Genetic lineage of the Amami islanders inferred from classical genetic markers

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Abstract

The peopling of mainland Japan and Okinawa has been gradually unveiled in the recent years, but previous anthropological studies dealing people in the Amami islands, located between mainland Japan and Okinawa, were less informative because of the lack of genetic data. In this study, we collected DNAs from 104 subjects in two of the Amami islands, Amami-Oshima island and Kikai island, and analyzed the D-loop region of mtDNA, four Y-STRs and four autosomal nonsynonymous SNPs to clarify the genetic structure of the Amami islanders comparing with peoples in Okinawa, mainland Japan and other regions in East Asia. We found that the Amami islanders showed genetically intermediate position between mainland Japan and Okinawa in mtDNA and Y-STR. However, the frequencies of several autosomal SNPs in the Amami islanders indicated significant difference from mainland Japanese, and it may be due to the gene flow from Okinawa but not natural selection. Furthermore, extremely high or low frequencies of several alleles implied the existence of founder effect in Kikai islanders. It should be noted that there is room for the interpretation of the results because of the small sample size and number of alleles in the present study, and geographically broad and detailed samplings and genome-wide analyses are awaited.

Keywords: Japanese, Amami islanders, Classical genetic markers,

1. Introduction

Peopling of Japan has been gradually unveiled in the recent years. The dual structure model (Hanihara, 1991) is a well-known hypothesis, which predicts that modern Japanese population was formed by the admixture of two populations, Jomon and Yayoi; Jomon peoples dwelled in the Japanese Archipelago since about 18,000 years ago and Yayoi peoples immigrated from Asian continent 2,000-3,000 years ago. This model is also supported largely by genomic/genetic studies (Jinam et al., 2012; Nakagome et al., 2015). According to this model, people living in the Ryukyu Archipelago (Fig. 1), southern end of Japan, are assumed to be strongly contributed from Jomon peoples as well as the Ainus who are indigenous in Hokkaido, northern most Japan (Fig. 1).

This model has been supported by previous genetic studies among the Ryukyuans, Ainus, mainland Japanese and other East Asian populations. Genetic similarity between the Ryukyuans and Ainus has been observed (Hammer et al., 2006; Koganebuchi et al. 2012), whereas the Ryukyuans are thought to be differentiated from mainland Japanese by the analyses of Y chromosome (Hammer & Horai, 1995; Hammer et al., 2006), mtDNA (Horai et al., 1996; Tanaka et al., 2004), autosomal markers (Omoto & Saitou, 1997) and genome-wide SNP data (Yamaguchi-Kabata et al., 2008). On the other hand, the Ryukyuans are rather genetically close to mainland Japanese than the Taiwan aborigines although Taiwan is geographically close to the Ryukyu Archipelago (Matsukusa et al., 2010). In addition, human skeletons in the late Pleistocene that are older than Jomon peoples were found in several sites in the Ryukyu Archipelago (Kobayashi et al., 1974; Nakagawa et al., 2010), but they seem to have no genetic relation with modern Ryukyuans (Sato et al., 2014). Furthermore,

genetic differences among the residents on the islands of the archipelago have been observed (Sato et al., 2014; Matsunami et al., 2021).

In those studies mentioned above, however, much attentions were not paid to the Amami islands which are located in the northern Ryukyu Archipelago (Fig. 1), and there have been historical differences between the Amami islands and the rest of the Ryukyu Archipelago (Okinawa prefecture). Some researchers claim that the Amami islands played important roles in the trade among East Asia including mainland Japan and China in around the 7–11th century and this distinguished the Amami islands from Okinawa (Takanashi, 2011; Yoshinari, 2011). In Kikai island, one of the Amami islands, there is the Gusuku Site group which is supposed to be local agency of the government of mainland Japan in the 9–13th century (Yoshinari, 2011; Takamiya et al., 2019), and this corresponds to the hypothesis that there was population migration into Ryukyu from the north via the Amami islands in around the 11–12th century (Takamiya, 2013). In the 15th century, the Amami islands were conquered by the Ryukyu Kingdom which was an independent country centered on main-island Okinawa (Yoshinari & Fuku, 2007), but since 1609, the Amami islands were directly ruled by the Satsuma-Han, one of the feudal domains in mainland Japan; this has brought about administrative and cultural differences between the Amami islands and Okinawa (Tsuha, 2012).

Despite these non-negligible historical and cultural features, few anthropological studies including genetic studies about the Amami islanders have been conducted. Dodo et al. (1998) exceptionally analyzed 22 cranial nonmetric traits of early modern Amami and Okinawa islanders and found that they shared morphological similarities. Nishiyama et al. (2012) indicated that there was a low but significant level of genetic difference between the Amami islanders and

mainland Japanese using SNP data, but their research was limited to only two islands, Tokuno island and Okinoerabu island (Fig. 1), in southern part of the Amami islands, and they concluded that studies which cover further north areas should be indispensable.

We then newly focused on two of the Amami islands, Amami-Oshima and Kikai island in this research. Amami-Oshima island (Fig. 1) is the largest island and has the largest population in the Amami islands (Kagoshima Prefecture, 2018a). Kikai island (Fig. 1) is a relatively small island with a population of about 7,000 (Kagoshima Prefecture, 2018b), located on the east of Amami-Oshima island, and assumed to had played a very important role in the relationship between the Ryukyu Archipelago and mainland Japan in around the 11–12th century (Takanashi, 2009; Yoshinari, 2011; Takamiya, 2013; Takamiya et al., 2019). Based on the previous studies and historical backgrounds described above, we hypothesized that people in these two islands might genetically be located in the intermediate position between Okinawa and mainland Japan. The purpose of this study is to clarify the genetic structure of the Amami islanders comparing with peoples in Okinawa, mainland Japan and other regions in East Asia.

2. Materials and methods

2.1. Subjects

A total of 104 Amami islanders (Fig. 1), 78 in Amami-Oshima island (49 males and 29 females) and 26 in Kikai island (15 males and 11 females), who were not genetically related to each other, were the subjects of this study. This research was approved by the Research Ethics Committee of the University of Tokyo, and all subjects provided informed consent. We collected nail or oral mucosa samples from the subjects. Their origins were confirmed by interview that at least one of the grandparents of each subject was born in the Amami islands.

2.2. DNA extraction

We extracted DNA from nail samples using ISOHAIR (Nippon Gene) and oral mucosa samples using High Pure PCR Template Preparation Kit (Roche).

2.3. Genotyping

We amplified the D-loop region (16024-16569 and 1-41 of the rCRS; Anderson et al., 1981; Andrews et al., 1999) of mtDNA by nested PCR method to avoid nuclear insert contaminations. The first PCR for 9 kb of mtDNA including the D-loop region was performed (primers: GENBANK_MT_26F & GENBANK_MT_5R) with an initial denaturation at 94°C for 2 min, followed by 25 cycles of denaturation at 98°C for 10 s, annealing at 72°C for 30 s, extension at 68°C for 10 min. The second PCR for the D-loop region was performed (primers: L15996F & H408R) with an initial denaturation at 94°C for 2 min, followed by 25 cycles of denaturation at 98°C for 10 s, annealing at 56°C for 30 s, extension at 68°C for 1 min. After confirmation by 2% agarose gel electrophoresis, PCR

products were purified using the PCR Clean-Up Mini Kit (FAVORGEN) and sequenced by Fasmac (Kanagawa, Japan) or Eurofins Genomics (Tokyo, Japan). The primers used for PCR amplifications and sequencing are listed in Supp. Table 1.

Four Y-STRs (DYS393, DYS19, DYS391 and DYS438) of which genetic diversities differed between Honshu (Fig. 1) and Okinawa (Uchihi et al., 2003) were selected. PCRs for these loci were performed with an initial denaturation at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 15 s, annealing at 53-59°C for 30 s, extension at 72°C for 1 min, followed by a final extension of 5 min at 72°C. After confirmed by 2% agarose gel electrophoresis, PCR products were purified using the PCR Clean-Up Mini Kit (FAVORGEN) and sequenced by Fasmac (Kanagawa, Japan) or Eurofins Genomics (Tokyo, Japan). The primers and annealing temperatures for each PCR amplification are listed in Supp. Table 2.

We genotyped four nonsynonymous autosomal SNPs (rs3827760, rs17822931, rs2070235 and rs14103) of which genotype frequencies differed between Hondo cluster which includes most of the individuals in mainland Japan and Ryukyu cluster which includes most of the individuals in Okinawa (Yamaguchi-Kabata et al., 2008) by PCR-RFLP. PCRs were performed with an initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 54-65°C for 30 s, extension at 72°C for 1 min, followed by a final extension of 5 min at 72°C. After confirmed by 2% agarose gel electrophoresis, PCR products were purified using the PCR Clean-Up Mini Kit (FAVORGEN) and digested with an appropriate restriction enzymes at 37°C for 1 hour. Thereafter, we electrophoresed these digested products on 4% agarose gel for genotype determination. The primers, annealing temperatures and restriction

enzymes for each locus are listed in Supp. Table 3.

2.4. Data analyses

The mtDNA sequences were aligned by using MEGA6 (Tamura et al., 2013) together with sequences of other Asian populations downloaded from NCBI (National Center for Biotechnology Information) database. Net genetic distances (d_A) between populations were calculated from these sequences under Tamura-Nei model (Tamura & Nei, 1993) with gamma distribution. Phylogenetic networks between populations based on d_A distances were constructed in the Neighbor-Net method (Bryant & Moulton, 2004) by using SplitsTree4 (Huson & Bryant, 2006). Pairwise F_{ST} between regions and nucleotide diversities within each population were calculated by using Arlequin ver 3.5.2.2 (Excoffier & Lischer, 2010), and Tajima's D (Tajima, 1989) within each population were calculated using DnaSP 5.10 (Librado & Rozas, 2009). Probabilities of Tajima's D were computed by 5,000 coalescence simulations.

We aligned the Y-STR sequences using MEGA6 and counted number of repeats. Pairwise R_{ST} between regions were calculated by using Arlequin ver 3.5.2.2 as well as gene diversities.

Fisher's exact test was used to compare the allele frequencies of the SNPs between populations.

3. Results

3.1. mtDNA

D-loop sequences (587 bp) were determined for a total of 102 subjects whose maternal grandmothers had been born in the Amami islands. Fig. 2 and Supp. Fig. 1 show the phylogenetic networks for 77 Amami-Oshima islanders, 25 Kikai islanders and other Asian populations. Amami-Oshima island and Kikai island were close to each other in both of the networks, and the Amami islanders did not form a clear cluster with the residents of Honshu, Kyushu, the Okinawa islands or any other populations. Nucleotide diversities and Tajima's D in each population are shown in Table 1. Nucleotide diversities in the Amami islands were almost same as those in other regions in Japan. Tajima's D values were significantly negative in the Amami islands (–1.613, P = 0.024) and Amami-Oshima island (–1.539, P = 0.038). Tajima's D value in Kikai island was negative but not significantly (–1.494, P = 0.050). Pairwise F_{ST} values between regions are shown in Table 2. F_{ST} value between Amami-Oshima island and Kikai island was not significant. F_{ST} values between Amami-Oshima island and Honshu, between Amami-Oshima island and Okinawa, between Amami-Oshima island and Ishigaki island and between Kikai island and Ishigaki island were significant.

3.2. Y-STR

Y-STR sequences were determined for 58 male subjects whose paternal grandfathers had been born in the Amami islands were determined. 25 different haplotypes were observed in the Amami islands (Supp. Table 4). Three of them were observed in Kikai island but not in Amami-Oshima island. The paternal

grandfather of one subject who had one of the three haplotypes (Ht11) had been born in Amami-Oshima island, and this subject was counted as Amami-Oshima samples in the Y-STR analyses. Comparing with data of other regions in Japan (Hashiyada et al., 2008), six haplotypes observed in Amami-Oshima island were not in Okinawa, and one haplotype was found only in Amami-Oshima island and Okinawa but not in any other regions. The allele frequency distributions of four loci were similar among regions (Supp. Table 5). Gene diversities of the four loci are shown in Supp. Table 6. In Amami-Oshima island, gene diversities of any loci did not significantly differ with Honshu or Okinawa. In Kikai island, gene diversities of the loci except DYS438 did not differ with other regions, and that of DYS438 was significantly higher than any other regions. Pairwise R_{ST} values between regions are shown in Table 3. R_{ST} value between Amami-Oshima island and Kikai island was significant. R_{ST} values between Amami-Oshima island and Okinawa and between Kikai island and Okinawa were not significant.

3.3. Autosomal SNPs

Genotypes of 91 subjects whose all four grandparents were born in the Amami islands were determined and allele frequencies were calculated.

The frequency of T allele in rs3827760 (*EDAR*) in Amami-Oshima island (0.385) was significantly higher than in Hondo cluster (0.222) ($P < 0.001$) and not significantly different from Ryukyu cluster (0.398) ($P = 0.426$). The frequency of T allele in Kikai island (0.647) was significantly higher than in Amami-Oshima island, Ryukyu cluster and Hondo cluster ($P = 0.005$, $P = 0.004$ and $P < 0.001$, respectively) (Table 4).

The frequency of G allele in rs17822931 (*ABCC11*) in Amami-Oshima island (0.311) was significantly higher than in Hondo cluster (0.121) ($P < 0.001$)

but not significantly different from Ryukyu cluster (0.258) (P = 0.123). The frequency of G allele in Kikai island (0.118) was significantly lower than in Ryukyu cluster (P = 0.044) and Amami-Oshima island (P = 0.015) but not significantly diffrent from Hondo cluster (P= 0.612) (Table 4).

The frequency of G allele in rs2070235 (*MYBL2*) in Amami-Oshima island (0.324) was significantly higher than in Hondo cluster (0.185) (P< 0.001) but not significantly different from Ryukyu cluster (0.291) (P= 0.228). The frequency of G allele in Kikai island was not significantly different from those in Amami-Oshima island, Ryukyu cluster and Hondo cluster (P = 0.324, P = 0.456 and P = 0.167, respectively) (Table 4).

The frequency of A allele in rs14103 (*C1orf212*) in Amami-Oshima island (0.791) was significantly lower than in Hondo cluster (0.855) (P= 0.022) and not significantly different from Ryukyu cluster (0.753) (P= 0.187), whereas, that in Kikai island was not different from those in Amami-Oshima island, Ryukyu cluster and Hondo cluster (P= 0.287, P= 0.126 and P= 0.562, respectively) (Table 4).

4. Discussion

Amami islanders showed a trend of Tajima's *D* values of mtDNA to be negative (Table 1). This trend is commonly observed in the populations of East Asia including main-island Okinawa, Miyako island, Ishigaki island and Honshu (Oota et al., 2002; Matsukusa et al., 2010). Purifying selection or recent population expansion would generally be a reason for the negative Tajima's *D* values (Tajima, 1989), but the former is unsuitable in this case because D-loop is a non-coding region. Therefore, this result suggests the presence of the past population expansion and/or gene flow from the surrounding regions, *i.e.* the Amani islanders were genetically not isolated for long.

Pairwise F_{ST} values (Table 2) and phylogenetic networks (Fig. 2 and Supp. Fig. 1) represent close genetic relations in the female lineages between Amami-Oshima island and Kikai island. The female lineages in Amami-Oshima island differed from Honshu, main-island Okinawa and Ishigaki island, and those in Kikai island differed from Ishigaki island (Table 2). On the other hand, the female lineages in Amami-Oshima island and Kikai island did not differ from those in Miyako island; however, no historical records that link geographically separated people in the Amami islands and Miyako island have been recognized.

Gene diversities (Supp. Table 6) and R_{ST} values (Table 3) of the four Y-STRs indicated that the male lineages in Amami-Oshima island located between those in Honshu and Okinawa but those in Kikai island showed some genetic deviation from these three populations including Amami-Oshima islanders. This may be due to the founder effect caused by the small population size of Kikai islanders (Kagoshima Prefecture, 2018b) and to the difference in the degree of contacts with mainland Japanese between Kikai and Amami-Oshima

islanders (Takanashi, 2009; Yoshinari, 2011; Takamiya, 2013; Takamiya et al., 2019).

Comparing the maternal (mtDNA) and paternal (Y-STR) genes, we found that the mean of pairwise R_{ST} values of Y-STR had a trend that the larger the value is, the smaller population size is, whereas the mean F_{ST} values of mtDNA did not (Table 5). This suggests that males are genetically more divergent than females because of patrilocality; in fact, female migration was suggested frequent in the Ryukyu Archipelago (Matsukusa et al., 2010), and we confirmed this in the Amami islands.

Four autosomal SNPs whose allele frequencies differed between Okinawa and the rest of Japan (Yamaguchi-Kabata et al., 2008) were selected to identify the Amami islanders' genealogic position and also to search for the traits of selective pressure if it ever exerted in these populations. The allele frequencies of the four SNPs in Amami-Oshima islanders were similar to Ryukyu cluster and significantly different from Hondo cluster (Table 4), and this may be due to gene flow from Okinawa. On the other hand in Kikai islanders, T allele frequency of rs3827760 (*EDAR*) was significantly higher than those in other three populations, and G allele frequency of rs17822931 (*ABCC11*) was significantly lower than those in Ryukyu cluster and Amami-Oshima islanders but not lower than that in Hondo cluster. These can be explained by the founder effect, as observed in Y-STR: DYS438. The presence of the selective pressure is hard to recognize in these loci.

In conclusion, populations in Amami-Oshima island and Kikai island showed genetically intermediate position between mainland Japan and Okinawa in mtDNA and Y-STR. However, the frequencies of several autosomal SNPs in the Amami islanders indicated significant difference from mainland Japanese. The *EDAR* and *ABCC11* of mainland Japanese are assumed to have been under some

kind of natural selection; however, those of the Amami islanders may have been affected by the gene flow from Okinawa but not natural selection. Furthermore, the extremely high or low frequencies of several alleles implied the existence of founder effect in Kikai island. It should be noted that there is room for the interpretation of the results because of small sample size (especially in Kikai island) and number of alleles in the present study, and geographically broad and detailed samplings and genome-wide analyses are awaited.

Declarations of interest: none

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References

Anderson, S., Bankier, A.T., Barrell, B.G., de Bruijn, M.H.L., Coulson, A.R., Drouin, J., Eperon, I.C., Nierlich, D.P., Roe, B.A., Sanger, F., Schreier, P.H., Smith, A.J.H., Staden, R., Young, I.G., 1981. Sequence and organization of the human mitochondrial genome. Nature 290, 457–465. https://doi.org/10.1038/290457a0

Andrews, R.M., Kubacka, I., Chinnery, P.F., Lightowlers, R.N., Turnbull, D.M., Howell, N., 1999. Reanalysis and revision of the Cambridge reference sequence for human mitochondrial DNA. Nat. Genet. 23, 147–147. https://doi.org/10.1038/13779

Ayub, Q., 2000. Identification and characterisation of novel human Y-chromosomal microsatellites from sequence database information. Nucleic Acids Res. 28, 8e – 8. https://doi.org/10.1093/nar/28.2.e8

Bryant, D., Moulton, V., 2004. Neighbor-Net: An Agglomerative Method for the Construction of Phylogenetic Networks. Mol. Biol. Evol. 21, 255–265. https://doi.org/10.1093/molbev/msh018

Butler, J.M., Schoske, R., Vallone, P.M., Kline, M.C., Redd, A.J., Hammer, M.F., 2002. A novel multiplex for simultaneous amplification of 20 Y chromosome STR markers. Forensic Sci. Int. 129, 10–24. https://doi.org/10.1016/S0379-0738(02)00195-0

de Knijff, P., Kayser, M., Caglià, A., Corach, D., Fretwell, N., Gehrig, C., Graziosi, G., Heidorn, F., Herrmann, S., Herzog, B., Hidding, M., Honda, K., Jobling, M., Krawczak, M., Leim, K., Meuser, S., Meyer, E., Oesterreich, W., Pandya, A., Parson, W., Penacino, G., Perez-Lezaun, A., Piccinini, A., Prinz, M., Schmitt, C., Schneider, P.M., Szibor, R., Teifel-Greding, J., Weichhold, G., Roewer, L., 1997. Chromosome Y microsatellites: population genetic and

evolutionary aspects. Int. J. Legal Med. 110, 134–140. https://doi.org/10.1007/s004140050052

Dodo, Y., Doi, N., Kondo, O., 1998. Ainu and Ryukyuan cranial nonmetric variation: Evidence which disputes the Ainu-Ryukyu common origin theory. Anthropol. Sci. 106, 99–120. https://doi.org/10.1537/ase.106.99

Excoffier, L., Lischer, H.E.L., 2010. Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. Mol. Ecol. Resour. 10, 564–567. https://doi.org/10.1111/j.1755-0998.2010.02847.x

Hammer, M.F., Horai, S., 1995. Y chromosomal DNA variation and the peopling of Japan. Am. J. Hum. Genet. 56, 951–962.

Hammer, M.F., Karafet, T.M., Park, H., Omoto, K., Harihara, S., Stoneking, M., Horai, S., 2006. Dual origins of the Japanese: Common ground for hunter-gatherer and farmer Y chromosomes. J. Hum. Genet. 51, 47–58. https://doi.org/10.1007/s10038-005-0322-0

Hanihara, K., 1991. Dual Structure Model for the Population History of the Japanese. Japan Rev. 2, 1–33.

Hashiyada, M., Umetsu, K., Yuasa, I., Tamura, A., Matsusue, A., Suzuki, K., Kashimura, S., Funayama, M., 2008. Population genetics of 17 Y-chromosomal STR loci in Japanese. Forensic Sci. Int. Genet. 2, 69–70. https://doi.org/10.1016/j.fsigen.2008.01.001

Hayashida, M., Ota, T., Iwao-Koizumi, K., Murata, S., 2010. Simple SNP genotyping of hair thickness-determining gene EDAR and human earwax-type gene ABCC11 for education of genetic tests. Bull. Mukogawa Women's Univ. Nat. Sci. 58, 43–47. https://doi.org/10.14993/00000508

Horai, S., Murayama, K., Hayasaka, K., Matsubayashi, S., Hattori, Y., Fucharoen,

G., Harihara, S., Park, K.S., Omoto, K., Pan, I.H., 1996. mtDNA polymorphism in East Asian populations, with special reference to the peopling of Japan. Am. J. Hum. Genet. 59, 579–590.

Huson, D.H., Bryant, D., 2006. Application of Phylogenetic Networks in Evolutionary Studies. Mol. Biol. Evol. 23, 254–267. https://doi.org/10.1093/molbev/msj030

Jinam, T., Nishida, N., Hirai, M., Kawamura, S., Oota, H., Umetsu, K., Kimura, R., Ohashi, J., Tajima, A., Yamamoto, T., Tanabe, H., Mano, S., Suto, Y., Kaname, T., Naritomi, K., Yanagi, K., Niikawa, N., Omoto, K., Tokunaga, K., Saitou, N., 2012. The history of human populations in the Japanese Archipelago inferred from genome-wide SNP data with a special reference to the Ainu and the Ryukyuan populations. J. Hum. Genet. 57, 787–795. https://doi.org/10.1038/jhg.2012.114

Kagoshima Prefecture. (2018a) Amami-Oshima Kakeroma shotō no gaiyō. http://www.pref.kagoshima.jp/ac07/pr/shima/gaiyo/amami/amami_top.html (accessed 19 April 2021). (in Japanese)

Kagoshima Prefecture. (2018b) Kikaijima no gaiyō. http://www.pref.kagoshima.jp/ac07/pr/shima/gaiyo/kikai/kikai_top.html (accessed 19 April 2021). (in Japanese)

Kayser, M., Caglià, A., Corach, D., Fretwell, N., Gehrig, C., Graziosi, G., Heidorn, F., Herrmann, S., Herzog, B., Hidding, M., Honda, K., Jobling, M., Krawczak, M., Leim, K., Meuser, S., Meyer, E., Oesterreich, W., Pandya, A., Parson, W., Penacino, G., Perez-Lezaun, A., Piccinini, A., Prinz, M., Schmitt, C., Schneider, P.M., Szibor, R., Teifel-Greding, J., Weichhold, G., De Knijff, P., Roewer, L., 1997. Evaluation of Y-chromosomal STRs: A multicenter study. Int. J. Legal Med. 110, 125–133. https://doi.org/10.1007/s004140050051

Kobayashi, H., Hirose, T., Sugino, M., Watanabe, N., 1974. University of Tokyo Radiocarbon Measurements V. Radiocarbon 16, 381–387. https://doi.org/10.1017/S0033822200059671

Koganebuchi, K., Katsumura, T., Nakagome, S., Ishida, H., Kawamura, S., Oota, H., Ohashi, J., Yamamoto, T., Nishida, N., Tajima, A., Tanabe, H., Umetsu, K., Saitou, N., Tokunaga, K., Suto, Y., Hirai, M., Kimura, R., Mano, S., Kawamura, S., Oota, H., Omoto, K., 2012. Autosomal and Y-chromosomal STR markers reveal a close relationship between Hokkaido Ainu and Ryukyu islanders. Anthropol. Sci. 120, 199–208. https://doi.org/10.1537/ase.120322

Librado, P., Rozas, J., 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25, 1451–1452. https://doi.org/10.1093/bioinformatics/btp187

Matsukusa, H., Oota, H., Haneji, K., Toma, T., Kawamura, S., Ishida, H., 2010. A genetic analysis of the sakishima islanders reveals no relationship with taiwan aborigines but shared ancestry with ainu and main-island Japanese. Am. J. Phys. Anthropol. 142, 211–223. https://doi.org/10.1002/ajpa.21212

Matsunami, M., Koganebuchi, K., Imamura, M., Ishida, H., Kimura, R., Maeda, S., 2021. Fine-scale genetic structure and demographic history in the Miyako Islands of the Ryukyu Archipelago. Mol. Biol. Evol. https://doi.org/10.1093/molbev/msab005

Nakagawa, R., Doi, N., Nishioka, Y., Nunami, S., Yamauchi, H., Fujita, M., Yamazaki, S., Yamamoto, M., Katagiri, C., Mukai, H., Matsuzaki, H., Gakuhari, T., Takigami, M., Yoneda, M., 2010. Pleistocene human remains from Shiraho-Saonetabaru Cave on Ishigaki Island, Okinawa, Japan, and their radiocarbon dating. Anthropol. Sci. 118, 173–183. https://doi.org/10.1537/ase.091214

Nakagome, S., Sato, T., Ishida, H., Hanihara, T., Yamaguchi, T., Kimura, R., Mano, S., Oota, H., 2015. Model-Based Verification of Hypotheses on the Origin of Modern Japanese Revisited by Bayesian Inference Based on Genome-Wide SNP Data. Mol. Biol. Evol. 32, 1533–1543. https://doi.org/10.1093/molbev/msv045

Nishiyama, T., Kishino, H., Suzuki, S., Ando, R., Niimura, H., Uemura, H., Horita, M., Ohnaka, K., Kuriyama, N., Mikami, H., Takashima, N., Mastuo, K., Guang, Y., Wakai, K., Hamajima, N., Tanaka, H., 2012. Detailed Analysis of Japanese Population Substructure with a Focus on the Southwest Islands of Japan. PLoS One 7, e35000. https://doi.org/10.1371/journal.pone.0035000

Omoto, K., Saitou, N., 1997. Genetic origins of the Japanese: A partial support for the dual structure hypothesis. Am. J. Phys. Anthropol. 102, 437–446. https://doi.org/10.1002/(SICI)1096-8644(199704)102:4<437::AID-AJPA1>3.0.CO;2-P

Oota, H., Kitano, T., Jin, F., Yuasa, I., Wang, L., Ueda, S., Saitou, N., Stoneking, M., 2002. Extreme mtDNA homogeneity in continental Asian populations. Am. J. Phys. Anthropol. 118, 146–153. https://doi.org/10.1002/ajpa.10056

Roewer, L., Epplen, J.T., 1992. Rapid and sensitive typing of forensic stains by PCR amplification of polymorphic simple repeat sequences in case work. Forensic Sci. Int. 53, 163–171. https://doi.org/https://doi.org/10.1016/0379-0738(92)90193-Z

Sato, T., Nakagome, S., Watanabe, C., Yamaguchi, K., Kawaguchi, A., Koganebuchi, K., Haneji, K., Yamaguchi, T., Hanihara, T., Yamamoto, K., Ishida, H., Mano, S., Kimura, R., Oota, H., 2014. Genome-wide SNP analysis reveals population structure and demographic history of the ryukyu islanders in the southern part of the japanese archipelago. Mol. Biol. Evol. 31, 2929–

2940. https://doi.org/10.1093/molbev/msu230

Stanford Genome Technology Center, 2002. List of primers used for resequencing the mitochondrial genome. http://insertion.stanford.edu/primers.html (accessed 19 April 2021).

Tajima, A., Sun, C.-S., Pan, I.-H., Ishida, T., Saitou, N., Horai, S., 2003. Mitochondrial DNA polymorphisms in nine aboriginal groups of Taiwan: implications for the population history of aboriginal Taiwanese. Hum. Genet. 113, 24–33. https://doi.org/10.1007/s00439-003-0945-1

Tajima, F., 1989. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics 123, 585–595.

Takamiya, H., 2013. Amami Okinawa shoto eno hito no idō, in Intoh, H. (Eds), Jinrui no idōshi. Rinsen Shoten, Kyoto, pp. 182–197. (in Japanese)

Takamiya, H., Katagiri, C., Yamasaki, S., Fujita, M., 2019. Human Colonization of the Central Ryukyus (Amami and Okinawa Archipelagos), Japan. J. Isl. Coast. Archaeol. 14, 375–393. https://doi.org/10.1080/15564894.2018.1501443

Takanashi, O., 2009. Doki dōtai kara kangaeru "Nihon bunka no nanzen", in Takanashi, O., Abe, M., Nakamoto, K., Yoshinari, N, Okinawa bunka wa doko kara kitaka: Gusuku jidai to iu kakki. Shinwasha, Tokyo, pp. 47–132. (in Japanese)

Takanashi, O., 2011. Nampō sekai eno hirogari: Ritsuryō kokka to Ryukyu-ko, in Arano, Y., Ishii, M., Murai, S. (Eds), Ritsuryō kokka to Higashi Ajia. Yoshikawakobunkan, Tokyo, pp. 319–333. (in Japanese)

Tamura, K., Nei, M., 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol. Biol. Evol. 10, 512–526. https://doi.org/10.1093/oxfordjournals.molbev.a040023

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol. Biol. Evol. 30, 2725–2729.

Tanaka, M., Cabrera, V.M., González, A.M., Larruga, J.M., Takeyasu, T., Fuku, N., Guo, L.J., Hirose, R., Fujita, Y., Kurata, M., Shinoda, K.I., Umetsu, K., Yamada, Y., Oshida, Y., Sato, Y., Hattori, N., Mizuno, Y., Arai, Y., Hirose, N., Ohta, S., Ogawa, O., Tanaka, Y., Kawamori, R., Shamoto-Nagai, M., Maruyama, W., Shimokata, H., Suzuki, R., Shimodaira, H., 2004. Mitochondrial genome variation in Eastern Asia and the peopling of Japan. Genome Res. 14, 1832–1850. https://doi.org/10.1101/gr.2286304

Tsuha, T., 2012. Okinawa gawa kara mita Amami no bunka henyō. Daiichi Shobo, Tokyo. (in Japanese)

Uchihi, R., Yamamoto, T., Usuda, K., Yoshimoto, T., Tanaka, M., Tokunaga, S., Kurihara, R., Tokunaga, K., Katsumata, Y., 2003. Haplotype analysis with 14 Y-STR loci using 2 multiplex amplification and typing systems in 2 regional populations in Japan. Int. J. Legal Med. 117, 34–38. https://doi.org/10.1007/s00414-002-0319-6

Vigilant, L., Pennington, R., Harpending, H., Kocher, T.D., Wilson, A.C., 1989. Mitochondrial DNA sequences in single hairs from a southern African population. Proc. Natl. Acad. Sci. U. S. A. 86, 9350–9354. https://doi.org/10.1073/pnas.86.23.9350

Yamaguchi-Kabata, Y., Nakazono, K., Takahashi, A., Saito, S., Hosono, N., Kubo, M., Nakamura, Y., Kamatani, N., 2008. Japanese Population Structure, Based on SNP Genotypes from 7003 Individuals Compared to Other Ethnic Groups: Effects on Population-Based Association Studies. Am. J. Hum. Genet. 83, 445–456. https://doi.org/10.1016/j.ajhg.2008.08.019

Yamaguchi-Kabata, Y., Tsunoda, T., Kumasaka, N., Takahashi, A., Hosono, N., Kubo, M., Nakamura, Y., Kamatani, N., 2012. Genetic differences in the two main groups of the Japanese population based on autosomal SNPs and haplotypes. J. Hum. Genet. 57, 326–334. https://doi.org/10.1038/jhg.2012.26

Yoshinari, N., 2011. Ryukyu no seiritsu: Ijū to kōeki no rekishi. Nanpou Shinsha, Kagoshima. (in Japanese)

Yoshinari, N., Fuku, H., 2007. Ryukyu ōkoku tanjō: Amami shotō shi kara. Shinwasha, Tokyo. (in Japanese)

Table 1. Nucleotide diversities and Tajima's D values of mtDNA

	Size	Nucleotide diversity	Tajima's *D*	*P* value	Ref.
Amami islands	102	0.019 ± 0.010	-1.613	0.024	Present study
Amami-Oshima island	78	0.019 ± 0.010	-1.516	0.042	Present study
Kikai island	24	0.020 ± 0.011	-1.494	0.050	Present study
Main-island Okinawa	95	0.019 ± 0.010	-1.972	0.004	Matsukusa et al., 2010
Miyako island	66	0.015 ± 0.008	-1.916	0.008	Matsukusa et al., 2010
Ishigaki island	63	0.016 ± 0.008	-1.582	0.039	Matsukusa et al., 2010
Honshu	89	0.015 ± 0.008	-2.050	0.002	Oota et al., 2002

For the analyses of mtDNA, one subject from Kikai island whose maternal grandmother was born in Amami-Oshima island was excluded from Kikai island samples and included in Amami-Oshima island samples.

Table 2. Pairwise F_{ST} values of mtDNA

	Amami-Oshima island	Kikai island	Main-island Okinawa	Miyako island	Ishigaki island	Kyushu	Honshu
Amami-Oshima island (n=78)	0						
Kikai island (n=24)	-0.007	0					
Main-island Okinawa (n=95)	**0.026**	0.003	0				
Miyako island (n=66)	0.004	-0.001	**0.034**	0			
Ishigaki island (n=63)	**0.044**	**0.028**	**0.013**	**0.043**	0		
Kyushu (n=104)	-0.001	-0.004	**0.036**	**0.013**	**0.055**	0	
Honshu (n=89)	**0.009**	0.010	**0.043**	**0.012**	**0.045**	**0.008**	0

Data of the regions except Amami-Oshima island and Kikai island were taken from Matsukusa et al. (2010). Significant differences are in bold ($p < 0.05$).

Table 3. Pairwise R_{ST} values of Y-STR

		Amami-Oshima island	Kikai island	Okinawa	Kyushu		Honshu		
					Fukuoka	Osaka	Tottori	Yamagata	Miyagi
Amami-Oshima island (n=48)		0							
Kikai island (n=10)		**0.113**	0						
Okinawa (n=83)		0.027	0.079	0					
Kyushu	Fukuoka (n=212)	-0.002	**0.102**	0.010	0				
Honshu	Osaka (n=131)	-0.010	**0.140**	**0.031**	0.001	0			
	Tottori (n=243)	-0.008	**0.117**	**0.017**	-0.003	-0.003	0		
	Yamagata (n=201)	-0.011	**0.161**	**0.047**	**0.010**	-0.004	0.002	0	
	Miyagi (n=296)	-0.004	**0.120**	**0.031**	0.001	-0.004	0.000	0.002	0

Data of the regions except Amami-Oshima island and Kikai island were taken from Hashiyada et al. (2008). Significant differences are in bold ($p < 0.05$).

Table 4. Allele frequencies of four autosamal SNPs

SNP	Gene	Allele frequencies								*P* value					
		Amami-Oshima island		Kikai island		Ryukyu		Hondo		Between Amami-Oshima and Kikai	Between Amami-Oshima and Ryukyu	Between Amami-Oshima and Hondo	Between Kikai and Ryukyu	Between Kikai and Hondo	Between Ryukyu and Hondo
		Allele 1	Allele 2	Allele 1	Allele 2	Allele 1	Allele 2	Allele 1	Allele 2						
rs3827760	*EDAR*	0.385	0.615	0.647	0.353	0.398	0.602	0.222	0.778	0.005	0.426	< 0.001	0.004	< 0.001	< 0.001
rs17822931	*ABCC11*	0.311	0.689	0.118	0.882	0.258	0.742	0.121	0.879	0.015	0.123	< 0.001	0.044	0.612	< 0.001
rs2070235	*MYBL2*	0.324	0.676	0.265	0.735	0.291	0.709	0.185	0.815	0.324	0.228	< 0.001	0.456	0.167	< 0.001
rs14103	*C1orf212*	0.791	0.209	0.853	0.147	0.753	0.247	0.855	0.145	0.287	0.187	0.022	0.126	0.562	< 0.001

Data of Ryukyu and Hondo clusters were taken from Yamaguchi-Kabata et al. (2008) for rs3827760 and rs17822931, and Yamaguchi-Kabata et al. (2012) for rs2070235 and rs14103.

Table 5. Mean of pairwise FST values of mtDNA and pairwise RST values of Y-STR

	mtDNA	Y-STR
	F_{ST}	R_{ST}
Amami islands	-0.007	0.113
Ryukyu Archipelago	0.019	0.073
Ryukyu and Kyushu	0.021	0.055
Japan	0.020	0.034

Data used for calculation were taken from Matsukusa et al. (2010) and Hashiyada et al. (2008) except those of the Amami islands.

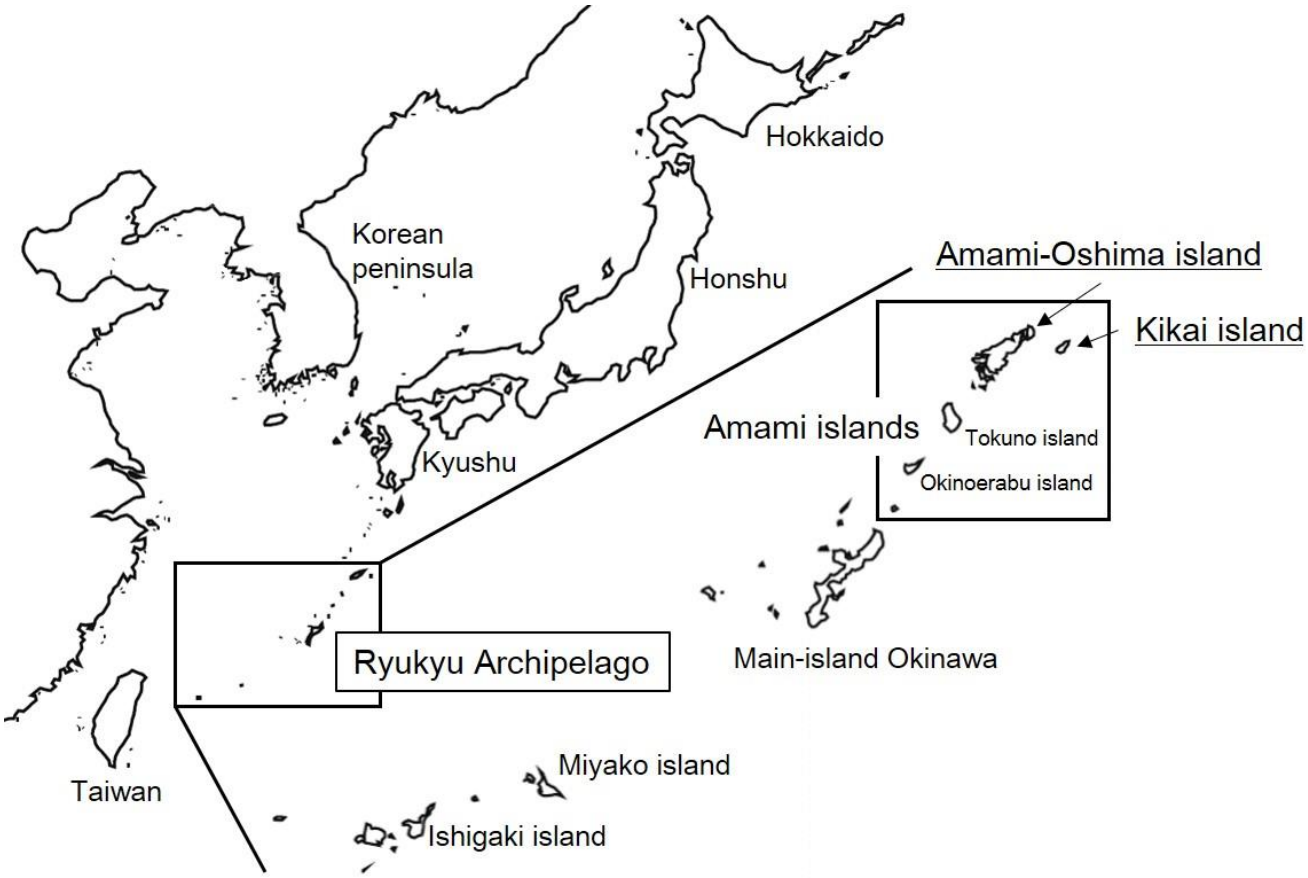


Fig. 1. Map of East Asia. Amami-Oshima island and Kikai island are placed on the northern edge of the Ryukyu Archipelago. Main-island Okinawa, Miyako island and Ishigaki island are included in Okinawa. Hokkaido, Honshu and Kyushu are included in mainland Japan (Hondo).

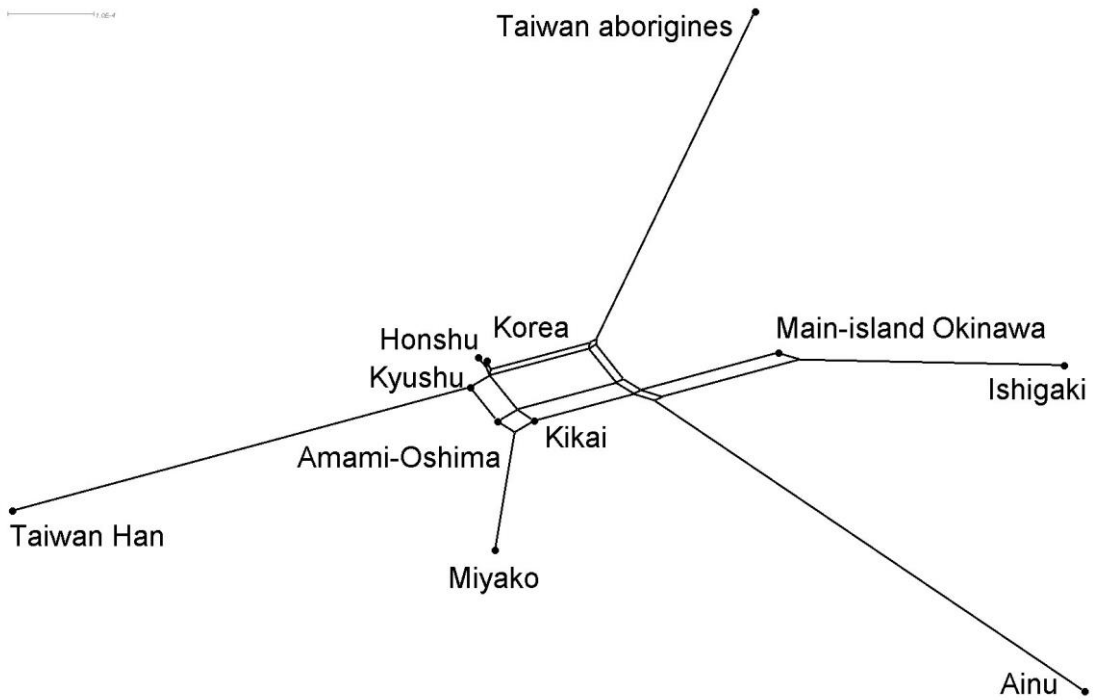


Fig. 2. Phylogenetic network between populations based on d_A distances calculated from 337 bp of D-loop region. Data of Miyako, Ishigaki and Main-island Okinawa were taken from Matsukusa et al. (2010). Data of Honshu were taken from Oota et al. (2002). Data of Ainu, Korea and Taiwan Han were taken from Horai et al. (1996). Data of Taiwan aborigines were taken from Tajima et al. (2003).

Supp. Table 1. Primers used for PCR and sequencing of mtDNA

Primer name	Sequence (5'-3')	Use	Ref.
GENBANK_MT_26F	CGGCTTCGACCCTATATCC	PCR	http://insertion.stanford.edu/primers.html
L15996F	CTCCACCATTAGCACCCAAAGC	PCR, sequencing	Vigilant et al., 1989
L16223F	AGCAAGTACAGCAATCAACC	Sequencing	Vigilant et al., 1989
H16401R	TGATTTCACGGAGGATGGTG	Sequencing	Vigilant et al., 1989
H408R	CTGTTAAAAGTGCATACCGCCA	PCR, sequencing	Vigilant et al., 1989
GENBANK_MT_5R	CCATAGGGTCTTCTCGTCTTG	PCR	http://insertion.stanford.edu/primers.html

Supp. Table 2. Primers used for PCR and sequencing of Y-STR

Primer name	Sequence (5'-3')	Annealing temperature	Ref.
DYS393-F	GTGGTCTTCTACTTGTGTCAATAC	53°C	Kayser, et al., 1997; de Kniff, et al., 1997
DYS393-R	AACTCAAGTCCAAAAAATGAGG		
DYS19-F	CTACTGAGTTTCTGTTATAGT	53°C	Roewer and Epplen., 1992
DYS19-R	ATGGCCATGTAGTGAGGACA		
DYS19-F2	ACTACTGAGTTTCTGTTATAGTGTTTTT	59°C	Butler et al., 2002
DYS19-R2	GTCAATCTCTGCACCTGGAAAT		
DYS391-F	CTATTCATTCAATCATACACCCA	53°C	Kayser, et al., 1997; de Kniff, et al., 1997
DYS391-R	GATTCTTTGTGGTGGGTCTG		
DYS438-F	TGGGGAATAGTTGAACGGTAA	53°C	Ayub et al., 2000
DYS438-R	GTGGCAGACGCCTATAATCC		
DYS438-F2	CCAAAATTAGTGGGGAATAGTTG	59°C	Butler et al., 2002
DYS438-R2	GATCACCCAGGGTCTGGAGTT		

Supp. Table 3. Primers and restriction enzymes used for PCR-RFLP of autosomal SNPs

Primer name	Sequence (5'-3')	Annealing temperature	Restriction enzyme	Ref.
EDAR-RFLP-F	AGGTCTTAGCCCCACGGAACTGCCAT	65°C	*Fnu4H I*	Hayashida et al., 2010
EDAR-RFLP-R	GGACTCCACAGCATCCAACCGCTC			
ABCC11-RFLP-F	TGCAAAGAGATTCCACCAGTT	54°C	*Dde I*	Hayashida et al., 2010
ABCC11-RFLP-R	AAGGTCTTCATTTTCTAGACAGC			
MYBL2-F	GGATGGCCACACCATCTCAG	62°C	*BanI*	Present study
MYBL2-R	GCCAGGTCTCGTTTTGCTCA			
LOC113444-F	CCAGCAGTGCACCAGTAAAC	58°C	*Nco I*	Present study
LOC113444-R	GGGCCCTATGGTCCTACTGT			

Supp. Table 4. Haplotypes observed in Amami islands and numbers of individuals in each region

Ht	DYS393	DYS19	DYS391	DYS438	Amami-Oshima island (n=47)	Kikai island (n=11)	Okinawa (n=83)	Kyushu		Honshu		
								Fukuoka (n=212)	Osaka (n=131)	Tottori (n=243)	Yamagata (n=201)	Miyagi (n=296)
1	12	14	10	10	2	0	0	1	0	1	1	3
2	12	14	10	11	1	1	0	4	6	5	6	7
3	12	15	10	10	1	1	2	10	4	12	1	8
4	12	16	10	10	5	0	2	8	3	9	7	8
5	12	17	10	10	3	0	2	6	1	12	2	4
6	13	13	10	10	1	2	1	0	0	7	2	3
7	13	14	10	10	1	0	0	2	3	2	4	4
8	13	15	10	10	1	0	3	13	12	10	16	28
9	13	15	10	11	1	1	0	2	1	4	1	7
10	13	15	10	13	8	0	10	38	21	31	43	51
11	13	15	11	10	0	1	4	3	0	1	1	5
12	13	16	10	10	2	0	10	17	4	10	13	24
13	13	16	10	13	2	0	1	9	12	18	16	11
14	13	16	10	14	2	0	0	0	0	0	1	1
15	13	16	11	10	2	0	1	0	0	3	0	1
16	13	17	10	10	1	2	16	24	14	23	25	32
17	13	17	11	10	1	0	2	3	2	7	4	3
18	14	13	10	10	3	0	6	6	4	8	6	2
19	14	15	10	9	0	1	1	1	0	0	1	0
20	14	15	10	10	3	0	0	5	5	12	5	8
21	14	15	10	13	3	0	2	1	1	5	2	1
22	14	16	10	9	1	1	3	4	0	0	0	1
23	14	16	11	13	2	0	4	0	0	0	0	0
24	14	17	10	9	1	0	2	1	0	0	0	0
25	15	13	10	10	0	1	2	2	0	0	0	0
others					0	0	9	52	38	63	44	84

Data of the regions except Amami-Oshima island and Kikai island were taken from Hashiyada et al. (2008).

Supp. Table 5. Allele frequencies of four Y-STRs

DYS393

Alleles	Frequency			
	Amami-Oshima island (n=48)	Kikai island (n=10)	Okinawa (n=87)	Hounshu (n=207)
11	0	0	0	0.024
12	0.250	0.200	0.230	0.222
13	0.479	0.500	0.506	0.618
14	0.271	0.200	0.218	0.126
15	0	0.100	0.046	0.010

DYS391

Alleles	Frequency			
	Amami-Oshima island (n=48)	Kikai island (n=10)	Okinawa (n=87)	Hounshu (n=207)
9	0	0	0	0.039
10	0.875	1	0.805	0.865
11	0.125	0	0.184	0.092
12	0	0	0.012	0.005

DYS19

Alleles	Frequency			
	Amami-Oshima island (n=48)	Kikai island (n=10)	Okinawa (n=87)	Hounshu (n=207)
13	0.083	0.300	0.081	0.048
14	0.083	0.100	0.058	0.058
15	0.375	0.300	0.299	0.488
16	0.333	0.100	0.287	0.217
17	0.125	0.200	0.276	0.184
18	0	0	0	0.005

DYS438

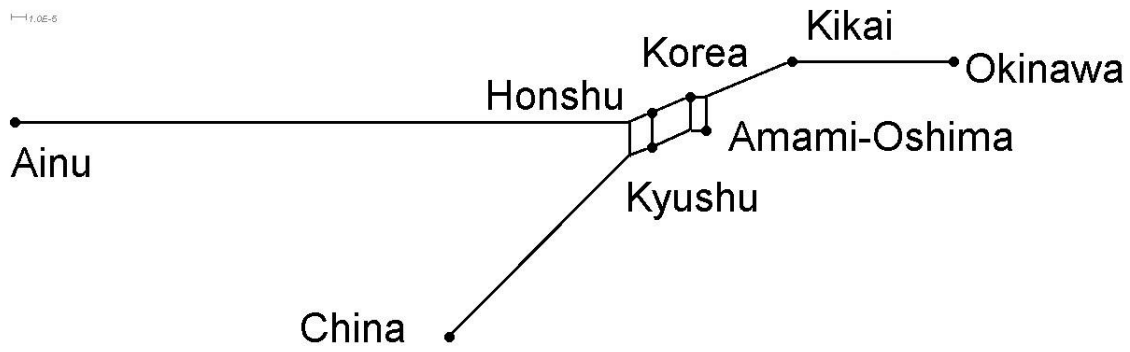
Alleles	Frequency			
	Amami-Oshima island (n=48)	Kikai island (n=10)	Okinawa (n=87)	Hounshu (n=207)
8	0	0	0	0.005
9	0.042	0.200	0.058	0.014
10	0.563	0.600	0.667	0.556
11	0.042	0.200	0.046	0.106
12	0	0	0	0.034
13	0.313	0	0.230	0.266
14	0.042	0	0	0.019

For the analyses of Y-STR, one subject from Kikai island whose paternal grandfather was born in Amami-Oshima island was excluded from Kikai island samples and included in Amami-Oshima island samples. Data of Honshu and Okinawa were taken from Uchihi et al. (2003).

Supp. Table 6. Gene diversities of Y-STRs

Locus	Gene diversity				*P* value					
	Amami-Oshima island (n=48)	Kikai island (n=10)	Okinawa (n=87)	Hounshu (n=207)	Between Amami-Oshima and Kikai	Between Amami-Oshima and Okinawa	Between Amami-Oshima and Honshu	Between Kikai and Okinawa	Between Kikai and Honshu	Between Okinawa and Honshu
DYS393	0.648	0.733	0.649	0.555	0.352	0.538	0.107	0.849	0.228	0.027
DYS19	0.734	0.844	0.751	0.679	0.194	0.354	0.316	0.199	0.062	0.046
DYS391	0.223	0.000	0.322	0.244	0.577	0.652	0.532	0.288	0.739	0.019
DYS438	0.593	0.622	0.503	0.611	0.035	0.320	0.351	0.026	0.041	0.045

Data of Honshu and Okinawa were taken from Uchihi et al. (2003).



Supp. Fig. 1. Phylogenetic network between populations based on d_A distances calculated from 487 bp of D-loop region. Data of the populations except Amami islands were taken from Horai et al. (1996).