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Composite and clinal distribution of *Glycine soja* in Japan revealed by RFLP analysis of mitochondrial DNA

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Abstract Wild soybean (*Glycine soja* Sieb. et Zucc.), regarded as the progenitor of cultivated soybean [*G. max* (L.) Merr.], is widely distributed in East Asia. We have collected 1097 *G. soja* plants from all over Japan and analyzed restriction fragment length polymorphisms (RFLPs) of mitochondrial DNA (mtDNA) in them. Based on the RFLPs detected by gel-blot analysis, using *coxII* and *atp6* as probes, the collected plants were divided into 18 groups. Five mtDNA types accounted for 94% of the plants examined. The geographic distribution of mtDNA types revealed that, in many regions, wild soybeans grown in Japan consisted of a mixture of plants with different types of mtDNA, occasionally even within sites. Some of the mtDNA types showed marked geographic clines among the regions. Additionally, some wild soybeans possessed mtDNA types that were identical to those widely detected in cultivated soybeans. Our results suggest that the analysis of mtDNA could resolve the maternal lineage among plants of the genus *Glycine* subgenus *Soja*.

Key words Wild soybean · *Glycine soja* · RFLP · Mitochondrial DNA · Geographic distribution

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Introduction

Wild soybean, (*Glycine soja* Sieb. et Zucc.) in an annual self-pollinated plant, and is distributed in East Asia, including the Japanese islands, the Korean peninsula, the Chinese continent, and Far East Russia near the Chinese border. Wild soybeans are thought to be a direct progenitor of cultivated soybeans, *G. max* (L.) Merr, and have much more genetic variation than cultivated soybeans (Hymowitz 1970; Kiang and Gorman 1983).

Variation of chloroplast DNA (cpDNA; Shoemaker et al. 1986; Close et al. 1989) and mitochondrial DNA (mtDNA; Sisson et al. 1978; Grabau et al. 1992; Hanlon and Grabau 1995; Moeykens et al. 1995), as well as nuclear DNA (Apuya et al. 1988; Keim et al. 1989; Skorupska et al. 1993), have been studied using restriction fragment length polymorphisms (RFLPs) in soybeans. Both cpDNA and mtDNA RFLP markers have been used to study the diversity of cytoplasm in an outcrossing soybean population (Lee et al. 1992, 1994). Close et al. (1989) defined six soybean plastome groups based on cpDNA RFLPs within the subgenus *Soja* consisting of *G. max* and *G. soja*. Compared with the chloroplast genome, the mitochondrial genome has been shown to have many variations within or between closely related species (Newton 1988; Palmer 1990). Accordingly, the analysis of mtDNA RFLP is thought to be useful for studying phylogenetic relationships within species. Grabau et al. (1992) showed that the mitochondrial genomes of 138 cultivated soybeans could be divided into four groups by using a cloned 2.3-kb *Hin*dIII mtDNA fragment from a cultivar 'Williams 82'. Hanlon and Grabau (1995) also surveyed a collection of old cultivars of soybean with the same 2.3-kb *Hin*dIII fragment, as well as a mtDNA fragment that contained *atp6*, to determine the sources of cytoplasmic diversity in their collection. They showed that the RFLP analysis of mtDNA with these probes is

useful for the classification of the mitochondrial genomes of soybean. Grabau and Davis (1992) roughly classified wild soybeans by using the 2.3-kb *Hin*dIII mtDNA fragment as a probe.

In terms of the polymorphisms of mtDNA and cpDNA, isozymes, and seed components, we have been studying the genetic diversity of wild and cultivated soybeans which have been collected from various regions of East Asia (Abe et al. 1992, 1993; Hirata et al. 1993; Kaneko et al. 1995; Hirata et al. 1996 a, b). In the present paper, we describe the diversity of mitochondrial genomes of wild soybeans in Japan based on the RFLPs of mtDNA that were detected using *coxII* and *atp6* as probes. The purpose of this work was to evaluate intra-species diversity in wild soybeans, and to determine the phylogenetic relationships of wild soybeans with cultivated soybeans as well as the evolutionary changes that have occurred in the mitochondrial genomes of wild soybeans.

Materials and methods

Plant materials

A total of 1097 plants were surveyed. The seeds used for these studies were collected individually from 349 natural habitats of various regions that cover almost the whole of Japan. The collection sites of wild soybeans are shown in Fig. 1 and were grouped into six geographic regions: Hokkaido (31 sites), North of Honshu (97), Central Honshu (87), South of Honshu (45), Shikoku (37), and Kyushu (52). Seeds were germinated in a greenhouse and the resultant plants were subjected to RFLP analysis.

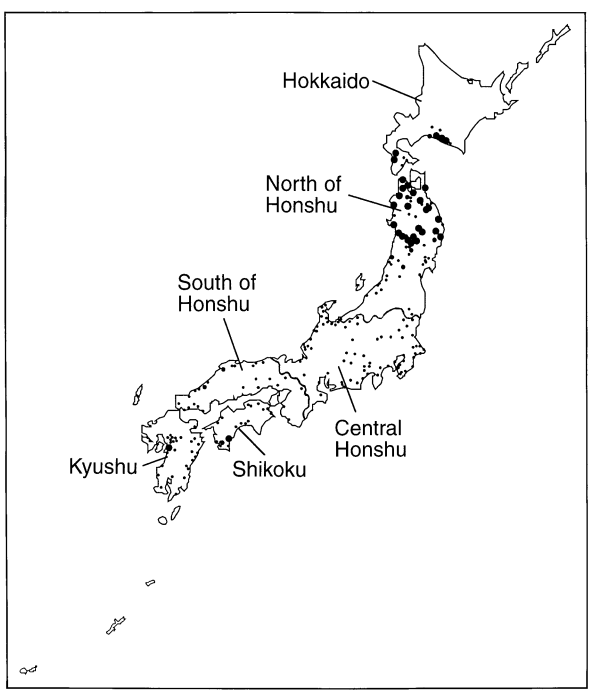

Fig. 1 Collection sites of the wild soybeans from Japan analyzed in this study. *Small*, *medium and large circles* indicate locations where 1–5, 6–10 and more than 11 individual plants were analyzed, respectively. Although the distribution of wild soybeans was extensively investigated, no plants were observed in Hokkaido other than in the southern part of this island. In Okinawa, the southwest islands of Japan, we failed to detect any wild soybeans in Okinawa island, Ishigaki island, and Miyako island, although wild soybeans have previously been recorded in Okinawa

Southern-blot analysis

Total cellular DNA was obtained from a few young leaves according to the method of Doyle and Doyle (1987). The DNAs were digested with the restriction enzymes *Bam*HI or *Hin*dIII and fractionated electrophoretically in 0.8% agarose gels for 1.5 h at 100 V. Then the DNAs were blotted onto nylon membranes (Hybond-N+; Amersham). The membranes were hybridized overnight at 42°C, with constant shaking, in a hybridization buffer that contained a labelled probe. Hybridization was carried out using an ECL direct nucleic acid labelling and detection system (Amersham). Two mtDNA fragments were used as probes. One was a 0.7-kb *Hin*dIII-*Nco*I fragment that contained *coxII* (a gene encoding mitochondrial cytochrome oxidase subunit II) from a wild soybean (Kato 1993) and the other was a 0.66-kb *Sty*I fragment that contained *atp6* (a gene encoding mitochondrial ATPase subunit 6) from *Oenothera* (Schuster and Brennicke 1987). After hybridization, the gel blot was rinsed twice with the first washing solution (6 M urea, 0.4% SDS and 0.5 × SSC) for 20 min at 42°C, followed by two rinses with a second washing solution (2 × SSC) for 5 min at room temperature. Labeling of the probe DNA and visualization of the probe-target DNA hybrid were carried out according to the supplier's instructions.

Results

Observed polymorphisms

We analyzed RFLPs of mtDNA for 1097 wild soybeans plants grown in Japan by using *coxII* and *atp6* as probes, because our preliminary screening revealed more RFLPs when both were used as probes (data not shown).

Seven types of hybridization patterns were detected when *Hin*dIII-digested DNAs were hybridized with *coxII* (Fig. 2; Table 1). The observed hybridization signals were mostly single and with various sizes: 1.2 kb, 1.3 kb, 1.6 kb, 1.7 kb, 3.5 kb, 5.8 kb and 8.5 kb. Some of the plants with the 3.5-kb or 5.8-kb hybridization signals also showed the 1.2-kb signal, which was, however, weak when compared with the major signals (data not shown).

When *Bam*HI-digested DNAs were hybridized with *coxII*, five polymorphic signals, having sizes of 5.8 kb, 7.0 kb, 8.1 kb, 8.5 kb and 15.0 kb, were detected (Fig. 3; Table 2). The observed RFLPs generated with *Hin*dIII and those generated with *Bam*HI were almost linked with each other: the plants that had the 1.6-kb, 1.3-kb, 1.2-kb, 3.5-kb, 1.7-kb, and 8.5-kb *Hin*dIII fragments always had the 5.8-kb, 7.0-kb, 8.5-kb, 8.1-kb, 5.8-kb, and 15.0-kb *Bam*HI fragments, respectively. Only plants with the 5.8-kb *Hin*dIII fragment had either the 8.1-kb or 15.0-kb *Bam*HI fragment.

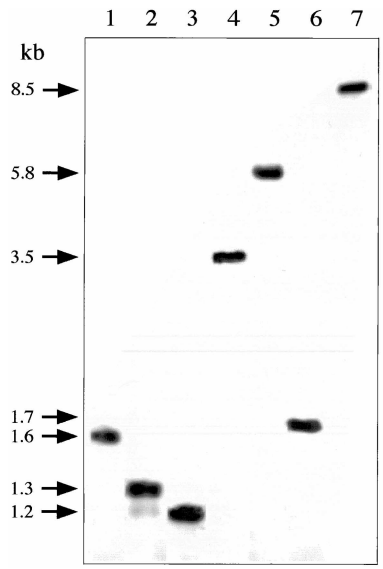

Fig. 2 Southern-blot analysis of total DNAs from wild soybeans digested with *Hin*dIII using *coxII* as a probe. *Lane 1*: type I (1.6 kb), *lane 2*: type II (1.3 kb), *lane 3:* type III (1.2 kb), *lane 4:* type IV (3.5 kb), *lane 5*: type V (5.8 kb), *lane 6:* type VI (1.7 kb), and *lane 7:* type VII (8.5 kb)

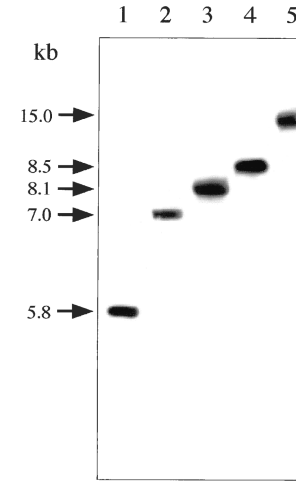

Fig. 3 Southern-blot analysis of total DNAs from wild soybeans digested with *Bam*HI using *coxII* as a probe. *Lane 1*: type I and type VI (5.8 kb), *lane 2*: type II (7.0 kb), *lane 3*: type IV and type V (8.1 kb), *lane 4*: type III (8.5 kb), and *lane 5*: type VII and type V′ (15.0 kb)

Eleven hybridization patterns were detected when DNAs were digested with *Bam*HI and hybridized with *atp6* (Fig. 4; Table 3). Most of them were composed of two or three fragments, suggesting the presence of two or three copies of *atp6* in the wild soybean mitochondrial genome. Grabau et al. (1988) actually detected two copies (copy 1 and copy 2) of *atp6* in mtDNA isolated from a soybean cultivar 'William 82'. RFLPs with *atp6* thus enable us to make a further subdivision of the eight mitochondrial haplotypes classified with *coxII*.

Classification of mitochondrial genomes

By combining the data of RFLP analyses using *coxII* and *atp6* as probes, we classified the plants tested into 18 haplotype groups in order to facilitate the evaluation of the variations detected. First, the hybridization patterns observed in the *Hin*dIII-digested DNAs with the *coxII* probe were designated types 'I' to 'VII': type I (1.6 kb), type II (1.3 kb), type III (1.2 kb), type IV (3.5 kb), type V (5.8 kb), type VI (1.7 kb), and type VII (8.5 kb; see Table 1). As mentioned earlier, the plants with the 5.8-kb *Hin*dIII fragment (type V) have either the 8.1-kb or 15.0-kb *Bam*HI fragment, so that type V was subdivided into two groups: type V (8.1 kb) and type V′ (15.0 kb; see Table 2). Second, the 11 hybridization patterns detected using *atp6* as a probe were designated alphabetically 'a' to 'k' (see Table 3). The data obtained using *coxII* and *atp6* as probes were combined and, as a consequence, provided 18 mitochondrial genome types as shown in Table 4. The genome types obtained with the *coxII* and *atp6* probes were associated with each other. For example, type I and type IV, both of which were predominant in the plants analyzed (see Table 5), were preferentially linked with types c and d, and types a and b, respectively.

Table 1 Fragments observed polymorphically in wild soybean in various regions of Japan (*coxII*/*Hin*dIII)

coxII/*Hin*dIII	Number of plants	Fragments hybridized (kb)							*H′*
		1.6 (I)	1.3 (II)	1.2 (III)	3.5 (IV)	5.8 (V)	1.7 (VI)	8.5 (VII)	
Hokkaido	212	92	0	0	87	33	0	0	1.47
North of Honshu	625	292	2	1	204	126	0	0	1.55
Central Honshu	92	62	0	1	24	4	1	0	1.23
South of Honshu	63	44	0	3	7	7	1	1	1.47
Shikoku	40	25	0	1	4	10	0	0	1.39
Kyushu	65	39	2	0	20	0	1	3	1.42
Total	1097	554	4	6	346	180	3	4	

H′: index of Shannon and Weaver (1949; see text)

Table 2 Fragments observed polymorphically in wild soybean in various regions of Japan (*coxII*/*Bam*HI)

coxII/*Bam*HI	Number of plants	Fragments hybridized (kb)					*H′*
		5.8 (I, VI)	7.0 (II)	8.1 (IV, V)	8.5 (III)	15.0 (V′, VII)	
Hokkaido	212	92	0	120	0	0	0.99
North of Honshu	625	292	2	327	1	3	1.08
Central Honshu	92	63	0	28	1	0	0.97
South of Honshu	63	45	0	14	3	1	1.13
Shikoku	40	25	0	14	1	0	1.09
Kyushu	65	40	2	20	0	3	1.31
Total	1097	557	4	523	6	7	

H′: index of Shannon and Weaver (1949; see text)

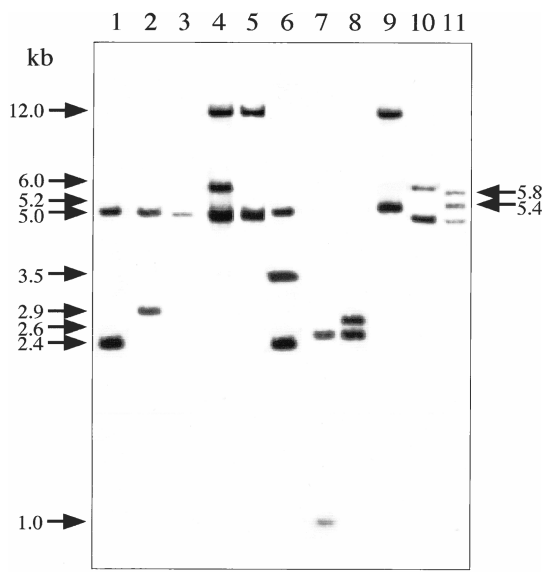

Fig. 4 Southern-blot analysis of total DNAs from wild soybeans digested with *Bam*HI using *atp6* as a probe. *Lane 1*: type a (2.4 and 5.0 kb), *lane 2*: type b (2.9 and 5.0 kb), *lane 3*: type c (5.0 kb), *lane 4*: type d (5.0, 6.0 and 12.0 kb), *lane 5*: type e (5.0 and 12.0 kb), *lane 6*: type f (2.4, 3.5 and 5.0 kb), *lane 7*: type g (1.0 and 2.6 kb), *lane 8*: type h (2.9 and 2.6 kb), *lane 9*: type i (5.2 and 12.0 kb), *lane* 10: type j (5.0 and 6.0 kb), and *lane 11:* type k (5.0, 5.4 and 5.8 kb)

Geographic distribution of mitochondrial genome types

The pattern of geographic distribution of the hybridization profiles and the extent of diversity are shown in Tables 1, 2 and 3. With regard to the fragments hybridized with *coxII*, plants with the 1.6-kb (type I) and 3.5-kb (type IV) *Hin*dIII fragments were distributed in all regions of Japan, although the frequency of the former was higher than the latter (Table 1). Plants with the 5.8-kb *Hin*dIII fragment were frequently observed in Hokkaido and North of Honshu, and plants with the other fragments were occasionally observed in some regions.

With regard to the results using *atp6* as a probe, plants with the 2.4-kb and 5.0-kb *Bam*HI fragments (type a) were frequently found in the northern part of Japan (Hokkaido, North of Honshu and Central Honshu), especially in Hokkaido (Table 3). Plants with the 5.0-kb, 6.0-kb and 12.0-kb *Bam*HI fragments were observed only in the northern part of Japan. On the other hand, plants with the 5.0-kb *Bam*HI fragment were observed frequently in the southern part of Japan, although this genome type was widely observed throughout Japan.

The extent of diversity was evaluated with the index of Shannon and Weaver (1949; *H′*) which is calculated as:

$$H' = -\sum_{i=1}^{n} p_i \log_2 p_i ,$$

in which p_i is the frequency of each mitochondrial genome type. This indicated differences in the extent of diversity among the six regions of Japan, although the order of the diversity among the regions was not conserved in the three hybridization experiments.

Table 5 presents the frequencies of the five most-observed mitochondrial genome types, namely, Ic, Id, IVa, IVb, and Va. These types were present in 94% of the plants tested. The other 13 types were region-specific with their frequencies being less than 1%. Most of the predominantly observed types showed a marked difference in frequency among different geographical regions of Japan. Type Ic and type Id were clearly characterized geographically. Type Ic was predominant in the southern part of Japan including South of Honshu, Shikoku and Kyushu, and there was a declining trend with increasing latitude. Type Id, on the other hand, showed a trend opposite to type Ic. Type Id was not observed at all in the South of Honshu, Shikoku and Kyushu, and its frequency increased with increasing latitude. Types IVa and IVb also showed a similar, but not so noticeable, cline to that found in types Ic and Id among the geographic regions. Type IVa was frequently observed in the northern part of Japan, whereas type IVb was observed at a low but appreciable frequency in the southern part of Japan.

Table 3 Fragments observed polymorphically in wild soybean in various regions of Japan (*atp6*/*Bam*HI)

atp6/*Bam*HI	Number of plants	Fragments hybridized (kb)											*H′*
		2.4, 5.0 (a)	2.9, 5.0 (b)	5.0 (c)	5.0, 6.0, 12.0 (d)	5.0, 12.0 (e)	2.4, 3.5 (f)	1.0, 2.6 (g)	2.6, 2.9 (h)	5.2, 12.0 (i)	5.0, 6.0 (j)	5.0, 5.4, 5.8 (k)	
Hokkaido	212	120	0	3	89	0	0	0	0	0	0	0	1.08
North of Honshu	625	306	14	34	257	1	3	2	0	4	3	1	1.56
Central Honshu	92	24	2	45	16	1	0	1	0	3	0	0	1.87
South of Honshu	63	8	8	44	0	0	0	2	0	1	0	0	1.37
Shikoku	40	10	5	25	0	0	0	0	0	0	0	0	1.30
Kyushu	65	1	10	45	0	0	0	6	3	0	0	0	1.40
Total	1097	469	39	196	362	2	3	11	3	8	3	1	

H′: index of Shannon and Weaver (1949; see text)

Table 4 Classification of mitochondrial genome types based on RFLPs using *coxII* and *atp6* as probes. Sizes of hybridization signals (kb) are shown

Mt type	Probe	*coxII*		*atp6*	Number of plants
	Enzyme	*Hin*dIII	*Bam*HI	*Bam*HI	
Ic		1.6	5.8	5.0	190
Id		1.6	5.8	5.0, 6.0, 12.0	361
Ie		1.6	5.8	5.0, 12.0	2
Ik		1.6	5.8	5.0, 5.4, 5.8	1
IIg		1.3	7.0	1.0, 2.6	4
IIIa		1.2	8.5	2.4, 5.0	1
IIIb		1.2	8.5	2.9, 5.0	4
IIId		1.2	8.5	5.0, 6.0, 12.0	1
IVa		3.5	8.1	2.4, 5.0	291
IVb		3.5	8.1	2.9, 5.0	35
IVc		3.5	8.1	5.0	6
IVf		3.5	8.1	2.4, 3.5, 5.0	3
IVh		3.5	8.1	2.6, 2.9	3
IVi		3.5	8.1	5.2, 12.0	8
Va		5.8	8.1	2,4, 5.0	177
V′j		5.8	15.0	5.0, 6.0	3
VIg		1.7	5.8	1.0, 2.6	3
VIIg		8.5	15.0	1.0, 2.6	4

We evaluated the diversity of mtDNA types in each region of Japan. *H′* (Shannon and Weaver 1949) for 18 mtDNA types showed that the most diverse region was North of Honshu (see Table 5) where 12 types were observed. In Hokkaido and Shikoku four mitochondrial genome types were present, in Central Honshu nine types were observed, and in the South of Honshu and Kyushu eight types were detected (see Tables 1–3).

Discussion

Grabau et al. (1992) and Hanlon and Grabau (1995) have classified the soybean accessions of the USDA germplasm collection into eight groups in terms of the RFLPs that were detected by using a 2.3-kb *Hin*dIII fragment of mtDNA and *atp6* as probes. Grabau et al. (1992) also surveyed RFLPs of mtDNA by using *coxII* as a probe, but failed to detect any polymorphism. In addition to wild soybeans, we also analyzed the RFLPs of five cultivated soybeans which had previously been determined for mitochondrial genome types by Grabau et al. (1989, 1992) and Hanlon and Grabau (1995): 'Minsoy' and 'Harosoy' of the Bedford type, 'Biloxi' and 'Palmetto' of the Arksoy type, and 'Peking' of the Soja-forage type. When analyzed with the *coxII* probe, all of the cultivars tested possessed the 3.5-kb *Hin*dIII fragment that was assigned to type IV in this study, being consistent with the results of Grabau et al. (1992). In combination with the result with the *atp6* probe, 'Minsoy' and 'Harosoy' which belong to the Bedford type were identified as type IVb, 'Bilox' and 'Plametto' of the Arksoy type were determined to be type IVc, and 'Peking' of the Soja-forage type were found to belong to type IVa (data not shown).

Grabau and Davis (1992) analyzed wild soybean accessions with the 2.3-kb *Hin*dIII fragment as a probe, and found that wild soybeans actually represent a set of cytoplasmic types that overlap with the types found in cultivated soybeans. In their analysis, 54 accessions from Japan exhibited two mitochondrial groups, the Arksoy-type (eight accessions) and the Soja-forage-type (46 accessions). Our study with both *atp6* and *coxII* probes revealed many more RFLPs in the wild soybeans distributed in Japan compared with the results of Grabau and Davis (1992).

Since many more RFLPs have been detected by using *coxII* and *atp6* as probes than by other mtDNA probes, this suggests that these mtDNA regions might be hot spots of variation in the mitochondrial genomes of the subgenus *Soja*. Furthermore, the observed polymorphisms for the *coxII* and *atp6* probes were partly associated with each other. This may be due to rearrangements of mtDNA around these two mitochondrial

Table 5 Frequency of five predominant mitochondrial genome types of wild soybean in various regions of Japan

Region	Number of plants	Mitochondrial genome types						H'
		Ic	Id	IVa	IVb	Va	Others	
Hokkaido	212	0.10	0.25	0.53	0.00	0.12	0.00	1.69
North of Honshu	625	0.17	0.38	0.22	0.03	0.15	0.05	2.39
Central Honshu	92	0.46	0.19	0.22	0.02	0.05	0.06	2.16
South of Honshu	63	0.68	0.00	0.02	0.10	0.07	0.13	1.72
Shikoku	40	0.65	0.00	0.00	0.09	0.23	0.03	1.36
Kyushu	65	0.73	0.00	0.02	0.10	0.00	0.15	1.44
Overall	1097	0.47	0.14	0.17	0.06	0.10	0.06	

genes. In fact, we have actually detected small repeated sequences that cause recombination between the upstream regions of *coxII* and *atp6* (Kanazawa et al. 1998).

In the present study, we have identified five predominant types of mtDNA in wild soybeans grown in Japan. Our analysis revealed that wild soybeans in Japan consist of a mixture of the plants with different types of mtDNA. This was occasionally true even within some collection sites. Conspicuous geographic clines of the predominant types were also detected. We have also analyzed cultivated soybeans in Japan with the *coxII* and *atp6* probes. Interestingly, the predominant type of mtDNA in cultivated soybeans, namely, IVb (Hirata et al. 1996 b), was also observed in wild soybeans in Japan. This observation may be important for analyzing the diversification of the subgenus *Soja* in Japan. In any case, the present results indicate that RFLP analyses of mtDNA are useful for clarifying the evolutionary process of diversification in the subgenus *Soja* through the maternal lineage. Our ongoing detailed analysis of mtDNA may provide explanations for the molecular mechanisms that generate the RFLPs observed in this study.

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