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3 Genetic characteristics of *Apodemus speciosus* at Akiyoshidai Quasi-National Park in  
4 Yamaguchi Prefecture

5

6 **Authors:**

7 Hiroyuki Imai<sup>1</sup>, Hiroshi Tanaka<sup>2</sup>, Taiki Matsuo<sup>3</sup>, Miho Seto<sup>1</sup>, Sumito Matsuya<sup>4</sup>,  
8 Muneyoshi Hyoto<sup>1</sup>, Kiyoshi Kano<sup>4</sup>, Ken Takeshi Kusakabe<sup>1</sup>

9

10 **Affiliation:**

11 1: Laboratory of Veterinary Anatomy, Joint Faculty of Veterinary Medicine, Yamaguchi  
12 University.

13 2: Yamaguchi Prefectural Museum

14 3: Laboratory of Veterinary Physiology, United Graduate School of Veterinary Science,  
15 Yamaguchi University.

16 4: Laboratory of Veterinary Embryology, Joint Graduate School of Veterinary Medicine,  
17 Yamaguchi University.

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19 **Running heads:** Genetics of *A. speciosus* in Akiyoshidai

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21 **Correspondence:** [himai@yamaguchi-u.ac.jp](mailto:himai@yamaguchi-u.ac.jp)

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24

25 **abstract**

26                                   The large Japanese field mouse (*Apodemus speciosus*) is a small  
27 rodent endemic to Japan. The mice have a genetic characteristic in which the number of  
28 chromosomes differs between those from western Japan and those from eastern Japan.  
29 *A. speciosus*, found throughout Japan, is used as a model animal for geogenetics and  
30 monitoring of radiation effects of wildlife. In this present study, to elucidate the genetic  
31 characteristics of the mice Akiyoshidai Quasi-National Park in Yamaguchi Prefecture,  
32 we investigated mitochondrial DNA and chromosome numbers. As a result, *A.*  
33 *speciosus* from Yamaguchi Prefecture were classified into the Honshu-Shikoku-Kyushu  
34 group and had a western Japan-type chromosome set of  $2n=46$ ; however, some  
35 Yamaguchi Prefecture mice formed a genetic cluster in Yamaguchi Prefecture,  
36 suggesting that continuous monitoring is needed to reveal the dynamics of genetic  
37 diversity.

38

39 **Key words:** chromosome, large Japanese field mouse, mitochondrial DNA

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41

## 42 **Introduction**

43           The large Japanese field mouse (*Apodemus speciosus*) is a small rodent species  
44 endemic to Japan. *A. speciosus* inhabit the entire Japanese islands except for Okinawa  
45 and is frequently used as a model for studies of geographic isolation. The genetics of *A.*  
46 *speciosus* is characterized by different chromosome numbers in the east and west of Japan  
47 within a species. This characteristic karyotype is caused by a Robertsonian translocation  
48 (Shimba and Kobayashi 1969) and these translocated chromosomes were detected by  
49 FISH analysis (Yamagishi *et al.* 2012), which indicate that the mice are important species  
50 for genetic research.

51           Recently, *A. speciosus* was used as animals to monitor the effects of radiation  
52 around nuclear power plants. Especially, *A. speciosus* was used to monitor  
53 spermatogenesis and chromosomal abnormalities in Fukushima Prefecture (Okano *et al.*  
54 2016; Takino *et al.* 2017; Ariyoshi *et al.* 2018; Fujishima *et al.* 2020). Decreases in the  
55 number of hematopoietic progenitor cells and chromosomal abnormalities were reported  
56 (Ariyoshi *et al.* 2020; Kawagoshi *et al.* 2017), indicating that *A. speciosus* was actually  
57 important in clarifying the effects of radiation on wildlife.

58           Although much genetic analysis has been performed, the sequence information  
59 is not sufficient to cover the whole of Japan. Many geogenetic studies were performed  
60 especially in the Japanese islands, but the information available on Honshu is not  
61 comprehensive. In the island genetics research, genetic diversity in the Seto inland sea  
62 region, Hokkaido and other remote islands were reported (Sato *et al.* 2017; Suzuki *et al.*  
63 2015). The research leads to the elucidation of the genetic diversity of *A. speciosus* on the  
64 islands; however, *A. speciosus* needs more genetic consideration in each region of Honshu.

65           In this present study, we focused on mitochondrial DNA (mtDNA) sequences  
66 and chromosome numbers to clarify the genetic information of *A. speciosus* in Yamaguchi  
67 Prefecture, mainly in Akiyoshidai Quasi-National Park. Since Akiyoshidai is also  
68 designated as a special natural monument by the Japanese government, which usually

69 restricts the collection of plants and animals, this present study could be an important

70 record of natural history.

71

## 72 **Materials and Methods**

### 73 *Study area and animals*

74           Eight Japanese field mice were captured in Yamaguchi city and Akiyoshidai  
75 Quasi-National Park using Sherman trap (Fig. 1). The captures were performed with the  
76 permission of Yamaguchi Prefecture and Mine city. Captured mice were euthanized by  
77 cardiac blood sampling under isoflurane anesthesia. All the procedures using animals  
78 were approved by the Experimental Animal Care and Use Committee of Yamaguchi  
79 University (protocol number: 432).

### 80 *DNA extraction and analysis*

81           Genomic DNA was isolated from tail skin using NucleoSpin Tissue XS  
82 (Takara Bio, Shiga) according to manufacturer's protocol. PCR was performed using  
83 PrimeSTAR HS (Takara Bio) and T100 Thermal Cycler (Bio-rad, CA). Primer  
84 sequences and their annealing temperatures were shown in Table S1. Fragments after  
85 electrophoresis were recovered using NucleoSpin Gel and PCR Clean-up (Takara Bio).  
86 The nucleotide sequences were determined by the Yamaguchi University Center for  
87 Gene Research. The obtained sequences were analyzed using ApE and MEGA X  
88 software (Tamura *et al.* 1993; Kumar *et al.* 2018; Stecher *et al.* 2020).

### 89 *Culture and chromosomal spread*

90           The tail tips were placed on a 24-well plate and cultured in DMEM (Fujifilm-  
91 Wako, Tokyo) supplemented with fetal bovine serum (10%, Thermo Fisher Scientific  
92 Japan, Tokyo) and Penicillin-Streptomycin Solution (1x, Fujifilm-Wako). Passages and  
93 expansion cultures were performed using the media and Trypsin-EDTA solution (1  
94 mmol/l EDTA-4Na, 0.25 w/v%, Fujifilm-Wako). The cells were arrested in metaphase  
95 by adding colchicine (Sigma-Aldrich Japan, Tokyo), suspended in hypotonic solution  
96 (1% sodium citrate), fixed in Carnoy's fixation solution, and expanded onto glass slides.  
97 Chromosomes were stained with Giemsa Stain Solution (Fujifilm-Wako). A set of 30-50  
98 chromosomes per individuals were observed.



## 100 **Result and Discussion**

101           The Cytb and D-loop regions of mtDNA in large Japanese field mice  
102 (*Apodemus speciosus*) were analyzed using genomic PCR. The nucleotide sequences  
103 obtained by direct sequencing were deposited in the DDBJ (Table S2). Comparing the  
104 Cytb sequences of the captured mice in this present study with those in the database, the  
105 sequences of *A. speciosus* in Yamaguchi prefecture formed a cluster (Fig. 2, bold line).  
106 However, *A. speciosus* in Yamaguchi prefecture captured in this present study (Fig. 2  
107 filled circles) and previous report (Fig. 2, opened circle; Suzuki *et al.* 2015) together  
108 were found to belong to the Honshu-Shikoku-Kyushu cluster. The distinct grouping of  
109 the mice in Hokkaido, Izu Islands, Sado Island, Nansei Islands and Tsushima Islands  
110 (Fig.2, fine, dotted, short-dashed, long-dashed and gray lines, respectively) might reflect  
111 previous report (Tsuchiya 1974) based on the findings of Imaizumi *et al.* Comparison of  
112 the sequences of the D-loop region with those of others from western Japan in the  
113 database showed that *A. speciosus* in Yamaguchi Prefecture did not constitute a distinct  
114 group (Fig. S1). These results indicated that *A. speciosus* in Yamaguchi prefecture can  
115 be classified into Honshu-Shikoku-Kyushu group.

116           Next, to count the number of chromosomes, we produced cultured cells from  
117 the tail tip tissues. As a result, we obtained fibroblast-like cells that migrated and  
118 proliferated from the tail tissues (Fig. S2). Chromosomal spreads were prepared by  
119 colchicine treatment of these cells (Fig. 3a). We counted the number of chromosomes  
120 per cell, which indicated that nearly 80% of the cell had a chromosome number of  
121  $2n=46$  (Fig. 3b). These results revealed that the chromosome number of *A. speciosus*  
122 was  $2n=46$  in Yamaguchi Prefecture. Previous reports have shown that the number of  
123 chromosomes in *A. speciosus* is different between the western and eastern parts of Japan  
124 (Shimba and Kobayashi 1969). The number of chromosomes in each region has not  
125 been analyzed in detail. Our present study is the first report showing that the number of

126 chromosomes of *A. speciosus* at Akiyoshidai Quasi-National Park in Yamaguchi  
127 Prefecture, is  $2n=46$ .

128           In summary, this present study investigated the mtDNA and chromosome  
129 number of *A. speciosus* in Yamaguchi Prefecture. Our results showed that *A. speciosus*  
130 in Yamaguchi Prefecture had a western Japan-type chromosome number and is  
131 classified into a cluster of the Honshu-Shikoku-Kyushu group. Focusing on *A.*  
132 *speciosus* in Yamaguchi Prefecture within Japan as a whole, it is possible that some of  
133 *A. speciosus* are genetically clustered. Therefore, it should be necessary to monitor the  
134 domestic invasion of *A. speciosus* involving the movement of natural persons and  
135 goods. Continuous monitoring of the large Japanese field mice population might be  
136 necessary to reveal the dynamics of genetic diversity around Yamaguchi Prefecture.  
137  
138



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145

146 **References**

- 147 Ariyoshi, K., Miura, T., Kasai, K., Goh, V.S.T., Fujishima, Y., Nakata, A., Takahashi, A.,  
148 Shimizu, Y., Shinoda, H., Yamashiro, H., Seymour, C., Mothersill, C. and Yoshida,  
149 M.A. 2020. Environmental radiation on large Japanese field mice in Fukushima  
150 reduced colony forming potential in hematopoietic progenitor cells without genomic  
151 instability. *International Journal of Radiation Biology* 1-12.
- 152 Ariyoshi, K., Miura, T., Kasai, K., Akifumi, N., Fujishima, Y. and Yoshida, M.A. 2018.  
153 Raddiation-induced bystander effect in large Japanese field mouse (*Apodemus*  
154 *speciosus*) embryonic cells. *Radiation and Environmental Biophysics* 57(3): 223-  
155 231.
- 156 Azuma, R., Hatanaka, Y., Shin, S.W., Murai, H., Miyashita, M., Anzai, M. and  
157 Matsumoto, K. 2020. Developmental competence of interspecies cloned embryos  
158 produced using cell from large Japanese field mice (*Apodemus speciosus*) and  
159 oocyte from laboratory mice (*Mus musculus domesticus*). *Journal of Reproduction*  
160 *and Development* 66(3): 255-263.
- 161 Fujishima, Y., Nakata, A., Ujiie, R., Kasai, K., Ariyoshi, K., Goh, V.S.T., Suzuki, K.,  
162 Tazoe, H., Yamada, M., Yoshida, M.A. and Miura, T. 2020. Assessment of  
163 chromosomal aberrations in large Japanese field mice (*Apodemus speciosus*) In  
164 Namie Town, Fukushima. *International Journal of Radiation Biology* 1-9.
- 165 Hanazaki, K., Tomozawa, M., Suzuki, Y., Kinoshita, G., Yamamoto, M., Irino, T. and  
166 Suzuki, H. 2017. Estimation of evolution rates of mitochondrial DNA in two  
167 Japanese field mouse species based on calibrations with quaternary environmental  
168 changes. *Zoological Science* 34(3): 201-210.
- 169 Hirota, T., Hirohata, T., Mashima, H., Satoh, T. and Obata, Y. 2004. Population structure  
170 of the large Japanese field mouse, *Apodemus speciosus* (Rodentia: Muridae), in  
171 suburban landscape, based on mitochondrial D-loop sequences. *Molecular Ecology*  
172 13(11): 3275-3282.

- 173 Kawagoshi, T., Shiomi, N., Takahashi, H., Watanabe, Y., Fuma, S., Doi, K., Kawaguchi,  
174 I., Aoki, M., Kubota, M., Furuhata, Y., Shigemura, Y., Mizoguchi, M., Yamada, F.,  
175 Tomozawa, M., Sakamoto, S.H., Yoshida, S. and Kubota, Y. 2017. Chromosomal  
176 aberrations in large Japanese field mice (*Apodemus speciosus*) captured near  
177 Fukushima Dai-Ichi nuclear power plant. *Environmental Science and Technology*  
178 51(8): 4632-4641.
- 179 Kumar, S., Stecher, G., Ki, M., Knyaz, C. and Tamura, K. 2018. MEGA X: Molecular  
180 Evolution Genetics Analysis across computing platforms. *Molecular Biology and*  
181 *Evolution* 35(6): 1547-1549.
- 182 Kuwahara, S., Mizukami, T., Omura, M., Hagihara, M., Inuma, Y., Shimizu, Y.,  
183 Tamada, H., Tsukamoto, Y., Nishida, T. and Sasaki, F. 2000. Seasonal changes in the  
184 hypothalamo-pituitary-testes axis of the Japanese wood mouse (*Apodemus*  
185 *speciosus*). *The anatomical Record* 260(4): 366-372.
- 186 Matsubara, K., Nishida-Umehara, C., Tsuchiya, K., Nukaya, D. and Matsuda, Y. 2004.  
187 Karyotypic evolution of *Apodemus* (Muridae, Rodentia) inferred from comparative  
188 FISH analysis. *Chromosome Research* 12(4): 383-395.
- 189 Matsunami, M., Endo, D., Saitou, N., Suzuki, H. and Onuma, M. 2017. Draft genome  
190 sequence of Japanese wood mouse, *Apodemus speciosus*. *Data Brief* 16: 43-46.
- 191 Meguro, K., Kamotsu, K., Ohdaira, T., Nakagata, N., Nakata, A., Fukumoto, M., Miura,  
192 T. and Yamashiro, H. 2019. Induction of superovulation using inhibin antiserum and  
193 competence of embryo development in wild large Japanese field mice (*Apodemus*  
194 *speciosus*). *Reproduction in Domestic Animals* 54(12): 1637-1642.
- 195 Okano, T., Ishiniwa, H., Onuma, M., Shindo, J., Yokohata, Y. and Tamaoki, M. 2016.  
196 Effects of environmental radiation on testes and spermatogenesis in wild large  
197 Japanese field mice (*Apodemus speciosus*) from Fukushima. *Scientific Reports* 6:  
198 23601.

- 199 Okano, T., Onuma, M., Ishikawa, H., Azuma, N., Tamaoki, M., Nakajima, N., Shindo, J.  
200 and Yokohata, Y. 2015. Classification of the spermatogenic cycle, seasonal changes  
201 of seminiferous tubule morphology and estimation of the breeding season of the  
202 large Japanese field mouse (*Apodemus Speciosus*) in Toyama and Aomori  
203 Prefectures, Japan. *Journal of Veterinary Medical Science* 77(7): 799-807.
- 204 Sakai, Y., Sakamoto, S.H., Kato, G.A., Iwamoto, N., Ozaki, R., Eto, T., Shinohara, A.,  
205 Morita, T. and Koshimoto, C. 2013. Rearing method to induce natural mating of the  
206 large Japanese field mouse, *Apodemus speciosus*. *Honyuruikagaku* 53(1): 57-65. (In  
207 Japanese, Abstract in English)
- 208 Sato, J.J., Tasaka, Y., Tasaka, R., Gunji, K., Yamamoto, Y., Takada, Y., Uematsu, Y.,  
209 Sakai, E., Tateishi, T. and Yamaguchi, Y. 2017. Effect of isolation by continental  
210 islands in the Seto inland sea, Japan, on genetic diversity of the large Japanese field  
211 mouse, *Apodemus Speciosus* (Rodentia: Muridae), inferred from the mitochondrial  
212 Dloop region. *Zoological Science* 34(2): 112-121.
- 213 Shimba, H. and Kobayashi, T. 1969. A Robertsonian type polymorphism of the  
214 chromosomes in the field mouse, *Apodemus speciosus*. *Japanese Journal of Genetics*  
215 44(3):117-122.
- 216 Stecher, G., Tamura, K. and Kumar, S. 2020. Molecular Evolutionary Genetics Analysis  
217 (MEGA) for macOS. *Molecular Biology and Evolution* 37(4): 1237-1239.
- 218 Suzuki, Y., Tomozawa, M., Koizumi, Y., Tsuchiya, K. and Suzuki, H. 2015. Estimating  
219 the molecular evolution rates of mitochondrial genes referring to quaternary ice age  
220 events with inferred population expansion and dispersals in Japanese *Apodemus*.  
221 *BMC Evolutionary Biology* 15: 187.
- 222 Takino, S., Yamashiro, H., Sugano, Y., Fujishima, Y., Nakata, A., Kasai, K., Hayashi, G.,  
223 Urushihara, Y., Suzuki, M., Shinoda, H., Miura, T. and Fukumoto, M. 2017. Analysis  
224 of the effect of chronic and low-dose radiation exposure on spermatogenic cells of

225 male large Japanese field mice (*Apodemus Speciosus*) after the Fukushima Daiichi  
226 nuclear power plant accident. *Radiation Research* 187(2): 161-168.

227 Tamura, K., and Nei, M. 1993. Estimation of the number of nucleotide substitutions in  
228 the control region of mitochondrial DNA in humans and chimpanzees. *Molecular*  
229 *Biology and Evolution* 10(3): 512-526.

230 Tsuchiya, K. 1974. Cytological and biochemical studies of *Apodemus speciosus* group  
231 in Japan. *The Journal of Mammalogical Society of Japan* 6(2):67-87. (In Japanese,  
232 Abstract in English)

233 Yamagishi, M., Matsubara, K. and Kasaizumi, M. 2012. Molecular cytogenetic  
234 identification and characterization of Robertsonian chromosomes in the large  
235 Japanese field mouse (*Apodemus speciosus*) using FISH. *Zoological Science* 29(10):  
236 709-713.

237

238

239 **Figure Legends**

240 *Table S1 Primer sequences*

241

242 *Table S2 The captured mice and their accession numbers*

243

244 *Fig. 1 Sampling locations in this present study.*

245 The left map showed a general view of the Japanese archipelago and Yamaguchi

246 Prefecture (dotted circle). The right map showed the locations of Yamaguchi city (filled

247 circle) and Akiyoshidai (opened circle).

248

249 *Fig. 2 Evolutionary analysis by Maximum Likelihood method*

250 The evolutionary history was inferred by using the Maximum Likelihood method and

251 Tamura-Nei model. The tree with the highest log likelihood is shown. Initial trees for the

252 heuristic search was obtained automatically by applying Neighbor-Join and BioNJ

253 algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model, and

254 then selecting the topology with a superior log likelihood value.

255

256 *Fig. 3 Chromosomal spreads and counts*

257 a) Giemsa stained images of chromosomal spreads of each mice.

258 b) Histogram of the number of chromosomes counted.

259

260

261 *Fig. S1 Phylogenetic tree analysis of D-loop region sequences.*

262 Maximum Likelihood method was used as in Fig. 2.

263

264 *Fig. S2 Recovery of cells from tail tip.*

265 The upper panels showed the initial stage of culture. The lower showed the cells after  
266 passaging. Asterisks indicated tail tips. Bar=100  $\mu\text{m}$ .  
267

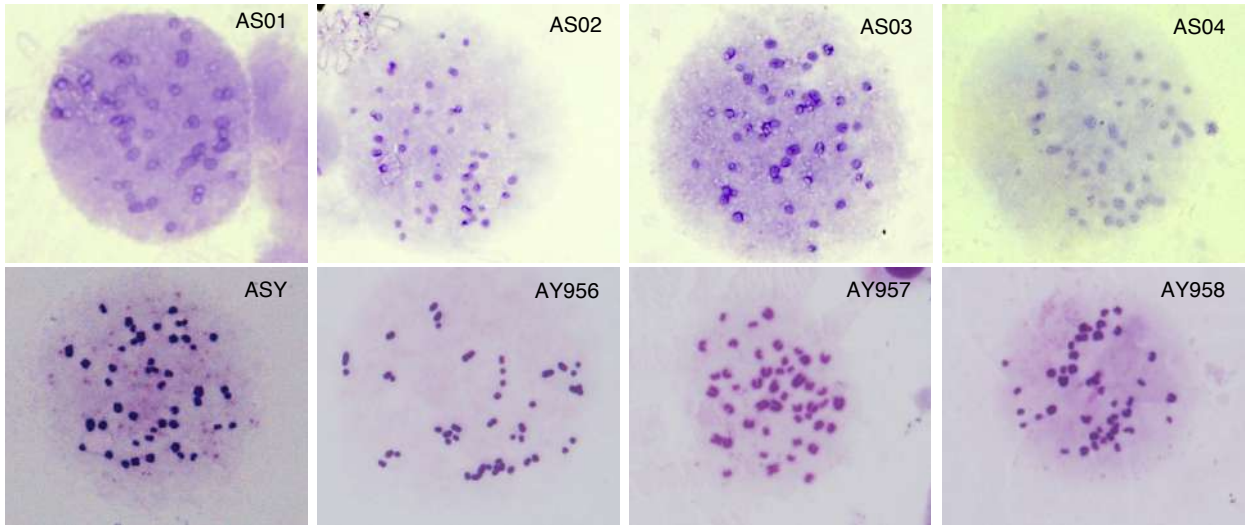


Figure1





a



b

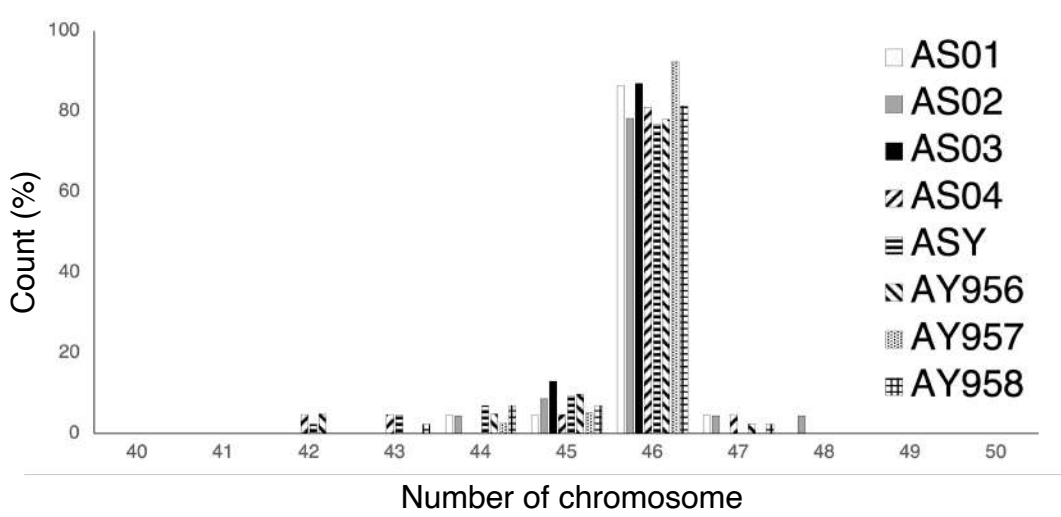
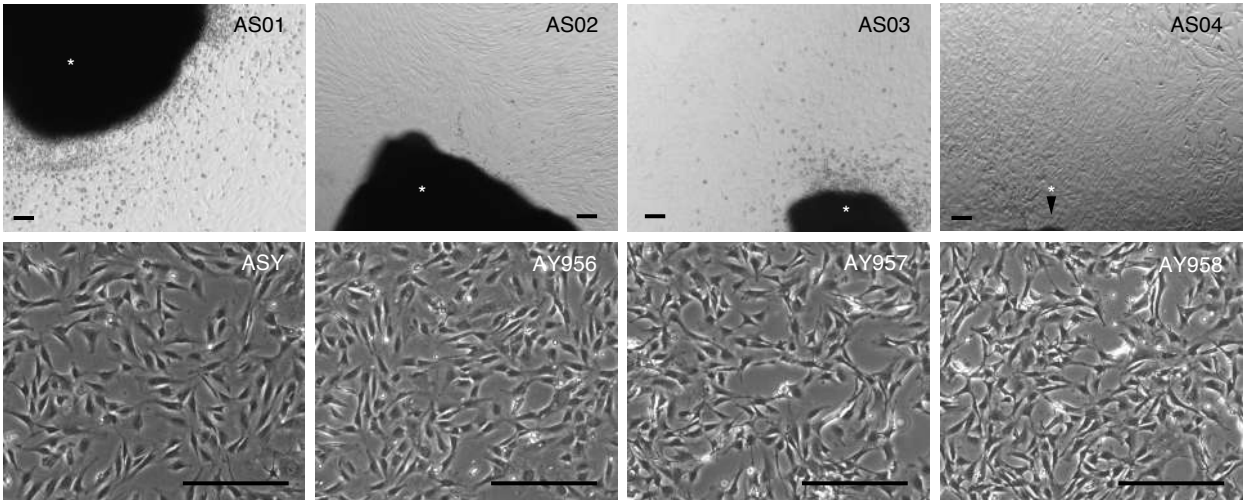


Figure3





FigureS2