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[Original report]

Species identification of 6,000-years-old beans from Sannai-Maruyama site, Aomori, Japan

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Abstract

Ancient beans were excavated at the 6,000-years-old layer in Sannai-Maruyama archaeological site, Aomori, Japan. Because their morphology did not allow definite taxonomic identifications, DNA of the ancient beans was extracted to identify their species on the basis of molecular data. The genomic DNAs were amplified using PCR technique for chloroplast microsatellites and plastid-subtype ID sequence between *rpl 16* and *rpl 14* genes. As comparative taxa, the following modern bean species were examined, mung beans, azuki beans, wild soybean, and cultivated soybean. The DNA analysis suggests that at least some ancient beans from the Sannai-Maruyama site may belong to soybeans. Although at least some of those ancient soybeans appear to be the wild type, there is no reason to dismiss the possibility that ancient villagers at the site practiced a primitive form of soybean domestication.

Key words: archaeology, bean, chloroplast microsatellite, DNA, Jomon era, PCR

Introduction

The Sannai-Maruyama archaeological site, located in Aomori, Japan, represents a large village prospered from 3,500 BC to 2,000 BC within the Jomon (Tsuji, 1999; reviewed by Habu, 2004). One open question is whether there was artificial food management in Jomon. In general, those people should gather edible plants, hunt mammals and fish (Habu, 2004.). Recent excavations at the Sannai-Maruyama site resulted in the discovery of numerous plant seeds along with bones of fishes and mammals (Minaki, 1995). Chestnuts (*Castanea crenata*) is one of edible plant seeds excavated from the Sannai-Maruyama site (Okada and Ito, 1995; Sato, 1997). Deoxyribonucleic acid (DNA) was amplified from those ancient chestnut seeds by using polymerase chain reaction (PCR) method to apply randomly amplified polymorphic DNA (RAPD) analysis (Yamanaka *et al*., 2000). The excavated seeds showed relatively uniform pattern than wild seeds collected, because of self-incompatilbility usually wild chestnut plants have. The data suggested that primitive selection of particular kinds of strains had been done at Sannai-Maruyama as primitive domestication (Yamanaka *et al*., 2000).

Many other seeds were identified as beans, grass seeds, grape seeds, elderberries, mulberries, raspberries, walnuts, and so on. All plant species were unknown yet by morphological identification. Plant species identification by DNA level, may

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inform us whether they had domesticated particular kinds of plant species. As for beans, there are some edible species in Asia such as azuki bean (*Viguna angularis*), mung bean (*Viguna radiate*), soybean (*Glycine max*) and so on (Okada and Ito, 1995). Wild form of soybean is known as *Glycine soja*, which is able to find in Japan. All these beans were candidates of ancient beans. Although wild form of mung bean could not be found in Japan, once the beans was presumed as ancient beans excavated from Triihama-Kaizuka which is another Jomon archaeological site. To consider about food use of beans, accurate species identification of ancient beans is critical to understand the domestication process of beans in Japan and also origin of cultivated beans. Species identification of ancient beans were performed by molecular tools, as PCR techniques allowed us to amplify small amount of DNA extracted from the ancient beans. One of target sequences is a linker sequence between *rpl14* and *rpl16* at chloroplast (cp) DNA to identify the plant taxa (Nakamura *et al*., 1997). Chloroplast microsatellites were other resources to compare plant species because they represented little or no variation among related species but between two other species (Ishii and McCouch, 2000).

Materials and methods

Bean samples-We examined eight seeds that were identified as beans on the basis of morphology and were labeled as "S1" through "S8" from a 6,000-years-old layer recorded as VI layer (Okada and Ito, 1995). These specimens were supplied by the Sannai-Maruyama Preservation Office. As shown in Fig. 1, all beans were preserved in charred state. Genomic DNA samples from the ancient beans were extracted by the method described by Dellaporta *et al*. (1983). Molecular samples in modern beans were used to compare with those in ancient beans (Table 1). Our comparative modern taxa consisted of azuki bean (*Phaseolus angularis*), wild azuki bean (*Phaseolus angularis*, strains, YBT1, YBT2, YBT3, YBT4), mung bean (*Vigna radiata*), wild soybean (*Glycine soja*, strains, WS1, WS2, and WS3) and cultivated soybean (*G. max* cv. Tanbaguro).

PCR (Polymerase chain reaction) technique-Plastid subtype -ID (PS-ID) sequence was used to distinguish plant species at chroloplast level (Nakamura *et al*., 1997). A single pair of primers was used to amplify target region for PSD-ID. The primers were as following; 5P primer : 5'- AAAGATCTAG ATTTCGTAAACAACATAGAGGAAGAA - 3' and 3P primer : 5'- ATCTGCAGCATTTAAAAGGGTC TGAGGTTGAATCAT - 3'. PCR condition was 35 rounds of one minutes (min.) at 94℃, 1 min. at 52 ℃, and 2 min. at 72℃ with 3 min., with 5min. pre-heat at 94℃ and 5 min. post-heat at 72℃. When initial PCR products were smear, the product was applied for subsequent PCR with a primer pair of 3P and another primer, G5P2 , 5'-GTAGCTGTTGTCAA ACCTGGGAAAATACTTTATGAAATG - 3' to obtain single products. Then, the PCR products were cloned into a vector, pBluescript II ™ (Stratagene) to sequence the cloned fragment with LICOR 4200S sequencer. Sequencing reaction was done with a SequiTherm Excel™ II (EPICENTRE Biotechnologies). Three microsatellites, Soycp, cpSSR2, and cpSSR3, were amplified with the protocol described by Xu *et al*. (2002) with modification. The reaction cycle was 32 rounds of 30 seconds (sec.) at 94℃, 30 sec. at 46 ℃, 30 sec. at 68℃. The amplification was applied with the following primers; 5'-CATAGATAGGTAC CATCCTTTTT -3' and 5'-CGCCGTATGAAAGCAA TAC - 3' for Soycp, 5'-TATCACTGTCAAGATTAA GAG - 3' and 5'-CTTTTATATGTATGGCGCAAC -3' for cpSSR2, and 5'-TCGATTCTATGCCCCTACTT -3' and 5'-AGACTCCCAAGTTTTCAGTCG -3' for cpSSR3. These PCR products were diluted four to ten folds with distilled water. Each 1.5-μL of PCR products were added with the same volume of loading buffer (95% form-amide and 5% bromo-phenol blue). These samples were denatured for 5min. at 95℃ and electrophoresed by using ABI 377 auto-sequencer (Applied Biosystems). The resulted electrophorogram were standardized using Gene scan Manage software (Perkin elmer).

Fig.1. Example of 6,000-years-old bean from Sannai-Maruyama archaeological site, Aomori, Japan. Note charred state of preservation. Scale is 1mm.

Results

Ancient seeds excavated were not identified their species based only on morphological features (Fig. 1). All beans looked similar to mung bean, wild azuki bean and wild soybean. However, they could not be defined as mung bean or wild soy (*G. soja*) bean. Thus, total DNA was extracted and used for PCR reaction with microsatellite primer pairs. In the first reaction, PCR products were smear (data not shown). The second PCR was then performed with a high annealing temperature (55℃). PCR products with Soycp were obtained from two ancient beans, S1 and S2. Amplified fragments were electrophoresed with sequence gel and shown as 92 bp fragment in size. The products of modern beans were also amplified to compare with those of the ancient beans (Table 1). The band amplified from the ancient beans was 92 base pair (bp) in size. The modern azuki bean showed a 92 bp fragment in size. Wild azuki beans showed, 92, 87 and 94 bp fragments in total. Wild soybean and mung bean showed 92, 94 and 95 bp fragments. Fragments amplified from cultivated soy beans ranged from 91 to 95 bp (Xu *et al*., 2002). S1, S2, S3 and S4 generated 134 and 135 bp fragments for cpSSR2, and S1, S2 and S3 generated 123 bp fragment for cpSSR3. All these fragments amplified from ancient beans showed similarity with those from cultivated and wild soybeans.

PS-ID sequence is a linker sequence between *rpl16* and *rpl14* genes. Conserved sequences were used to amplify the linker fragments (total of approximately 550bp in size) in modern beans

Table1. Plant materials and variation of chroloplast SSR genotypes

Strain	Common name	collecting site	SoycpSSR(bp)	cpSSR2(bp)	cpSSR3(bp)
(Charred seed)					
S1	Charred bean	Sannai-Mauryama	92	134	123
S2	Charred bean	Sannai-Mauryama	92	134	123
S3	Charred bean	Sannai-Mauryama	NA	135	123
S4	Charred bean	Sannai-Mauryama	NA	134	NA
S5-8	Charred bean	Sannai-Mauryama	NA	NA	NA
(Vigna angularis)					
YBT1	Wild azuki bean	Ibaraki	87	130	NA
YBT2	Wild azuki bean	Nagasaki	92	127	NA
YBT3	Wild azuki bean	Aomori	92	123	NA
YBT4	Wild azuki bean	Aomori	94	106	124
(Vigna angularis)					
AZ	Azuki bean	Shimane(Oki)	49	123	122
(Vigna radiata)					
RT	Mung bean	unknown	92	123	121
(Glycine soja)					
WS1	B02178	Sannai-Mauryama	94	134	123
WS2	B02179	Sannai-Mauryama	92	134	123
WS3	B02138	Yamagata	95	134	123
Reference	Soybean	(Xu et al. 2002)	91-95	134-136	121-123

NA : not available.

Table2. PS-ID sequence of beans

Species	Strains obtained sequence*	Sequence
Unknown(Charred beans)	S1, S2, S4, S5	TAATAAATTA GAACCAAAGG AAAGAGGTCT TTAAGATGAA AACAAAAAAT
Glycine max	Tanbaguro	TAATAAATTA GAACCAAAGG AAAGAGGTCT TTAAGATGAA AACAAAAAAT
Glycine soja	B02178, B02179, B02138	TAATAAATTA GAACCAAAGG AAAGAGGTCT TTAAGATGAA AACAAAAAAT
Vigna angularis	YBT1-4	TAAAAATGAG AACCAAAGGA AAGAGAAGAG GTCTTTAGGA TGCAAAAAAA
Vigna angularis	AZ	TAATAAAAGA GAACCAAAGG AAATAGGTCT TTAGAATGAA AAGAAAAAAG
Vigna radiata	RT	TAATAAAAGA GAACCAAAGG AAATAGGTCT TTAGAATGAA AACAAAAAAG

*Other charred beans were not avilable to amplify PS-ID.

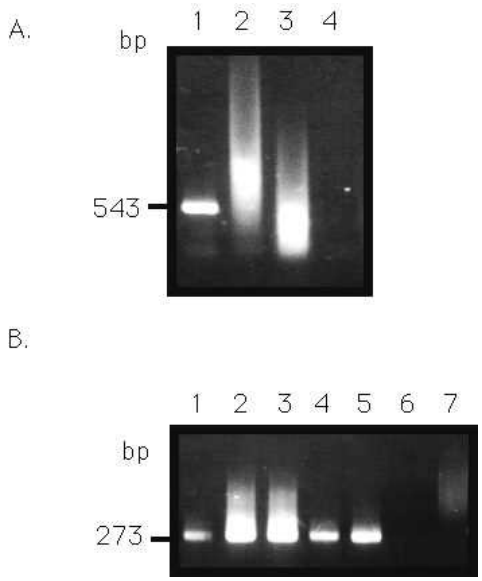

Fig.2. Amplified fragments of DNA from ancient bean from Sannai-Maruyama site and modern wild soy bean (*Glycine soja*). Panel A; 543 bp rice PS-ID and corresponding fragment from amplified modern wild soy bean genomic DNA (lane 1), second PCR product amplified from 10^{-4} and 10^{-6} diluted first PCR products from ancient bean (S1) revealing smear bands (lanes 2 and 3), and water as negative control revealing no band (lane 4). Panel B ; 273bp fragment from modern wild soy bean (WS3) amplified with G5P2 and 3P primers (lane 1), second PCR fragment revealing relatively broad bands with 10^{-4} diluted solutions from the first PCR products of S1 (lane 4) and S2 (lane 5). As negative control, PCR mixture without any DNA template was loaded in lane 6. The first PCR product without DNA template was added to PCR mixture without any DNA template and the second PCR product was loaded in lane 7.

including cultivated soy bean (Tanbaguru), wild soybean (WS1-WS3), wild azuki bean (YBT1-YBT4) and mung bean. These amplified fragments were cloned and sequenced. The first 50 bp sequences were used to compare sequences (Table 2). PS-ID sequences between azuki and mung beans, and between cultivated and wild soybeans were identical, where there were 7bp differences between two pairs of bean taxa. The PS-ID sequences were amplified for the ancient beans. However, the first PCR failed, so the second PCR was conducted using the first PCR products as DNA templates, which were diluted into 10^{-4} to 10^{-6}. Although four of the eight ancient bean samples were succeeded to amplify PS-ID fragments, the bands of the second PCR products looked smear (Fig. 2A, lanes 2 and 3). Therefore, a new primer, G5P2, was designed within the sequence based on genomic sequence information of soybean, azuki bean, and mung beans. By using a combination of two primers, G5P2 and 3P, the second PCR was re-performed on diluted first PCR products. Then, clear bands, 273 bp in size were amplified with the 10^{-4} and 10^{-6} diluted solutions (Fig. 2B, lanes 2 to 5). As negative controls, we added water without any DNA fragments as well as the PCR product of the negative control in the first PCR. Even PCR reaction was performed, both negative controls did not show any clear bands as shown in Fig. 2B (lanes 6 and 7). Clear bands obtained with ancient beans as template were sequenced directly after purified the DNA fragments. The PS-ID sequences of the ancient beans were identical to those of cultivated and wild soybeans (Table 2), but different from cultivated and wild azuki beans, and also mung bean.

Phylogeny tree was created by CLC2 Free workbench 2.5.2 (free software) based on the sequence data which represented as first 50 nucleotides from the stop codon of *rpl16*. Then, the tree clearly revealed genetic similarity between azuki and mung beans, and among cultivated and wild soybeans, and ancient beans sequenced.

Discussion

Japanese agriculture is thought to have started from the Yayoi Era about less than 3,000 years ago, reviewed by Habu (2004). The Sannai-Maruyama site is a Jomon period site where continuous occupation took place for a long period of time about 5,500～4,000 years ago from early Jomon to middle Jomon (Tsuji, 1999).

People in the Jomon Era at Sannai-Maruyama apparently obtained their foods through fishing, hunting, and gathering. Te evidence for fishing and hunting comes from artificial mounds of dumped materials found in the site, including bones of tunas, halibuts, salmons, whales, seals, and various other animals. The evidence of gathering comes from collected remains of walnuts, chestnuts, elderberries, mulberries, raspberries and grapes, although whether they were wild or domesticated has been difficult to ascertain. However, one DNA-based study on ancient chestnuts from the Sannai-Maruyama

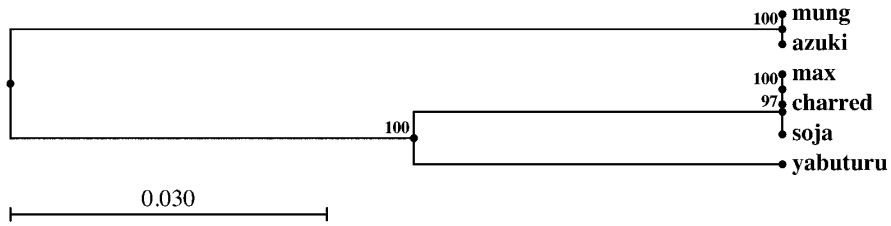

Fig.3. A proposed phylogeny of the PS-ID sequences. The tree represented the distance among respective PS-ID sequences drawn by UPGM method (CLC-free work bench software, ver2.5.2). Bootstrap score of 100 means that the corresponding branch occurs in all 100 trees tried. The scale was the relative distance. Cultivated soy bean (max), wild soy bean (soja), mung bean (mung), azuki bean (azuki), wild azuki (yabuturu), and ancient beans (S1, S2, S4, and S5 ; charred) .

site (Yamanaka *et al*., 2000) showed a narrower range of genetic diversity in them compared to that in chestnuts from natural forests, possibly indicating an early domestication stage of chestnuts. Yamanaka *et al*. (2000) suggested that the ancient villagers likely maintain chestnut trees for yielding stable production and making the nut collection easier.

Another evidence of primitive agriculture in Japan comes from the beans found in the Toriihama-Kaizuka archaeological site (Maeda, 1987). The beans were identified as mung beans (*Vigna radiata*) based on morphology (Maeda, 1987), because a primitive domesticated form of mung beans is known from India (Vavilov, 1951). However, the taxonomic identification of beans from the Toriihama-Kaizuka should be viewed with care, especially because morphology-based taxonomic identifications of ancient beans are difficult (Sato and Ishikawa, 2004). However, our study shows that such identifications are possible based on molecular data. The lack of any genetic distances (within the length of examined PS-ID sequence) among the two soybean taxa and ancient bean (Fig. 3) strongly suggest that the ancient beans belong to soybeans, although we could not conclude whether the ancient beans belong to cultivated or wild soybean. The distribution of wild soybeans ranges from south Shiberia in Russia to south China, Korea and Japan. Domestication of soybeans is thought to have originated from north China approximately 5,000 years ago (Hymowitz, 1970). In the initial phase of soybean domestication, wild soybean would be collected throughout Asia where wild soybean were widely distributed. As mature pods scattered beans, people would collect and boiled them for consumption. Although we have no evidence for primitive cultivation of wild soybeans, it is plausible to think that ancient people in the expanding village over 2,000 years in early Jomon Era could have begun to nurse wild soybean fields around the village just as their nursery chestnut forests.

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