Conserv Genet (2007) 8:1331–1337
DOI 10.1007/s10592-006-9281-z

ORIGINAL RESEARCH ARTICLE

Low genetic diversities in isolated populations of the Asian black bear (*Ursus thibetanus*) in Japan, in comparison with large stable populations

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Received: 14 July 2006 / Accepted: 24 December 2006 / Published online: 2 March 2007

Abstract Populations of the Asian black bear (*Ursus thibetanus*) are relatively large and continuous in central Honshu, the main island of Japan, but they are isolated in western Honshu. To clarify the degree of genetic isolation of the populations in western Honshu, we compared the genetic diversities of four populations in western Honshu with that of one of the continuous populations of central Honshu. Three of the four western Honshu populations were isolated and the other was continuous with the central Honshu populations on a geographical distribution basis. The genotypes at 10 microsatellite loci of the sampled individuals were determined and the genetic structures of the populations examined. Genetic diversities were significantly lower in the isolated populations than in the continuous populations. The continuous population in central Honshu had high levels of genetic diversity, comparable to those in populations of the American black bear (*Ursus americanus*) and the brown bear (*Ursus arctos*). The genetic distances between the two continuous populations were smallest, even though their geographic distance was largest (>200 km) among all the pairs of neighboring populations examined. Low genetic diversity within the isolated populations suggested genetic drift due to the small population size; the genetic differentiation among the populations indicated low rates of gene flow among them.

Keywords Microsatellite DNA · Habitat fragmentation · Genetic drift · Heterozygosity · Gene flow

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Introduction

The Asian black bear *Ursus thibetanus* (G. Cuvier, 1823) inhabits two main islands, Honshu and Shikoku, in Japan (Fig. 1). The distribution of the bear is continuous from eastern to central Honshu, except in the northernmost peninsula, but in western Honshu the distribution is fragmented and population sizes are relatively small (Japan Wildlife Research Center [JWRC] 1993; JWRC 1999; Ministry of the Environment 2002; Biodiversity Center of Japan 2004).

The population sizes of the western Chugoku (WC), eastern Chugoku (EC), and southern Kinki populations, which are geographically isolated from each other, are estimated to be 280–680 (JWRC 2000), 150–200 (JWRC 1993), and about 200 (JWRC 1993, 1999), respectively. A few dozen bears are thought to be alive on Shikoku (JWRC 1996). These four populations and the population in northernmost Honshu are recognized as endangered local populations (Mammalogical Society of Japan 1997; Ministry of the Environment 2002). On Kyushu the bear is thought to be extinct (Biodiversity Center of Japan 2004).

The reasons for the decreases in size and fragmentation of the bear populations are thought to be hunting, nuisance kills, and habitat loss (JWRC 1993, 1999). In

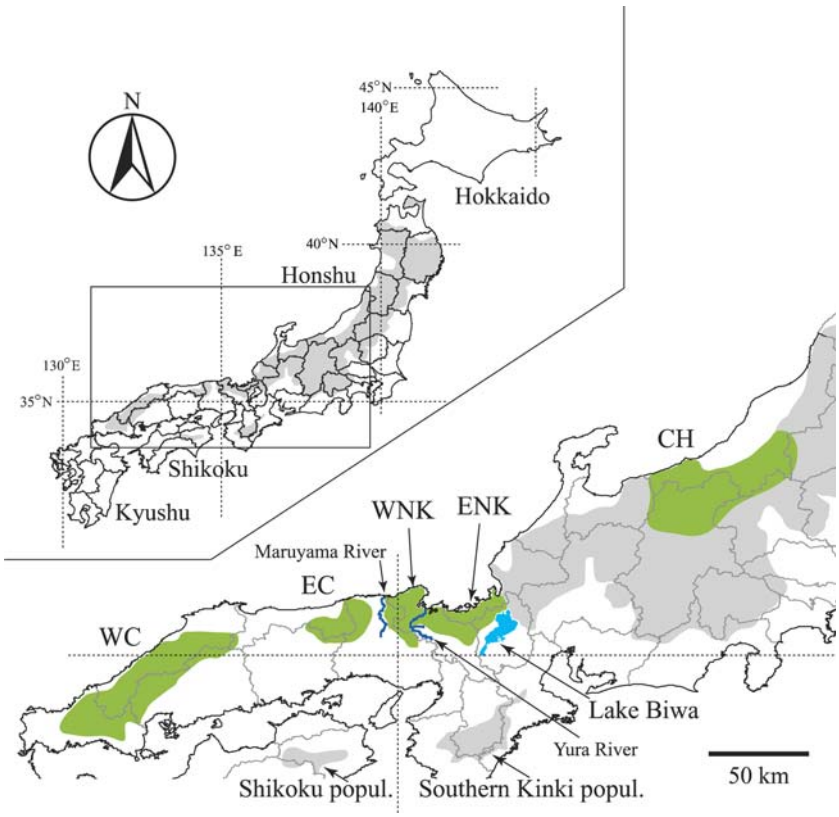

Fig. 1 Distribution of Asian black bears in Japan (light shading) and the studied populations (dark shading in lower diagram). Distributions were estimated by the Biodiversity Center of Japan (2004). Gray lines indicate prefectural boundaries. Local populations: WC, western Chugoku; EC, eastern Chugoku; WNK, western northern Kinki; ENK, eastern northern Kinki; CH, central Honshu. The Yura River forms the border between WNK and ENK

western Honshu, broad-leaved forests, which are the main habitats for bears, have been deforested or converted into conifer forests for commercial use to a greater extent than in eastern Honshu (Forestry Agency 2002). In particular, the forests between WC and EC were stripped by about 150 years ago (Nishikawa 1995).

In the prefectures in western Honshu that have these isolated populations, bear hunting has been prohibited since 1994. However, many bears there are still culled as pests. For example, on average about 40 bears were culled every year between 1995 and 2001 in WC (JWRC 2003).

The western part of the northern Kinki population (WNK) and the eastern part of the northern Kinki population (ENK) (Fig. 1), which are separated by the Yura River, were thought to constitute one population and to be connected to the more eastern populations. However, Saitoh et al. (2001) compared the population genetic structures of four local populations (WC, EC, WNK, and ENK) by analyzing five microsatellite loci and revealed that the genetic structures were significantly different among all the populations. Moreover, mitochondrial DNA (mtDNA) analysis revealed phylogenetic differentiation between ENK and the other three populations (Ishibashi and Saitoh 2004). As a result of these studies, WNK and ENK are now recognized as different populations.

Saitoh et al. (2001) also found that the genetic diversity within these four populations was relatively low. The easternmost population, ENK, had the highest genetic diversity of the four. However, it is unclear whether the level of genetic diversity in ENK is as high as the standard level of diversity in large populations of Japanese black bears. We therefore evaluated the genetic diversities of ENK and the three isolated western populations (WC, EC, and WNK) by comparing them with one of the continuous populations in central Honshu (CH). In the central and eastern parts of Honshu, broad-leaved trees, which are preferred by bears, are widespread, and there are many bear populations (JWRC 1993, Mammalogical Society of Japan 1997, JWRC 1999). We predicted that the genetic diversities of the populations in central Honshu was relatively high, because these populations are thought to be linked with each other and frequent gene flow may occur among them (Saitoh et al. 2001). In terms of geographical distribution, ENK appears to be connected with the central large population, so we expected that the genetic diversity in ENK would be as high as that in CH.

Methods

Samples

Bears captured for pest control and hunting between 1991 and 2004 were sampled. Sample numbers of each population were 72 from WC (Shimane and Tottori prefectures), 46 from EC (Tottori and Hyogo prefectures), 50 from WNK (Hyogo and Kyoto prefectures), 50 from ENK (Kyoto prefecture), and 56 from CH (Nagano and Niigata prefectures) (Fig. 1).

DNA extraction and genetic typing

Genomic DNA was extracted from tissues by using the phenol–chloroform method (Sambrook et al. 1989) and from hairs by using a QIAmp DNA mini kit (QIAGEN). Genotypes at 10 microsatellite DNA loci (G1A, G1D, G10B, G10L, G10M, G10X, MSUT-1, MSUT-2, MSUT-6, and MSUT-7; Paetkau et al. 1995; Kitahara et al. 2000) were determined for all individuals by the PCR technique. PCR amplification was carried out by using DNA thermal cyclers (Perkin Elmer 9700 and MJ Research, Inc. PTC-100) with 10 μl of reaction mixture containing 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris–HCl (pH 8.3), 0.2 mM dNTP, 0.5 μM of each primer, and 0.5 units *Taq* DNA polymerase (Applied Biosystems AmpliTaq Gold). After denaturation of the sample at 95°C for 10 min, PCR amplification was performed for 30 cycles under the following conditions: 30 s at 95°C, 30 s at the locus-specific annealing temperature, and 30 s at 72°C. The locus-specific annealing temperatures were 65°C for G1A and G10B, 58°C for G1D and G10L, 54°C for G10M and G10X, and according to Kitahara et al. (2000) for MSUT-1, -2, -6, and -7. PCR products were analyzed with a PRISM 377 or 3100-Avant Genetic Analyzer (Applied Biosystems), and genotypes were determined with GENOTYPER 2.1 or GENEMAPPER 3.5 software (Applied Biosystems).

Statistical analyses

Departure of the observed heterozygosity from the Hardy–Weinberg equilibrium was tested by the Markov chain method with 10,000 permutations (Guo and Thompson 1992). The allelic richness of 40 individuals as the sample size was estimated according to Petit et al. (1998). Differences in the mean observed and expected heterozygosities and values of allelic richness were evaluated between populations using a paired *t*-test.

Recent population bottlenecks were tested by the method of Cornuet and Luikart (1996) and Luikart et al. (1998) using BOTTLENECK (ver. 1.2.02; Piry et al. 1999). As recommended by Piry et al. (1999), we used the Two Phase Model with 95% single-step mutations and 5% multiple-step mutation, and statistical significance was tested by the Wilcoxon test (two-tailed).

Genetic distance, F_{ST} (Wright 1951), between populations was calculated according to Weir and Cockerham (1984) using MICROSATELLITE ANALYZER (Dieringer and Schlötterer 2003). Another genetic distance, R_{ST} (Slatkin 1995), was calculated with RSTCALC VER. 2.2 (Goodman 1997). By means of a permutation test (10,000 replicates), both programs examined statistically whether the genetic distances differed from zero.

A Mantel test (Manly 1997) with 10,000 permutations was performed to analyze the correlation between genetic and geographic distances by using GENEPOP 3.1c (Raymond and Rousset 1995).

Differences in genotypic frequencies at each locus were tested for all population pairs using a *G*-based exact test (Goudet et al. 1996), in which the null hypothesis was identical genotypic frequency distributions. These statistical tests were performed using GENEPOP 3.1c (Raymond and Rousset 1995).

An assignment test was carried out by the method of Paetkau et al. (1995) using DOH (Brzustowski 2002).

The sequential Bonferroni method was used to adjust significance values for all multiple comparisons (Rice 1989).

Results

Genetic diversity

Observed and expected heterozygosities (H_O and H_E, respectively) and values of allelic richness (A_R) of each locus in each population are shown in Table 1. The mean H_O was lower than the mean H_E in all populations, but in no case did H_O differ significantly from the Hardy–Weinberg equilibrium. The means of both H_E and H_O in CH were highest among all populations, and the mean H_E in CH was significantly higher than those in EC and WNK ($P < 0.05$). No significant difference was observed among the means of H_O. Among the four western populations, the means of both H_E and H_O in ENK were highest, but a significant difference in H_E was observed only between ENK and WNK ($P < 0.05$). The means of both H_O and H_E were lowest in EC among all the populations. The mean A_R in CH was also highest among all populations and was significantly higher than in each of the four western populations ($P < 0.05$). The mean A_R in ENK was higher than those in the other three western populations, although

Table 1 Expected/observed heterozygosities and values of allelic richness (in parentheses) in the five Asian black bear populations analyzed, in a continuous population (West Slope in Canada) of the American black bear (*Ursus americanus*) and in a continuous population (Kluane in Canada) and an isolated population (Yellowstone in U.S.A) of the brown bear (*U. arctos*); from Paetkau et al. (1998)

Locus	WC (n = 72)	EC (n = 46)	WNK (n = 50)	ENK (n = 50)	CH (n = 56)	West Slope *U. am.* (n = 116)	Kluane *U. ar.* (n = 50)	Yellowstone *U. ar.* (n = 57)
G1A	0.810/0.831 (7.11)	0.723/0.700 (6.96)	0.766/0.837 (6.93)	0.724/0.714 (6.75)	0.870/0.887 (11.85)	0.740/ – (6.92)	0.733/ – (6.95)	0.670/ – (4.61)
G10B	0.734/0.786 (4.00)	0.540/0.500 (3.00)	0.606/0.633 (6.45)	0.743/0.620 (5.95)	0.767/0.696 (6.14)	0.798/ – (5.34)	0.809/ – (7.79)	0.681/ – (4.99)
G1D	0.515/0.569 (2.91)	0.499/0.500 (2.00)	0.481/0.380 (2.00)	0.626/0.673 (3.82)	0.692/0.482 (4.69)	0.731/ – (8.70)	0.845/ – (9.80)	0.805/ – (6.70)
G10L	0.403/0.347 (3.00)	0.420/0.366 (5.95)	0.453/0.429 (3.00)	0.662/0.653 (3.00)	0.647/0.554 (4.71)	0.832/ – (11.43)	0.613/ – (5.00)	0.407/ – (2.00)
G10M	0.667/0.569 (5.91)	0.482/0.561 (4.00)	0.681/0.680 (5.76)	0.786/0.760 (5.96)	0.806/0.679 (9.34)	0.840/ – (9.34)	0.816/ – (6.96)	0.642/ – (4.97)
G10X	0.649/0.611 (3.00)	0.607/0.500 (4.00)	0.602/0.540 (4.00)	0.673/0.620 (5.00)	0.640/0.589 (5.82)	0.850/ – (11.11)	0.733/ – (6.96)	0.101/ – (2.00)
MSUT-1	0.462/0.431 (3.80)	0.113/0.093 (3.92)	0.618/0.600 (3.80)	0.601/0.680 (3.00)	0.541/0.500 (3.00)			
MSUT-2	0.041/0.042 (1.91)	0.155/0.167 (2.00)	0.236/0.220 (3.00)	0.368/0.400 (5.92)	0.750/0.768 (7.89)			
MSUT-6	0.448/0.444 (2.00)	0.475/0.395 (2.93)	0.413/0.440 (2.99)	0.608/0.560 (3.99)	0.771/0.830 (7.26)			
MSUT-7	0.559/0.500 (3.00)	0.599/0.500 (3.00)	0.132/0.140 (2.00)	0.298/0.280 (2.00)	0.544/0.442 (4.77)			
Mean[a]	0.529/0.513 (3.67)	0.461/0.428 (3.78)	0.499/0.490 (3.99)	0.610/0.596 (4.54)	0.703/0.643 (6.55)			
Mean (G)[b]	0.630/0.619 (4.32)	0.545/0.521 (4.32)	0.598/0.583 (4.69)	0.702/0.673 (5.08)	0.737/0.648 (7.09)	0.799/ – (8.81)	0.758/ – (7.24)	0.551/ – (4.21)

WC, western Chugoku; EC, eastern Chugoku; WNK, western northern Kinki; ENK, eastern northern Kinki; CH, central Honshu; n, number of individuals examined

[a] Mean of 10 loci

[b] Mean of six loci (G1A, G10B, G1D, G10L, G10M, and G10X)

statistical significance was not detected ($P > 0.05$). The means of A_R were similar among the three western populations (WC, EC, and WNK).

H_Es and A_Rs at six loci were compared with those in continuous populations of the American black bear (*Ursus americanus*) and the brown bear (*Ursus arctos*) and an isolated population of the brown bear (Paetkau et al. 1998) (Table 1). Compared with those of the American black bear population, the means of H_E for EC, WNK, and ENK and the means of A_R in the four western populations were significantly lower ($P < 0.05$). The mean H_E and mean A_R for CH did not differ significantly from those of the American black bear population ($P > 0.05$). Although the means of H_E of the five populations of the Asian black bear were lower than that of the continuous population of the brown bear, only those of EC and WNK were significantly lower ($P < 0.05$). The means of A_R in WC, WNK, and ENK were significantly lower ($P < 0.05$) than that of the continuous population of the brown bear. No significant difference was observed between EC and the continuous brown bear population, although A_R in EC was as low as that in WC. The mean A_R for CH was lower than that of the continuous population of the brown bear, but the difference was not significant ($P > 0.05$). The means of H_E and A_R in the five populations of the Asian black bear, excluding H_E in EC, were higher than that of the isolated population of the brown bear, although statistical significance was not detected ($P > 0.05$).

No recent population bottleneck was detected by the BOTTLENECK results, indicating that there was no significant excess or deficiency of heterozygosity for all analyzed populations.

Genetic differentiation among populations

All of the pairwise genetic distances (F_{ST} and R_{ST}) between the five populations were significantly higher than zero ($P < 0.05$) (Table 2). The values of both the F_{ST} and the R_{ST} estimator were highest between EC and WNK and lowest between ENK and CH. Neither of the genetic distances was correlated with geographic distance (Mantel test: $P > 0.05$).

Table 2 Pairwise genetic distances (F_{ST}: lower-left, R_{ST}: upper-right) between the five Asian black bear populations

	WC	EC	WNK	ENK	CH
WC		0.114	0.160	0.111	0.145
EC	0.187		0.215	0.182	0.169
WNK	0.199	0.230		0.098	0.158
ENK	0.162	0.133	0.105		0.037
CH	0.165	0.201	0.154	0.078	

Genotypic frequencies did not differ significantly in six cases: the pairs WNK– ENK and WNK–CH for MSUT-1, the pairs WC–EC, WC–WNK, and EC–WNK for MSUT-6, and the pair WC–EC for G10L. In all the remaining cases (94) the frequencies were significantly different ($P < 0.05$).

Over 90% of bears were correctly assigned to their original populations by their genotypic frequencies; in particular, assignment was completely correct in WC (Table 3).

Discussion

Genetic status of Asian black bear populations in Japan

Among the five populations analyzed, we clarified the fact that CH, a continuous population in central Honshu, had the highest genetic diversity and ENK, which may be connected with the continuous populations, had the second-highest genetic diversity. Consequently, we conclude that the genetic diversity was considerably lower in the three isolated populations of western Honshu than in the two continuous populations.

By analyzing five microsatellite loci, Saitoh et al. (2001) revealed that ENK had the highest genetic diversity among the four populations in western Honshu. However, it was unclear whether the level of genetic diversity in ENK was similar to that of large populations of Japanese black bears. In central Honshu, many populations are connected with each other and gene flow is likely to occur frequently. ENK appears to be the westernmost of these continuous populations (Fig. 1), and we therefore expected that the genetic diversity in ENK would be as high as that in the other continuous populations and would not differ from that of CH. However, we found that ENK had lower diversity than CH, even though it had the highest diversity in western Honshu. On the other hand, considering the fact that CH is located in the middle of the continuous populations, it would be reasonable to conclude that the genetic diversity in CH is representative of that of the continuous black bear populations of Japan.

Note also that the pair ENK–CH had the lowest genetic distances of all pairs of populations analyzed, even though the geographical distance between these two populations was largest (> 200 km) among all pairs of the populations examined. This result implies that gene flow between these two continuous populations occurs the most frequently. On the basis of the present geographical distribution, it is thought that ENK is a continuum of populations that contains CH. A stepping-stone-like gene flow might be modeled in the exchange of nuclear genes between ENK and CH, because the geographic distance between the two populations is too far for individual bears to disperse.

The genetic diversity in CH was no less than that in the continuous populations of North American bears, i.e. the West Slope population of the American black bear (*U. americanus*) and the Kluane population of the brown bear (*U. arctos*) (Paetkau et al. 1998). The fact that these two populations are recognized to be stable (Servheen et al. 1999; COSEWIC 2002) suggests that the genetic diversity of CH may be high enough to maintain the stability of this population.

The genetic diversities within the isolated populations of the Asian black bear were close to that of the isolated Yellowstone population of the brown bear. The situation of the Yellowstone population of the brown bear resembles that of the WC and EC populations of the Asian black bear; the Yellowstone population has been isolated for over 100 years, and the population size is estimated at 350–450 (Servheen et al. 1999). This isolation is assumed to have caused the drop-off in genetic diversity of the Yellowstone population (Paetkau et al. 1998). In the light of this argument, it is reasonable to suppose that genetic drift has decreased the genetic diversity within the three Japanese western populations since their isolation occurred.

Table 3 Results of assignment test. The expected frequency of each genotype was calculated and animals were assigned to the population in which their genotype was most likely to occur

Assigned population	Source population				
	WC	EC	WNK	ENK	CH
WC	72 (100)	1 (2.2)	0	0	0
EC	0	43 (93.5)	0	0	0
WNK	0	2 (4.3)	46 (92.0)	4 (8.0)	1 (1.8)
ENK	0	0	3 (6.0)	46 (92.0)	2 (3.6)
CH	0	0	1 (2.0)	0	53 (94.6)
Total	72	46	50	50	56

Values are numbers (percentages) of animals from each population analyzed. Underlines indicate correct assignments

Ishibashi and Saitoh (2004), however, suggested an additional reason for the low rates of diversity in microsatellite loci within the three isolated populations. They revealed by phylogeographical analysis that two mtDNA lineages are separated by the Yura River. They suggest that the three isolated populations (WC, EC, and WNK) on the western side of the river and ENK on the eastern side of the river originate from two populations that retreated in different refugia during the last glacial period. Because of the smaller size of the western refugium, genetic diversity may have been lost more there than in the other refugium, and this past loss might have caused the lower rates of microsatellite variation within the three isolated populations than in ENK (Ishibashi and Saitoh 2004). However, this does not explain the large genetic differences among the isolated populations. Considering the small genetic distance between ENK and CH, we conclude that the present differences in genetic features and the present genetic distances among the three isolated populations were generated after the isolation. Population isolation and size reduction may have caused an additional decrease in genetic diversity. Further studies are required to clarify the relative importance of isolation as a cause of low genetic diversity, in comparison with effects in the past.

Our results support two points noted by Saitoh et al. (2001): the four western populations are genetically fragmented and the three isolated populations have low genetic diversities. In addition, our results revealed that heterozygosities were lowest in EC and the value of allelic richness was as low as that of WC. The genetic distance between EC and WNK was greater than that between EC and WC, even though the geographic distance between EC and WNK is shorter than that between EC and WC. Saitoh et al. (2001) argue that this large genetic distance is related to the barrier effect of the Maruyama River: this river may prevent gene flow between EC and WNK. However, some migrations of male bears between EC and WNK have been suggested by Ishibashi and Saitoh (2004), and these may contribute to gene flow in terms of nuclear DNA. Considering the small population size of EC (150–200), the influence of genetic drift may be strong (Ishibashi and Saitoh 2004) and thus probably overwhelms the influence of gene flow from WNK.

Implications for conservation

We revealed that the genetic variations in the isolated populations were lower than in the continuous populations. Loss of genetic diversity is said to decrease adaptive diversity and the potential for evolution under variable and unpredictable environments (Crandall et al. 2000; Frankham et al. 2002), and this reduction might increase the extinction risk. Thus, there is a pressing need to maintain and increase genetic diversity within isolated populations.

We propose that two points are important for the conservation of genetic diversity in these isolated populations. First, we should design preferred corridor habitats for bears to move among the isolated populations, as Ishibashi and Saitoh (2004) proposed. Considering the lowest genetic distance between the geographically farthest populations (ENK–CH), if the western four populations were to be linked with each other by habitat corridors then gene flow among them would increase dramatically.

Next, we emphasize that the key to increasing genetic diversity in the isolated populations is to increase genetic immigration from the more easterly continuous populations. Gene flow from the easterly populations to the isolated western populations must be mediated by the populations to the north of Lake Biwa, the area that links the isolated populations and continuous populations (Fig. 1). Because this area is so narrow, gene flow from the more easterly populations to the westerly populations might dramatically decrease if the preferred habitat of this area were to be damaged. It is thus essential to conserve suitable habitat for bears to the north of Lake Biwa.

Acknowledgments We thank T. Shimada and Y. Segawa for their support in collecting samples. R. Kishimoto, E. Kitahara, N. Nishi, M. Yokoyama, H. Kanamori, and the Niigata branch of the Japan Hunting Association contributed samples. D. Paetkau kindly helped us to compile references. Helpful comments on earlier drafts of the manuscript were received from Y. Miyazaki and two anonymous referees. This study was supported partly by a Grant-in-Aid for Scientific Research (no. 15780119) from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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