al. 2017; Nunome et al. 2010), and found no sign of acceleration of recombination attributable to the 5S cluster (Fig. 2A). Thus, our results suggest that meiotic recombination is not accelerated by the 5S cluster. Instead, the cluster could be characterized as a cold spot for meiotic recombination. Accounting for the available genetic map length in the mouse genome databases (assembly GRCm38.p4; 0.09 cM) for a chromosome region (*Cdk10-D8MIT13*; ~0.6 Mb) harboring the 5S cluster (~0.24 Mb; Table 1), the recombination rate is estimated to be 0.15 cM/Mb (Supplementary Material, Fig. S2), which is lower than the standard rate of 0.52 cM/Mb (Jensen-Seaman et al. 2004). Thus, one may conclude that recurrent unequal crossover during meiosis is unlikely the major mechanism driving sequence similarity within and among the 5S rDNA clusters. The low recombination during meiosis is consistent with previous studies evaluating human U2 snRNA (Liao et al. 1997) and yeast rDNA (Petes and Botstein 1977).

Here we addressed whether genetic exchanges occur between 5S clusters on different chromosomes. Our genotyping of the 5S spacer suggests that its two variations, the 68-bp duplication and C132T, are characteristic of the lineages of MUS and CAS, which now co-occur in the Japanese Islands (Supplementary Material, Fig. S4). These two lineages are suggested to have interbred in northern Japan for a substantial period of time (Kuwayama et al. 2017; Nunome et al. 2010). Although it may be expected that the 5S rDNA clusters originating from MUS and CAS exchanged their subspecies-specific variants via certain genetic mechanism(s), the mice that were homozygous for subspecies-specific haplotypes in flanking genes of both sides exhibited subspecies-specific patterns for the 5S variants (Table 2), although the resolution was not high. Hence, our present data did not provide robust evidence to support the hypothesis that the 5S clusters are subjected to intercluster genetic exchanges via meiotic recombination processes during the course of evolution.

Given the considerations mentioned above, the intracellular mechanism underlying 5S sequence similarity within an rDNA cluster most likely operates within a 5S cluster and is independent of other 5S clusters. Unequal sister chromatid exchanges and gene conversion are the most plausible mechanisms of homogenization processes (James et al. 2016; Ganley and Kobayashi 2007). Alternatively, the amplification of a specific repeating unit and removal of divergent repeating units by a deletion event lead to increased sequence similarity within a 5S cluster (Freire et al. 2010; Ganley and Kobayashi 2011; Nei and Rooney 2005; Scoles et

al., 1998). A possible explanation for gene amplification is the rolling cycle model (Cohen et al. 2010; Ide et al. 2013), in which tandem DNA arrays are produced by gene duplication and incorporated through a specific type of DNA replication. According to Ide et al. (2013), a large increase in copy number could have occurred with just a few cell divisions. To better understand which mechanisms are involved in shaping the sequence similarity within a rDNA cluster, it will be valuable to assess the mutation profile by a population survey, as has been attempted in yeast (James et al. 2016).

Natural selection of 5S clusters in a population

The process resulting in sequence similarity between repeating units is likely to be specific to single 5S clusters and independent of other clusters, as discussed above. Together with the notion that gene sequences within the spacer region evolve at a higher rate (Schlotterer et al. 1994; Suzuki et al. 1994a), individual 5S clusters are expected to be substantially differentiated within a population. However, the opposite situation holds, where a higher level of sequence similarity within the same population has been achieved.

One possible explanation is that positive natural selection has led to the sharing of unique, favorable rDNA clusters among members of a population (Gibbons et al. 2015). In this study, low level of genetic diversity observed in the region (*e.g.*, *Rhou*) immediately flanking the 5S cluster was evident in both the survey of natural populations (Fig. 2B) and the comparison of genome sequences (Fig. 2C). Given the restricted recombination of the 5S cluster, as discussed above, the low level of genetic diversity in both sides of the flanking regions could be explained by the effect of selection (Charlesworth et al. 1993, 1994; Braverman et al. 1995; Maynard Smith and Haigh 1974; Nielsen 2005). Such an effect can be generated either by background selection (Charlesworth et al. 1993, 1994) or selective sweep (Maynard Smith and Haigh 1974; Nielsen 2005), in which purifying selection and positive selection act, respectively, to reduce diversity levels at linked sites with low rates of meiotic recombination (Kellogg and Appels 1995). The HKA test showed a significant departure from neutrality ($P < 0.05$), suggesting that the 5S cluster was involved in selective processes for low genetic diversity in the flanking regions, whereas we could not exclude the possibility that the flanking regions play a role. In contrast, however, Tajima's D and Fu's F_s tests provided no evidence for the involvement of

positive selection (Supplementary Material, Table S2).

Whether positive or negative selection is in operation, an important question is which factors affect selection in the 5S clusters. 5S rDNA is a housekeeping gene that produces 5S rRNA very efficiently (Kobayashi 2011). We presume that the quality of the 5S cluster tends to decay over time due to the accumulation of mutations, and that only clusters retaining relatively high quality are distributed throughout the population. Selection may occur for copy number (Gibbons et al. 2015; Ide et al. 2013), deleterious mutations on the 5S coding region, and spacer composition, such as those in the promoter region. To better understand the evolution of 5S rDNA, further studies are needed to determine how natural selection contributes to sequence identity within a population.

Conclusion

Our efforts clarified the evolutionary pattern of rodent 5S rDNA as a tandem array, at an evolutionarily conserved single chromosome position, which was consistent with those predicted in analyses of humans (Stults et al. 2008). Our results suggest that crossover between the clusters on different chromosomes during meiosis is less frequent than crossover in ordinary chromosome regions, and that an intrachromosomal mechanism is responsible for sequence similarity among members of the repeating units within a cluster. Our results, together with previous results (Suzuki et al. 1994a), allow us to propose that the 5S cluster evolves under selective pressure, which causes sequence similarity between 5S clusters within the same reproductive population. Further investigation will provide insight into the factors that shape sequence similarity within a 5S cluster and items, such as copy number, over selective processes. Finally, we would like to emphasize that the 5S rDNA array could be useful as a phylogeographic marker, as the spacer regions evolved rapidly (Campo et al. 2009; Suzuki et al. 1994a), but with a reduction in heterogeneity within each genome and each population. It is worthwhile to note that interchromosomal recombination is less frequent in the 5S cluster, indicating the utility of the 5S cluster as a genealogical marker, similar to mitochondrial DNA, in which no recombination generally occurs.

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Figure Legends

Figure 1. Inference of 5S rDNA cluster length by pulsed-field gel electrophoresis and Southern hybridization. **A** 5S rDNA cluster lengths were inferred in eight laboratory mouse strains: AKR/J (lane 3), BALB/cA (lane 4), BALB/cBy (lane 5), CBA/J (lane 6), C3H/HeJ (lane 7), C57BL/6J (lane 8), DBA/2J (lane 9) and SM/J (lane 10), using *Bgl*III and the mouse 5S rDNA probe (Suzuki et al. 1994a). The size markers are lambda *Hind*III digests (lanes 1, 11) and lambda cocatemers (lanes 2, 12). **B** 5S rDNA cluster length variety in BALB/cBy: *Eco*R1 (lane 2), *Bam*HI (lane 3), *Hind*III (lane 4), *Pvu*II (lane 5), *Bgl*III (lane 6), *Sac*I (lane 7), *Pst*I (lane 8). The latter two cut the repeating units at least once (Suzuki et al. 1994a). The size markers are lambda *Hind*III digests (lane 9) and lambda cocatemers (lanes 1, 10).

Figure 2. **A** Estimated relative recombination rates (number of recombination signals per megabase) across the chromosomal regions comprising the following six gene regions: *Afg3l1* (1), *Dbnnd1* (2), *Gas8* (3), *Gm19600* (4), *Rhou* (5), and *6030466F02Rik* (6). The rates were obtained from haplotype patterns, shown in Table 2, determined by a population survey of the wild mouse from Northern Japan, where haplotypes represented by two subspecies groups are considered to have mingled for several hundred generations (Kuwayama et al. 2017; Nunome et al. 2010). The areas defined by the nearest gene markers are designated a, b, c, d, and e. **B** Nucleotide diversities (π ; mean and standard error) of the six genes in each of the three subspecies groups and the combined data set. The values were calculated using the dataset based on mice from outside Japan shown in Table 2. **C** Nucleotide diversities between two mouse subspecies genome sequences from chromosome regions including the six genes. The genome sequences of C57BL6 and MSM/Ms representing *Mus musculus domesticus* and *M. m. musculus* were obtained from NIG_MOG2 (<http://molossinus.lab.nig.ac.jp/mog2/>). The values were plotted at a 10 kb interval.

Supplementary Figures

Figure S1.

Schematic view of the predicted migration routes of two house mouse subspecies groups, *Mus musculus castaneus* (CAS) and *M. m. musculus* (MUS), to the Japanese Islands (Suzuki et al. 2013; Kuwayama et al. 2017). According to the mtDNA analyses, each of the subspecies groups is thought to have migrated to Japan through two rapid expansion events 7,000–8,000 years ago and 2,000–4,000 years ago. In the nuclear gene sequences analyses, certain portions of the CAS elements found in northern Japan differ from those found on the continent (Kuwayama et al. 2017; Nunome et al. 2010) and southern Japan (this study), which is exemplified in the pattern of the 5S rDNA (i.e., “132T” variation). Notably, the allele of *Afg3ll* is a *M. m. domesticus* (DOM) type when its adjacent gene of *Dbnndl* is CAS. Thus it has been postulated that DOM moved to the CAS homeland before the initial rapid expansion event of CAS (Kodama et al. 2015). In northern Japan, admixture of the two lineages is estimated to have started 700–800 generations ago and is ongoing (Kuwayama et al. 2017; Nunome et al. 2010). In this study, we addressed 1) the meiotic recombination events between the parental subspecies haplotypes mediated by the 5S rDNA clusters, 2) homogenization events between the subspecies-specific rDNA clusters, and 3) the presence or absence of the selective sweep signature, determined by measuring reduction, using the six linked genes on mouse chromosome 8.

Figure S2.

A Physical (in Mb) and linkage (in cM) maps for mouse chromosome 8, which includes the 5S rDNA cluster, based on the GRCm38.p4 assembly. The number of recombinations is counted using simple sequence length polymorphism (SSLP) markers of D8MIT12, D8MIT13 and D8MIT42. A segment harboring the 5S cluster is shown to emphasize that the predicted recombination frequency between two markers of the immediately flanking regions of the 5S cluster is low, based on information available on the Mouse Genome Information website. The average size (0.24 Mb) of the 5S rDNA cluster predicted in this study is shown. **B** Example of Southern blot analysis (lanes 1–20) with cross-breeding of inbred strains of BALB/c (B) and MOA (M), using

the mouse 5S probe (Suzuki et al. 1994a) and the restriction enzyme *Pst*I. The size marker is a lambda *Eco*T14I digest (lane 21).

Figure S3.

Network trees with resultant allelic sequences of the following six regions: *Afg311*, *Dbn11*, *Gas8*, *Gm19600*, *Rhou*, and *6030466F02Rik*. Assessment of the *Mus musculus*, CAS, DOM, and MUS subspecies groups was performed based on the geographic origins of the haplotypes. Alleles are denoted by letters representing the subspecies groups [CAS (*c*), DOM (*d*) and MUS (*m*)], followed by numbers. Open circles indicate the following outgroups: O1, *M. caroli*; O2, *M. terricolor*; O3, *M. spretus*; O4, *M. macedonicus*. Bars indicate genetic distances.

Figure S4.

Schematic representation of the mouse 5S repeating units, with special emphasis on the two mutations. The majority of the 5S copies of Japanese CAS and MUS had their own mutations in the 5' part of the spacer. The CAS repeating unit possesses a single nucleotide mutation of C to T at site 132 from the start site of the spacer. The MUS repeating unit has a 68-bp duplication at site 143 of the spacer. Representative chromatograms are shown.

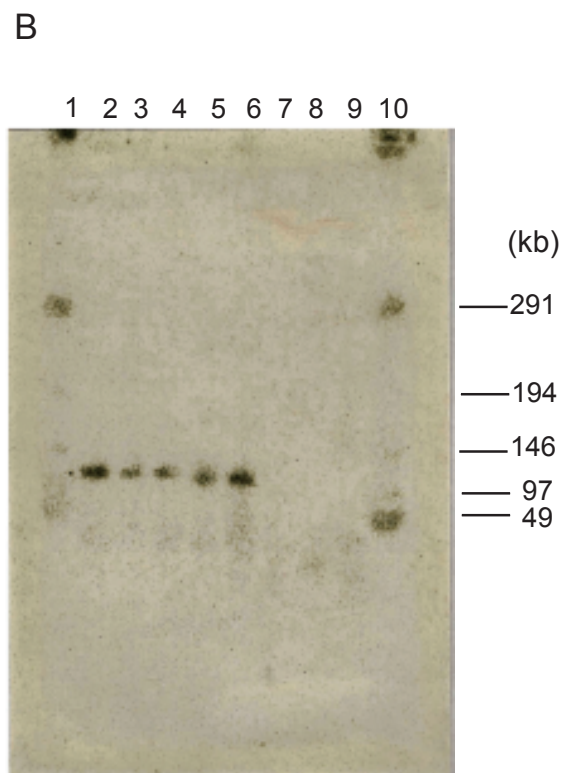
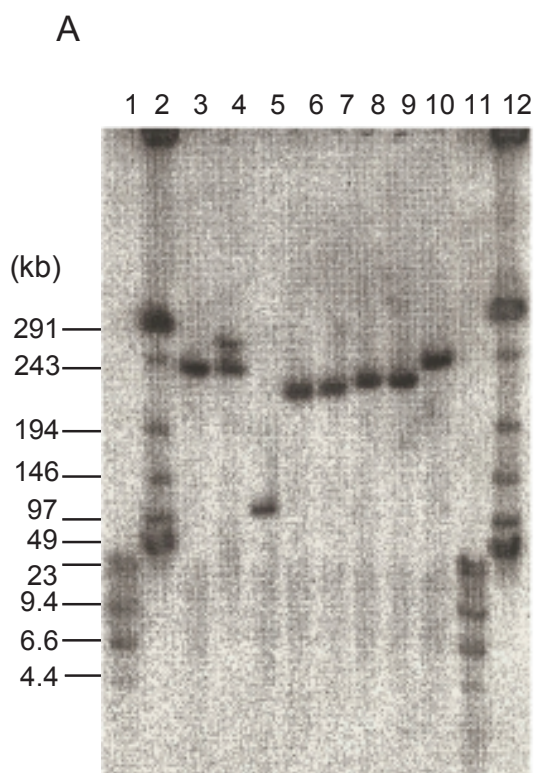
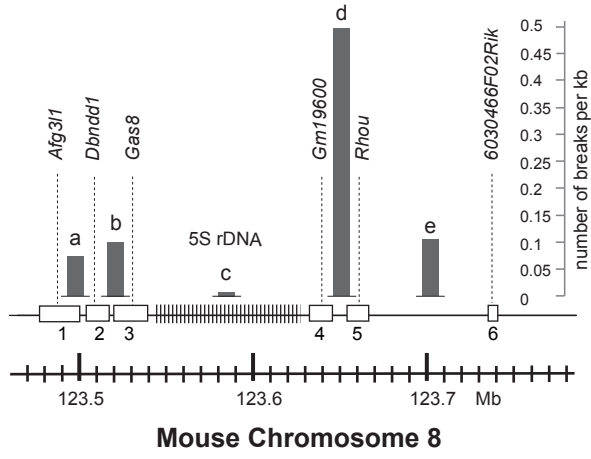
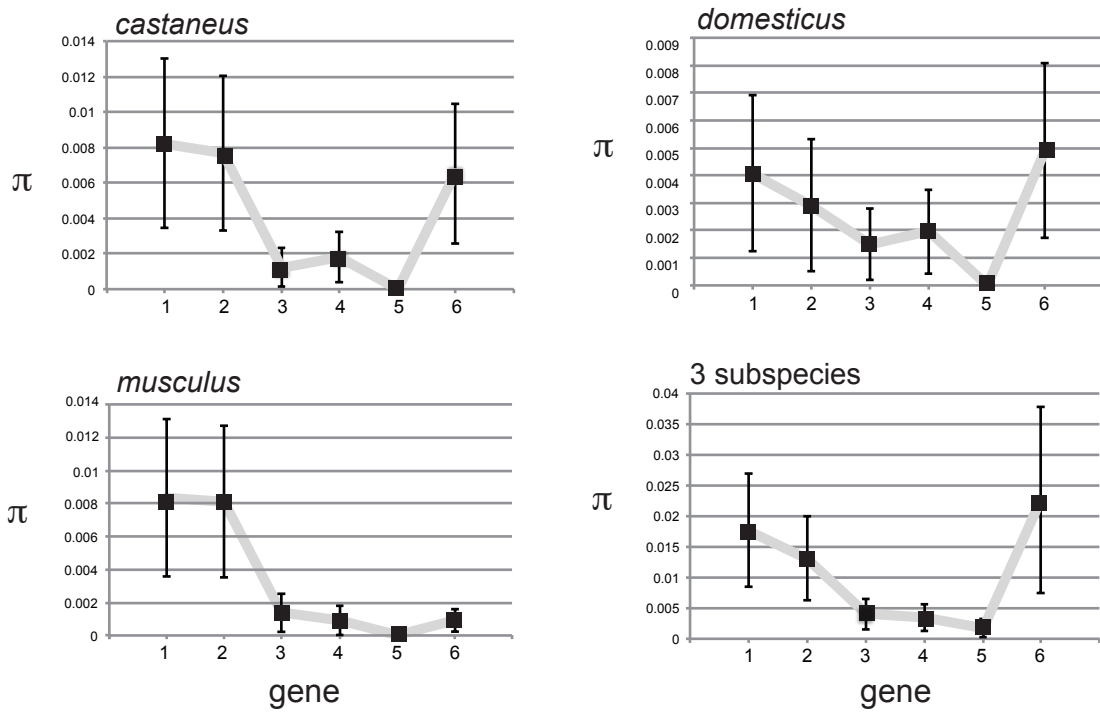


Fig. 1

A



B



C

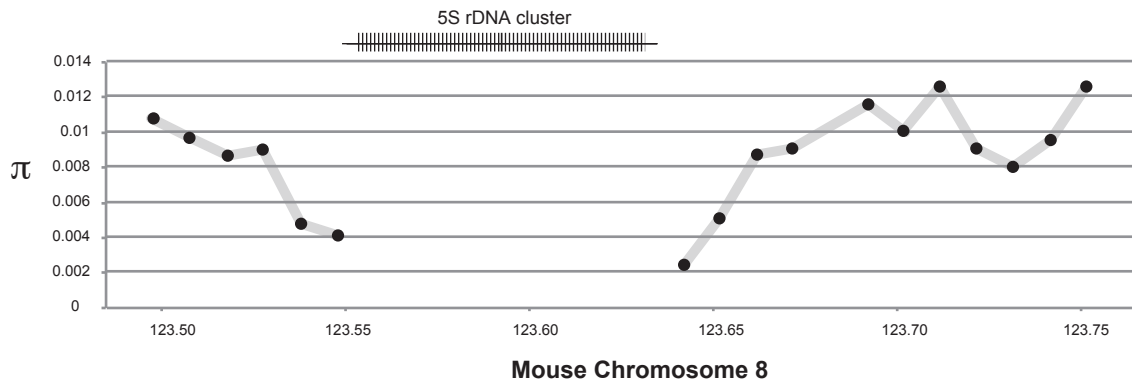


Fig. 2

Table 1

List of laboratory strains used and 5S rDNA copy numbers assessed.

Experiment	Strain	Restriction enzyme	Fragment length (kb)	Presumed copy number
1	AKR/J	<i>Bgl</i> II	250	142
	BALB/cA		290/250	165/142
	BALB/cBy		115	63
	CBA/J		225	128
	C3H/HeJ		225	128
	C57BL/6J		240	136
	DBA/2J		240	136
	SM/J		243	138
2	BALB/cBy	<i>Eco</i> RI, <i>Bam</i> HI,	125	69
		<i>Pvu</i> II, <i>Bgl</i> II	120	66

Table 2. List of samples used in this study and genetic typing in the 5S rDNA and its flanking gene regions.

Locality*	DNA code	Afg31l	Dbndd1	Gas8	5S rDNA**		Gm 19600	Rhou	6030466 F02Rik	
					132T	duplication				
1 Hokkaido	Kushiro	HS1946	d2	d1	d1	0	0	d1	d1	d1
			m1	m10	m1	0	33	m1	m1	m1
2	Asahikawa	HS1947	m1	m10	m1	0	57	m1	m1	m1
			d2	c2	c1	50	0	c1	m1	m1
3	Obihiro	HS2781	m1	m10	m1	0	40	m1	m1	m1
			m1	m10	m1	0	40	m1	m1	m1
4	Otaru	HS2340	d2	c2	c1	40	0	c1	c1	m1
			d2	c2	c1	40	0	c1	c1	m1
5	Date	HS2451	m1	m10	m1	0	28	m1	c1	m1
			m1	m10	m1	0	28	m1	c1	m1
6	Kyowa	HS2779	d1	d2	d2	0	0	d2	d1	d1
			m1	m10	m1	0	50	m1	m1	m1
7	Kyowa	HS2780	m1	m10	m1	0	30	m1	m1	m1
			m1	m10	m1	0	30	m1	m1	m1
8	Rankoshi	HS3834	d1	c2	c1	42	0	c1	m1	m1
			d1	c2	c1	42	0	c2	m1	m1
9	Onuma	HS394	d2	m10	c1	40	0	c1	m1	m1
			d2	c2	c1	40	0	c1	m1	m1
10	Hakodate	HS2323	d1	c2	c1	38	0	c1	c1	m1
			d1	c2	c1	38	0	c2	c1	m1
11 Honshu	Otsuchi	HS2454	m1	m10	m1	0	39	m1	m1	m1
			m1	m10	m1	0	39	m1	m1	m1
12	Sendai	HS2456	m1	m10	m1	0	40	mc1	m1	m1
			m1	m10	m1	0	40	m1	m1	m1
13	Sakata	HS2457	m1	m10	m1	0	30	m1	m1	m1
			d2	c2	m1	0	30	c1	m1	m1
14	Sakekawa	HS2461	d2	c2	c1	43	0	m1	c1	m1
			d2	c2	c1	43	0	c1	c1	m1
15	Tendo	HS2458	m1	m10	m1	0	33	m1	m1	m1
			d2	c2	c1	50	0	c1	m1	m2
16	Atsugi	HS3839	d1	d1	d1	0	0	d3	d1	d1
			m1	m10	m1	0	28	m1	m1	m1
17	Mishima	HS3950	m1	m10	m1	0	25	m1	m1	m1
			m1	m10	m1	0	25	m1	m1	m1
18	Kanazawa	HS2816	m1	m10	m1	0	30	m1	m1	m1
			m1	m10	m1	0	30	m1	m1	m1
19	Misasa	HS4097	m1	m10	m1	0	50	m1	m1	m1
			c1	c1	c1	0	0	c1	c1	c1
20	Sado	HS4229	m1	m10	m1	0	38	m1	m1	m1
			m1	m10	m1	0	38	m1	m1	m1
21 Kyushu	Miyazaki	HS2472	m1	m10	m1	0	50	m1	m1	m1
			m1	c2	c1	0	0	c1	c1	m1
22	Kagoshima	HS3603	m1	m10	m1			m1	m1	c2
			c1	c1	c1			c1	c1	c3
23	Aktobe, Kazakhstan	HS1464	m5	m4	m1	0	0	m1	m1	m1
			m19	m4	m1	0	0	m2	m1	m1
24	Irkutsk, Russia	HS3608	m8	m5	m1			m1	m1	m3
			m15	m5	m1			m1	m1	m3
25	Gorno-Altaysk, Russia	HS3605	m8	m5	m1			m1	m1	m3
			m8	m5	m1			m1	m1	m3
26	Tomsk, Russia	HS3604	m8	m5	m1			m1	c1	m4
			m8	m5	m1			m1	c1	m4
27	Khabarovsk, Russia	HS1461	m11	m4	m1			m1	c1	m3
			m8	m5	m1			m1	c1	m3
28	Kraskino, Russia	HS1412	m12	m4	m2			m1	c1	m3
			m12	m4	m2			m1	c1	m3
29	Tallin, Estonia	MG3044	m2	m2	m2			m1	c1	m3
			m14	m4	m2			m1	c1	m3
30	Khasan, Russia	HS1335	m21	m4	m2	0	0	m1	m1	m3
			m22	m4	m2	0	0	m1	m1	d1
31	Denmark	BRC1437	m25	m4	m2			m3	m1	m3
			m25	m4	m2			m1	m1	m3
32	Gyeongju, Korea	HS1368	m1	m9	m1	0	0	m1	m1	m1
			m1	m9	m1	0	0	m1	m1	m1
33	Busan, Korea	HS3540	m1	m9	m1	0	19	m1	m1	m1
			m1	m9	m1	0	19	m1	m1	m1
34	Balkhash, Russia	HS3602	m16	m8	m3			m1	m1	m3
			m16	m8	m3			m1	m1	m3
35	Toshkent, Uzbekistan	HS1338	m17	m9	m4			u1	m1	m3
			m17	m9	m4			u1	m1	m3
36	Lanzhou, China	MG509	m2	m9	m1			m1	m1	d3
			m2	m11	m1			m1	m1	d3
37	Bogor, Indonesia	HS3736	c1	c1	c1			c3	c1	c6
			c1	c1	c1			c3	c1	c6
38	Los Banos, Philippines	HS3882	c3	c1	c1			c1	c1	c9
			c3	c1	c1			c2	c1	c9
39	Islamabad, Pakistan	HI179	c4	c8	c2			c2	c1	c9
			c5	c9	e2			c5	c1	c9
40	Lahore, Pakistan	HI266	c6	c11	c2			c4	c1	c5
			c7	c5	e2			c4	c1	c5
41	Mysore, India	HI273	c3	u1	e2			c1	c1	c10
			c3	u1	e2			c2	c1	c10
42	Taitung, Taiwan	HS2400	c1	c1	c1	0	0	c1	c1	c1
			c1	c1	c1	0	0	c2	c1	m1
43	Kunming, China	HS506	c1	c1	c1	0	0	c1	c1	c1
			c3	c1	c1	0	0	c2	c1	m1
44	Kunming, China	HS507	m24	m9	m1	0	0	c1	c1	m1
			c1	c1	c1	0	0	c2	c1	m1
45	Kathmandu, Nepal	HS1467	c1	c1	c1	0	0	c1	c1	c7
			e2	c1	c1	0	0	c2	c1	c8
46	Aizawl, India	HS3441	c1	c1	c1	0	0	c1	c1	c4
			c1	c1	c1	0	0	c2	c1	c4
47	Haifa, Israel	MG378	c3	c7	d2			d4	m1	d2
			c3	c7	d2			d5	m1	d2
48	Aosta, Italy	HSS89	d1	d1	d1			d4	d1	d1
			d2	c4	d1			d4	d1	d1
49	Coppeck, Peru	BRC1523	d1	d1	d2			d4	d1	d4
			d1	d1	d2			d4	d1	d4
50	Corse, France	MG385	d3	c10	d1			d4	d1	d1
			d3	c6	d3			d4	d1	d1
51	Cairo, Egypt	MG382	d8	c7	d2			d3	m1	d4
			d8	c7	d2			d3	m1	d4
52	Moscow, Russia	BRC173	d1	d1	d3			d4	d1	d1
			d3	d2	d3			d4	m1	u1

* The recombination rates and population nucleotide diversity were assessed using two data sets from Japan (Localities 1–22) and outside Japan (Localities 23–52), respectively.

In the former analyses, predicted break points for recombination between haplotypes are marked with thick bars. Abbreviations for each of the subspecies groups:

c, *M. m. castaneus*; d, *M. m. domesticus*; m, *M. m. musculus*; u, unknown.

**Frequencies of the two variants (in %) of the 5S spacer based on peak heights in the chromatograms. See text for details of the variants.

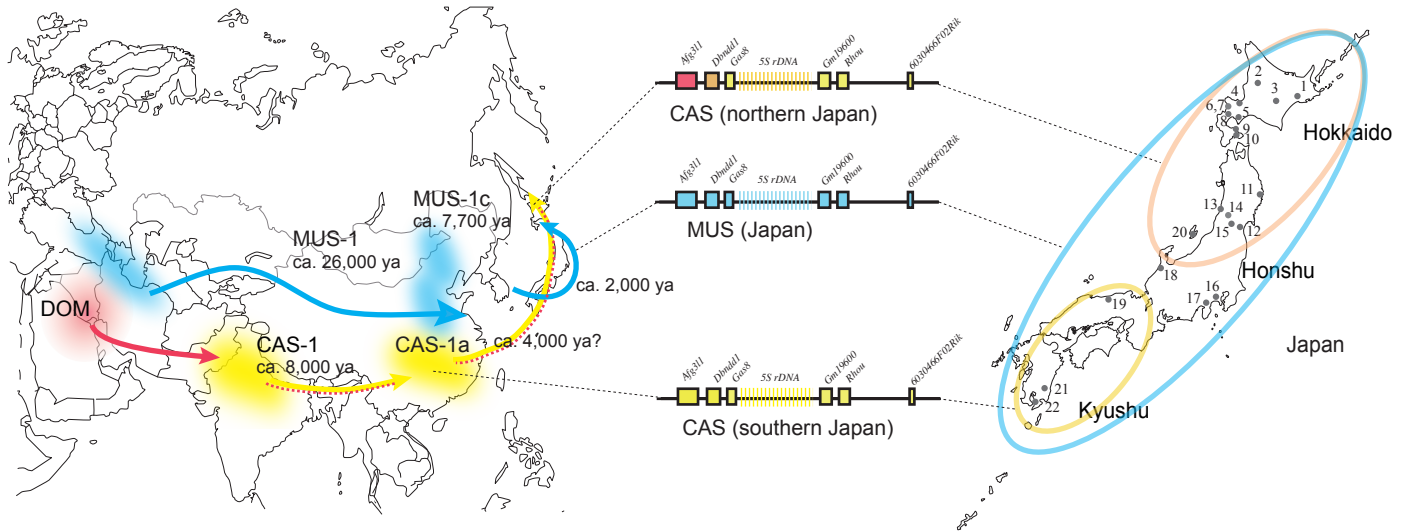


Figure S1.

Schematic view of the predicted migration routes of two house mouse subspecies groups, *Mus musculus castaneus* (CAS) and *M. m. musculus* (MUS), to the Japanese Islands (Suzuki et al. 2013; Kuwayama et al. 2017). According to the mtDNA analyses, each of the subspecies groups is thought to have migrated to Japan through two rapid expansion events 7,000–8,000 years ago and 2,000–4,000 years ago. In the nuclear gene sequences analyses, certain portions of the CAS elements found in northern Japan differ from those found on the continent (Kuwayama et al. 2017; Nunome et al. 2010) and southern Japan (this study), which is exemplified in the pattern of the 5S rDNA (i.e., “132T” variation). Notably, the allele of *Afg3l1* is a *M. m. domesticus* (DOM) type when its adjacent gene of *DbnDD1* is CAS. Thus it has been postulated that DOM moved to the CAS homeland before the initial rapid expansion event of CAS (Kodama et al. 2015). In northern Japan, admixture of the two lineages is estimated to have started 700–800 generations ago and is ongoing (Kuwayama et al. 2017; Nunome et al. 2010). In this study, we addressed 1) the meiotic recombination events between the parental subspecies haplotypes mediated by the 5S rDNA clusters, 2) homogenization events between the subspecies-specific rDNA clusters, and 3) the presence or absence of the selective sweep signature, determined by measuring reduction, using the six linked genes on mouse chromosome 8.

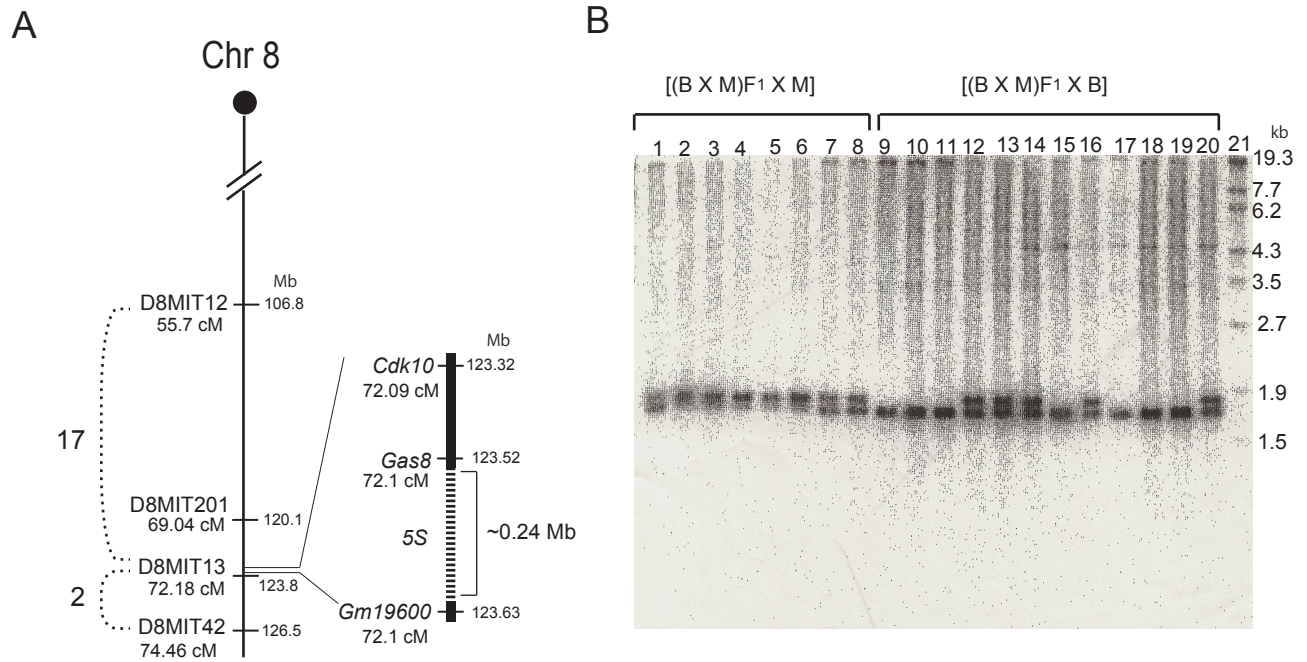
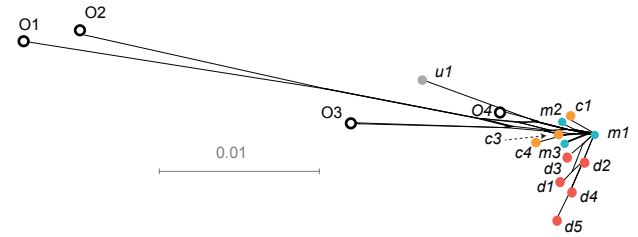
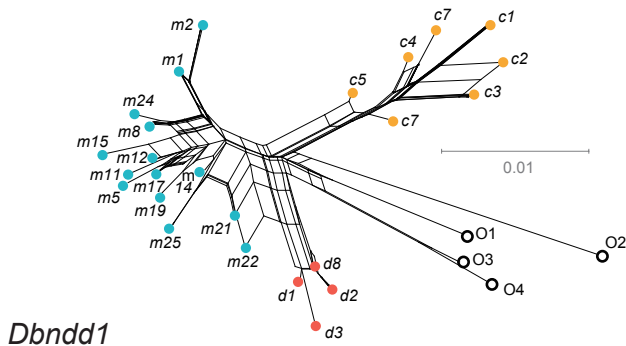


Figure S2.

A Physical (in Mb) and linkage (in cM) maps for mouse chromosome 8, which includes the 5S rDNA cluster, based on the GRCm38.p4 assembly. The number of recombinations is counted using simple sequence length polymorphism (SSLP) markers of D8MIT12, D8MIT13 and D8MIT42. A segment harboring the 5S cluster is shown to emphasize that the predicted recombination frequency between two markers of the immediately flanking regions of the 5S cluster is low, based on information available on the Mouse Genome Information website. The average size (0.24 Mb) of the 5S rDNA cluster predicted in this study is shown. B Example of Southern blot analysis (lanes 1–20) with cross breeding of inbred strains of BALB/c (B) and MOA (M), using the mouse 5S probe (Suzuki et al. 1994a) and the restriction enzyme PstI. The size marker is a lambda EcoT14I digest (lane 21).

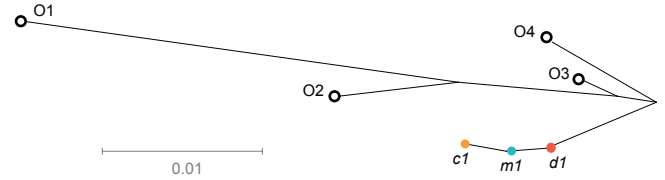
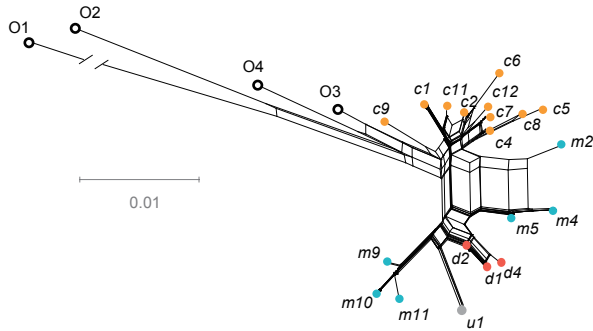
Afg3l1

Gm19600



Dbnbd1

Rhou



Gas8

6030466F02Rik

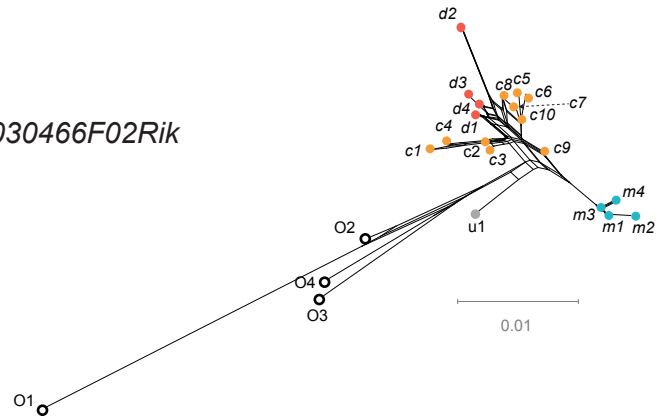
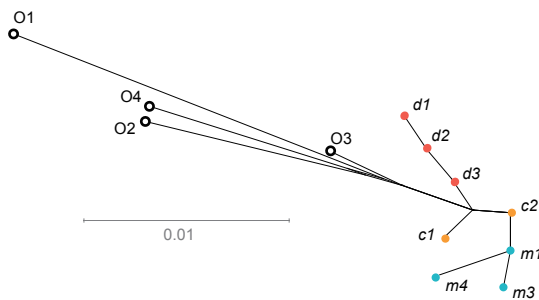


Figure S3.

Network trees with resultant allelic sequences of the following six regions: *Afg3l1*, *Dbnbd1*, *Gas8*, *Gm19600*, *Rhou*, and *6030466F02Rik*. Assessment of the *Mus musculus*, CAS, DOM, and MUS subspecies groups was performed based on the geographic origins of the haplotypes. Alleles are denoted by letters representing the subspecies groups [CAS (c), DOM (d) and MUS (m)], followed by numbers. Open circles indicate the following outgroups: O1, *M. caroli*; O2, *M. terricolor*; O3, *M. spretus*; O4, *M. macedonicus*. Bars indicate genetic distances.

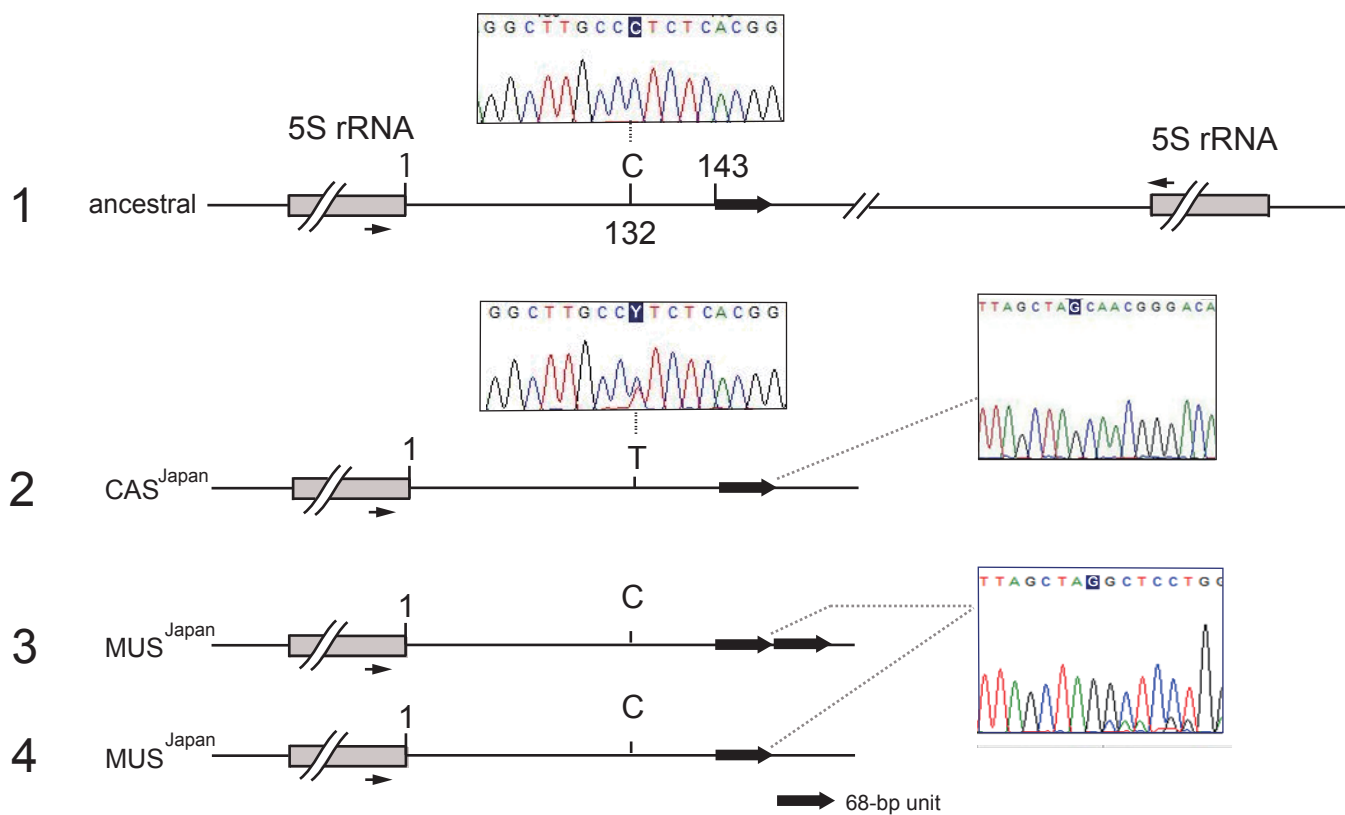


Figure S4.

Schematic representation of the mouse 5S repeating units, with special emphasis on the two mutations. The majority of the 5S copies of Japanese CAS and MUS had their own mutations in the 5' part of the spacer. The CAS repeating unit possesses a single nucleotide mutation of C to T at site 132 from the start site of the spacer. The MUS repeating unit has a 68-bp duplication at site 143 of the spacer. Representative chromatograms are shown.

Supplementary material. Table S1. List of primers used in this study

Gene	Primer code	Sequence	Location of 3' end*	Size (bp)			Cycle Condition
				Exon	Intron	Total	
5S rDNA							
<i>5S rDNA (5S ribosomal RNA gene)</i>							
	5S_F	ACCGCCTGGGAATACCGGGTGTCTAGG	-	-	-	-	95°C (30 sec). 70°C (1 min). 72°C (2 min)
	5S_R	GCGCGTTCAGGGTGGTATGGCCGTAGA	-				
Genes flanking the 5S cluster							
<i>Afg3l1 (AFG3-like gene 1)</i>							
	Afg_F	ACTGACTTCATTTTCCCCGAGG	123,480,414	0	482	482	95°C (30 sec). 57°C (30 sec). 72°C (30 sec)
	Afg_R	TTGGAGGTACTGAATTCCTGG	123,481,139				
<i>Dbndd1 (Dysbindin (dystrobrevin binding protein 1) domain contig 1)</i>							
	Dbn_F	AATACCAGCACCAGGGTTCCTG	123,509,861	0	555	555	95°C (30 sec), 57°C (30 sec), 72°C (30 sec)
	Dbn_R	AGAGAGGAGACGCTGCTCAGAG	123,509,170				
<i>Gas8 (Growth arrest-specific protein 8)</i>							
	Gas8_F1	TGAGAGGAGGAAACCTCAGC	123,530,232	120	429	549	95°C (30 sec). 51°C (60 sec). 72°C (60 sec)
	Gas8_R1	TCTAAAGGGGTGGGGTTAGG	123,531,197				
	Gas8_F2	AAGAAAGCAGGCTGAGCAAG	123,530,378				
	Gas8_R2	GCAAGCCACAAGAGGACAGT	123,531,166				
<i>Gm19600 (predicted gene, 19600)</i>							
	Gm_F	CAGAAGTGCCAGGTGAGACA	123,633,555	554	0	554	95°C (30 sec). 57°C (30 sec). 72°C (30 sec)
	Gm_R	GAAACACGCGAAGACAGACA	123,634,228				
<i>Rhou (Ras homolog gene family, member U)</i>							
	Rhou_F	CTACGGCCTTCGACAACTTC	123,654,212	0	414	414	95°C (30 sec). 60°C (30 sec). 72°C (30 sec)
	Rhou_R	TAGAGACTGGCCACGGAGAC	123,654,703				
<i>6030466F02Rik (RIKEN cDNA 6030466F02 gene)</i>							
	60Rik_F	AGTACCTGGAGCGGACACTA	123,739,293	518	0	518	95°C (30 sec). 58°C (30 sec). 72°C (30 sec)
	60Rik_R	CAGCCACACAAAGGGGCTTA	123,739,844				

*Numerical numbers indicate locations of the 3' end of primers, referring the GRCm38 mouse assembly.

Supplementary material. Table S2

Genetic diversity indices of the six nuclear markers.

Group	<i>Afg3l1</i>	<i>Dbnddl</i>	<i>Gas8</i>	<i>Gm19600</i>	<i>Rhou</i>	<i>6030466F02Rik</i>
All samples						
n	60	60	60	60	60	60
S (Allele no)	37 (24)	25 (22)	9 (9)	13 (10)	2 (3)	19 (14)
pi	0.017657	0.013104	0.004029	0.003429	0.001729	0.022605
SE	0.009182	0.006906	0.002502	0.002199	0.001458	0.011515
Tajima's <i>D</i>	0.90481	0.92215	0.39561	-1.41571	1.14354	0.34108
<i>P</i> -value	0.0853	0.8581	0.6996	0.0586	0.8607	0.6984
Fu's <i>F_s</i>	-2.91663	-2.85166	-0.4909	-1.82018	1.31664	5.07515
<i>P</i> -value	0.1962	0.1947	0.4544	0.2145	0.757	0.9322
MUS						
n	29	28	28	26	25	29
S (Allele no)	14 (14)	10 (7)	4 (4)	2 (3)	0 (1)	1 (2)
pi	0.008319	0.008112	0.001388	0.000931	0	0.00055
SE	0.004764	0.004594	0.001169	0.000903	-	0.000652
Tajima's <i>D</i>	0.41947	2.3344	-0.67705	-1.22394	-	-0.75285
<i>P</i> -value	0.71025	0.992	0.2945	0.0792	-	0.2199
Fu's <i>F_s</i>	-3.80213	2.10475	-0.30798	0.02175	-	0.53201
<i>P</i> -value	0.0553	0.8315	0.3877	0.3983	-	0.3969
CAS						
n	21	25	20	20	28	16
S (Allele no)	12 (6)	17 (10)	3 (3)	3 (4)	0 (1)	9 (8)
pi	0.008233	0.007657	0.001237	0.001818	0	0.006534
SE	0.004802	0.004381	0.001099	0.001424	-	0.003959
Tajima's <i>D</i>	0.71643	-0.75413	-0.5264	-0.69264	-	0.5585
<i>P</i> -value	0.7941	0.2447	0.3354	0.2671	-	0.7371
Fu's <i>F_s</i>	1.9874	-0.7048	0.38186	0.00331	-	-1.13465
<i>P</i> -value	0.834	0.392	0.5417	0.484	-	0.2539
DOM						
n	10	5	12	12	7	14
S (Allele no)	5 (4)	4 (4)	2 (3)	4 (3)	0 (1)	6 (4)
pi	0.004066	0.002909	0.00149	0.001949	0	0.004757
SE	0.002838	0.002397	0.00129	0.001522	-	0.00309
Tajima's <i>D</i>	0.42681	-1.0938	0.68788	-0.6603	-	-0.07717
<i>P</i> -value	0.6893	0.1129	0.7857	0.2957	-	0.5016
Fu's <i>F_s</i>	0.5426	-1.40478	0.28954	0.83595	-	1.65128
<i>P</i> -value	0.616	0.0691	0.4706	0.673	-	0.825

N: sample size, S: number of polymorphic sites

Supplementary Table S3. HKA test on combined data of the gene sequences of the 5S flanking regions

Subspecies	Genes	Number of segregating sites		Number of differences		χ^2
		Observed	Expected	Observed	Expected	(<i>P</i> -value)
MUS	Control region: <i>Afg311</i> , <i>Dbndd1</i>	26	19.88	40.61	46.73	3.947
	Region tested: <i>Gas8</i> , <i>Gm19600</i>	4	10.12	29.91	23.79	(0.0469)
CAS	Control region: <i>Afg311</i> , <i>Dbndd1</i> , <i>6030466F02Rik</i>	22	15.27	38.64	45.38	4.175
	Region tested: <i>Gas8</i> , <i>Rhou</i> , <i>Gm19600</i>	4	10.73	38.64	31.92	(0.04)