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Estimation of the Process Invaded by Accidentally Introduced Strains of *Abutilon theophrasti* into Japan: Temporal Change of Chloroplast DNA Haplotype Frequencies across a Century

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Abstract

Thirty-nine herbarium specimens of *Abutilon theophrasti* collected between 1883 and 2000 were examined for chloroplast DNA (cpDNA) analysis in order to examine the invasion process by cpDNA haplotype B, which was mixed in imported grain and accidentally introduced into Japan. By using nested PCR, all of the 39 specimen materials prepared were determined to be either of two haplotypes (the total numbers of haplotype A and B were 21 and 18, respectively). The first specimen of haplotype A was the oldest of all the samples; it had been collected in Yamagata in 1883. The collection times of the haplotype A specimens ranged evenly over the surveyed period. The first specimen of haplotype B was also old; it had been collected in Tokyo in 1893. Compared to haplotype A, the collection times of haplotype B concentrated on the 1960s and 1970s. The frequency of haplotype B has significantly increased after 1946 (before 1945: 27.8%; after 1946: 61.9%). These results may suggest that the accidental introduction of haplotype B has been increasing after World War II, reflecting the increase in the amount of grain imports.

Discipline: Agricultural environment
Additional key words: alien species, forage crop, herbarium specimen, velvetleaf, weed

Introduction

Invasive organisms are a threat throughout the world, causing destructive modification of natural ecosystems, reduction of agricultural productivity, and genetic modification of indigenous ecotypes. Understanding the invasion processes and the mechanisms of invasive alien species is important for the development of risk management strategies. Especially in the case of useful crop species, adequate risk management is needed for sustainable utilization if the species is subject to high invasion risk.

Abutilon theophrasti is an annual plant in the family Malvaceae. The species originated in either India[2,15] or China[18]. It first appeared in Japanese literature in "Honzo-Wamyo," a Chinese-Japanese dictionary about natural medicines compiled in 918[3]. Therefore, it may have been introduced to Japan before 918. We can also find many descriptions of the cultivation of *A. theophrasti* as a fiber crop and for other uses[5,7,11,13,19]. After the record of cultivation compiled by the former Ministry of Agriculture and Commerce in 1880, the cultivation of *A. theophrasti* almost stopped[19]. The species was not recognized as a

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weed until the 1980s[6]. In the mid-1980s, however, it suddenly became one of the most troublesome exotic weeds in forage crop fields all over Japan[16].

There were two possible reasons for the outbreak of *A. theophrasti* in Japanese forage crop fields: (1) the accidental escape of a cultivar; and (2) an invasion of a new strain from abroad. Some *A. theophrasti* seeds were found in imported sorghum and soybean from the United States and lupine from Australia, which supports the second scenario[1,17]. The contaminants detected in imported grain showed strong weedy growth, and were genetically different from crop types indigenous to Japan[8,9]. Chloroplast DNA (cpDNA) variations, which distinguish two haplotypes (A and B), were used to distinguish three genotypes (Type I, II, and III) with capsule color variations (ebony and ivory) as a morphological marker[10]. All of the samples from the United States and the samples taken from grain imports to Japan were found to be Type III (haplotype B, ebony capsule) in contrast to all Japanese cultivars, which were Type I (haplotype A, ivory capsule)[10]. Since most of the weedy types distributed in Japan were Type III, it was argued that they were introduced as seed in imported grain, that is, the second outbreak scenario[10]. In order to understand the process of invasion by accidental introduction, it is useful to know the temporal changes in genotype frequencies over a century.

Recent remarkable progress in molecular biology has allowed past events to be inferred by directly analyzing ancient DNA (aDNA) from historical specimens. Even in the cryptic invasion of exotic genotypes, aDNA analysis could help visualize the process of such changes[14]. Herbarium specimens, one type of historical material, have several useful properties for the study of invasion processes in plants. We can directly use the records of each herbarium specimen to understand its temporal and spatial changes. Although the time-scale is restricted to the last several hundred years, most recent drastic changes accompanying plant invasion took place within this time scale.

To clarify the process by which *A. theophrasti* invaded Japan, specifically with regard to when or where the invasive genotype invaded, it is necessary to compare the frequencies of genotypes over time. In order to do this, herbarium specimens could be useful materials for increasing our understanding of past events.

The collection method of herbarium specimens was probably not random sampling because, in general, botanists are unlikely to collect crop or weed plants from crop fields. Nevertheless, two cpDNA haplotypes of herbarium specimens must have been collected randomly because two cpDNA haplotypes of *A. theophrasti* in this study cannot be discriminated by morphology. Although it may be difficult to infer directly the situation in the crop fields, herbarium specimens can be considered to be valuable materials for our objectives.

In this study, as a first step in comparing the frequencies of genotypes over time, a DNA extraction method and PCR conditions for cpDNA haplotype discrimination were developed for the historical herbarium specimens of *A. theophrasti* collected from 1883 to 2000. Thus, we tried to answer the following two questions in order to understand the invasion process of *A. theophrasti*: 1) When did haplotype B increase in Japan?; and 2) What was the expanding pattern of the haplotype B distribution? According to Muranaka (2008)[12], the number of exotic plants has drastically increased since World War II. If haplotype B was introduced in the same way as other exotic plants, the frequencies of haplotype B should have also increased since World War II.

Materials and methods

In this study, we examined the herbarium specimens of *Abutilon theophrasti* preserved in four major herbaria of Japan: the National Science Museum (TNS), the University of Tokyo (TI), Tokyo Metropolitan University (MAK), and Kyoto University (KYO). Seed materials for cpDNA analysis were kindly provided by the curators of these herbaria. We selected the specimens, all of which had mature capsules containing seeds that were suitable for DNA extraction. In total, 39 specimens of *A. theophrasti* were used in this study (Table 1). Some specimens from different herbaria showed the same serial number, but all were analyzed because the existence of roots in each specimen showed them to be different individuals. The collection period of the specimens ranged from 1883 to 2000.

To determine the portion of herbarium specimens most suited for DNA extraction, we performed a preliminary experiment using a DNeasy Plant Mini Kit (QIAGEN, Tokyo, Japan) as follows: Adult whole plants of *A. theophrasti* were collected from a maize field infested with *A. theophrasti* in Tochigi Prefecture. The specimens were dried at 70°C for three days after the whole plants had been pressed. DNA samples were obtained from mature seeds, immature seeds, and the leaves of the herbarium specimens. As a control sample, the total DNA was also extracted from fresh leaves. The suitability of the material was judged based on the degree of DNA degradation. As a result, the mature seeds were determined to be the most suitable portion because relatively long fragments of DNA were detected in the electrophoresis gel. The following method of DNA extraction was determined

Table 1. Herbarium specimens of *A. theophrasti* collected in Japan and their cpDNA haplotype

Sample no.	Capsule color	cpDNA type	Herbarium	Specimen no.	Collected date	Name of collector	Locality	Habitat	Notes
1	Ebony	A	TNS	53705	1883/9/?	T. Nagasawa	Yamagata, Uzen (Yamagata)		cultivated
2	Ebony	B	MAK	73420	1893/10/3	T. Makino	Musasi, Tokyo (Tokyo)		
3	Ebony	B	KYO	2492	1897/10/5	Faurie	Akita (Akita)		
4	Ebony	B	KYO	?	1897/7/?	Kinashi	Wakayama (Wakayama)		
5	Ebony	B	MAK	73418	1907/9/5	M. Nagai	Ajigasawa, Kidukuri town, Nisitsugaru, Aomori (Aomori)		
6	Ebony	A	TI	71400	1910/8/?	T. Makino	Osumi, Prov. (Kagoshima)		
7	Ebony	A	MAK	71400	1910/8/?	T. Makino	Osumi (Kagoshima)		
8	Ebony	A	KYO	71400	1910/8/?	T. Makino	Ohsumi, Kagoshima (Kagoshima)		
9	Ivory	A	TI	?	1924/9/15	H. Muramatsu	Komaba (Tokyo)		Ichibi, Chingma, Kiriasa, Bouma (Japanese old name of the species)
10	Ivory	A	MAK	73372	1926/7/30		Tatesina, Sinano (Nagano)		
11	Ebony	A	MAK	73421	1933/?/?	T. Makino	Seihoden, Harima (Hyogo)		
12	Ebony	A	MAK	73422	1934/?/?	T. Makino	Ohsio, Harima (Hyogo)		
13	Ebony	B	KYO	?	1934/7/?	A. Sobajima	Arashima, Shima-gun, Mie (Mie)	wild plain	
14	Ebony	A	TNS	44527	1934/9/20	Y. Kiuchi	Ryobun Town, Katori, Shimofusa (Chiba)		
15	Ebony	A	KYO	?	1936/8/4	S. Hosomi	Yokota, Hikami Town, Hikami, Hyogo (Hyogo)		
16	Ebony	A	MAK	71397	1940/9/2	T. Makino	Ohizumi, Musasi (Tokyo)		
17	Ebony	A	MAK	71399	1940/10/7	T. Makino	Ohizumi, Musasi (Tokyo)		
18	Ebony	A	TI	?	1942/9/24	M. Hara	Sapporo City, Hokkaido (Hokkaido)		cultivated
19	Ebony	B	TNS	81998	1949/8/2	M. Togashi	Zyusoh, Osaka, Setsu (Osaka)		
20	Ebony	B	TNS	117253	1949/11/1	T. Kamino	Campus of Faculty of Education of Ehime University, Matuyama City (Ehime)	campus	If this plant is spontaneous, this is the first record. It may have disappeared after collecting
21	Ebony	A	KYO	?	1954/9/30	S. Kitamura, G. Murata	Kamigamo experimental field of Kyoto University, Kyoto (Kyoto)		cultivated
22	Ebony	A	KYO	27874	1955/8/7	S. Yamamoto	Matsuyamaminami School, Suehiro, Matsuyama City, Ehime (Ehime)	schoolyard	

Table 1. (continued)

Sample no.	Capsule color	cpDNA type	Herbarium	Specimen no.	Collected date	Name of collector	Locality	Habitat	Notes
23	Ebony	B	TNS	146136	1960/9/4	T. Ogata	Kajo, Yamagata City, Uzen (Yamagata)		
24	Ebony	B	KYO	?	1961/8/?	S.Okamoto	Experimental field of Kyoto University, Tokuyama City, Yamaguchi (Yamaguchi)		
25	Ebony	B	KYO	69	1966/10/8	Y. Nomura	Minatoura, Ikata Town, Nishiuwa, Ehime (Ehime)		
26	Ebony	B	TNS	253863	1969/8/3	F. Kazami	Higashikata, Yokohama City, Kanagawa (Kanagawa)		
27	Ivory	A	TI	?	1969/8/15	K. Enomoto	Mogi, Fukuoka (Fukuoka)		
28	Ebony	B	TNS	272693	1970/7/18	Y. Shimada	Ohe, Kumamoto City (Kumamoto)	roadside	rare
29	Ebony	B	KYO	6	1971/11/6	M. Naruhashi, M. Takahashi, Y. Naito	Southern part of Higashihoda, Miki City, Hyogo (Hyogo)		
30	Ebony	B	TNS	010090	1976/8/23	H. Konta	Kamimanno, Mannoharasinden, Fujinomiya City, Shizuoka, SW. foot of Mt. Fuji, Elevation ca. 300 m. (Shizuoka)	grassy cultivated field	
31	Ebony	B	TI	1326171	1979/9/23	K. Midorikawa	Yamagata Pref. Yonezawa-shi, Narushima (Yamagata)		
32	Ebony	A	TI	11802	1981/9/5	N. Kurosaki	Honshu, Pref. Hyogo: Mikageyamate, Higashinada-ku, Kobe City (Hyogo)		
33	Ebony	A	KYO	11802	1981/9/5	N. Kurosaki	Mikageyamate, Higashinada, Kobe City, Hyogo (Hyogo)		
34	Ebony	A	KYO	12277	1983/9/6	N. Fukuoka	Pref. Hyogo: Ikuno, Soei Junior College, Higashinada-ku, Kobe City (Hyogo)	in herb-grown place in light shade	
35	Ebony	B	KYO	1091	1986/7/11	S. Fujii	Higashitarumi, Tojo Town, Kato, Hyogo (Hyogo)		
36	Ebony	A	TNS	13334	1990/10/21	H. Ohota	Dogasan, Yokkaichi City (Mie)		
37	Ebony	B	TI	13334	1990/10/21	H. Ohota	Dogasan, Yokkaichi City (Mie)		
38	Ebony	A	TNS	19710	1999/9/28	H. Konta	Yumenoshima, Yumenoshima Park, Koto-ku, Tokyo, Elevation ca 10m. (Tokyo)		
39	Ebony	B	TNS	20385	2000/9/10	H. Konta	Riverside of Tonegawa, Abiko Town, Chiba (Chiba)	after cultivation	

Parentheses show the present prefecture of the collected locality

from the result of this preliminary experiment:

One or two seeds from each specimen were immersed in 400 μL of AP1 buffer (provided by manufacturer) containing 4 μL of RNase A (100 mg/mL) for 10 min at room temperature in a 1.5-mL micro test tube (DNeasy Plant Mini Kit, QIAGEN). Using a micro homogenizer (S-203, Ikeda Scientific Co., Ltd., Tokyo, Japan), the softened seeds were completely homogenized. We then followed the protocol recommended by the manufacturer. The extracted DNA solution was evaporated and diluted by 20 μL of distilled water. The solution was used as a template DNA for the following polymerase chain reaction (PCR) analysis.

The preliminary experiment described above revealed that all DNA extracted from the herbarium specimens was degraded due to the heat used in air-drying. In order to analyze such degraded DNA, three primers (primer 1: GTCTTGTGATATATTGTGAT, primer 2: ACGTACAAATCCCTTTAATA, primer 3: ATATGGATTATGAATCATTC) were designed to amplify the 6-base insertion/deletion shown in the non-coding region located between *trn*L and *trn*F. The primer sets can be used to discriminate haplotypes A and B by nested PCR (Fig. 1, see also Reference no. 10). The first amplification reactions were performed in a volume of 10 μL consisting of 1 μL of 50-fold diluted genomic DNA extract, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.25 mM of each dNTP, 0.25 U of Takara Ex Taq DNA polymerase (Takara Bio, Inc., Otsu, Japan), and 0.5 μM each of primers 1 and 3. The reaction products were diluted 10-fold and used as a template in the second amplification step, which was performed in a volume of 10 μL with the same constituents as described above, except for 0.5 μM each of primers 2 and 3, rather than 1 and 3. Primer 3 for the second PCR was labeled by Cy5 fluorescence at the 5' end. Both PCRs were performed under the same program: initial denaturation for 5 min at 94ºC, followed by 45 cycles of 30 s at 94ºC, 30 s at 45ºC, 30 s at 72ºC, and a final 7-min extension at 72ºC in a GeneAmp 9700 (Applied Biosystems Japan, Tokyo, Japan). The amplified cpDNA fragment was separated using ALFexpressII (Amersham Biosciences Corp, Piscataway, NJ, USA). The 6-base difference between haplotypes A and B was identified using an ALFwin Fragment Analyzer ver.1.00 (Amersham).

Results and discussion

In the previous study, we revealed that two cpDNA haplotypes (A and B) of *Abutilon theophrasti* could be distinguished by amplification of the fragments (approx. 460 base pairs) containing 6 base of an insertion or deletion polymorphism[10]. For successful amplification of this polymorphic site, it was necessary to devise a primer pair for a nested PCR technique. We developed new primer pairs flanking short fragments containing the polymorphic site. By using mature seeds as material and these primer pairs in the nested PCR step, all of the 39 specimen materials prepared were discriminated into two types of cpDNA haplotypes (Fig. 2). The numbers of specimens for each of the haplotypes A and B were 21 and 18, respectively.

The first specimen of cpDNA haplotype A was collected in Yamagata in 1883, although the label indicated that this specimen was cultivated. This specimen type was evenly collected in all decades of the period surveyed except for the 1890s, 1900s, and 1970s (Table 1). Since haplotype A occurs also in forage crop fields now[10], these results suggest that voluntary strains that escaped from cultivated plants have persisted in the wild. In contrast, the first specimen of cpDNA haplotype B was collected in Tokyo in 1893. Although specimens of this haplotype were also detected in most decades of the period surveyed, the collection times concentrated on the 1960s and 1970s (Table 1).

In Japan, the number of exotic plant species has drastically increased with the rapid growth in the amount of imports[12]. Especially in animal production, the amount

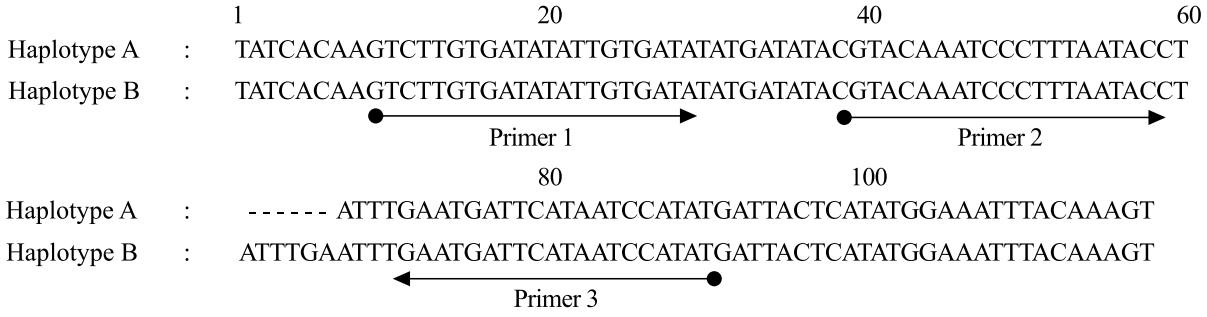

Fig. 1. Positions of the 6 base-pair insertion/deletion site and three primers for nested PCR performed to discriminate cpDNA haplotypes in herbarium specimens of *Abutilon theophrasti*

of grain imports for concentrated feed has also rapidly grown, increasing the chances of exotic weeds invading Japan. Totaling the numbers of herbarium specimens collected before 1945 and after 1946, we compared the percentages of cpDNA haplotypes A and B in the specimens collected before and after World War II by a Chi-square test. It was found that the percentage of cpDNA haplotype B in the specimens collected after 1946 was 61.9%, which is significantly higher than before 1945 (27.8 %) (χ^2 =4.54, P<0.05) (Fig. 3). Therefore, the cpDNA haplotype B of *A. theophrasti* tended to increase in number after World War II, approaching current conditions in crop fields (83.8% of the *A. theophrasti* samples mainly collected from corn fields in 1998 were cpDNA haplotype B[10]).

Based on the label, we could also plot the collection sites of herbarium specimens on a map. According to the cpDNA haplotypes determined, the distributions of the specimens were as shown in Fig. 4. The distribution of *A. theophrasti* includes 18 of 47 prefectures in Japan and does not show consecutive distribution as observed with indigenous wild plants. The distribution of cpDNA haplotype A ranged widely between Hokkaido (N43°) and Kagoshima (N32°) prefectures (Fig. 4-a, c), and that of cpDNA haplotype B also ranged widely between Aomori (N41°) and Kumamoto (N33°) prefectures (Fig. 4-b, d). Any notable differences in distribution patterns between cpDNA haplotypes A and B were not seen in the maps. The labels of three herbarium specimens (samples 1, 18, and 21 in Table 1) with the haplotype A of cpDNA noted them to be "cultivated," indicating distributions accompanied by cultivation. On the other hand, although some important trading ports for grains, such as Yokkaichi or Yokohama ports, were located near the sites where some specimens of haplotype B were collected, the relationship between grain trading and the distribution of haplotype B could not be clarified by the distribution patterns. In addition, the temporal changes in their distribution did not show a trend. Those sporadic patterns might be characteristic of crop species cultivated anywhere in Japan or transportation of accidentally introduced exotic weeds by human activity.

In the past several hundred years, the world has seen a huge number of exotic species invading new geographical areas beyond their natural range. People have recently become increasingly concerned about the problems caused by invasive species. In Japan, a new law, "the Invasive Alien Species Act," was promulgated as of June 2, 2004, to regulate the introduction of potential invasive species. However, it is difficult to evaluate a species' risk of invasion based only on the biological traits that may contribute to successful invasion. Their degree of potential invasiveness lies not in the species' traits themselves, but is a result of the process of invasion itself[4]. Understanding the mechanism of the invasion process might help us to evaluate which species has the strongest potential of becoming the next invasion threat. In order to understand the invasion processes of plants, it is important to elucidate the evolutionary and ecological changes in the plants that occurred over the last century. Combining herbarium specimens and the method described in our study can elucidate floristic change occurring over the last century. In particular, when we investigate taxa that are difficult to discriminate morphologically, molecular genotyping can provide valuable information. This study provided us with some suggestions to help us understand

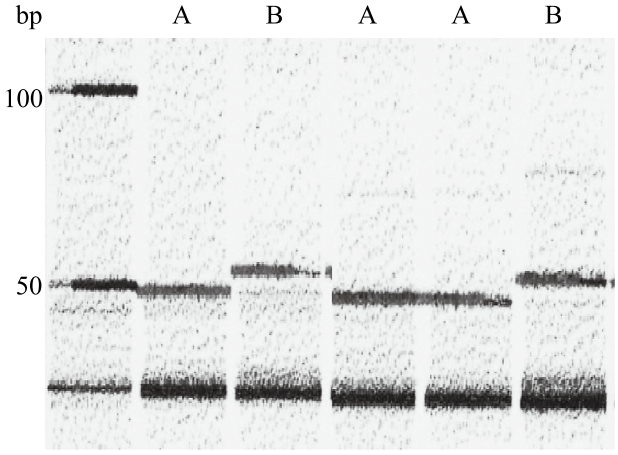

Fig. 2. An example of gel image-discriminated haplotypes of *Abutilon theophrasti* herbarium specimens
Note: A: herbarium specimen discriminated as haplotype A, B: herbarium specimen discriminated as haplotype B.

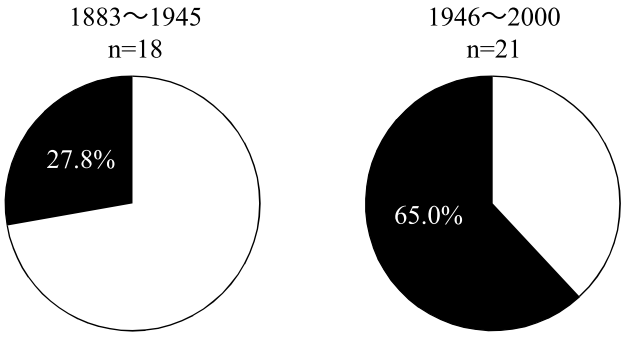

Fig. 3. Temporal distributions of each haplotype based on the cpDNA analysis of *Abutilon theophrasti* herbarium specimens over a period of 120 years
□ : Haplotype A, ■ : Haplotype B.

the invasion mechanism of *A. theophrasti*. The results showing the rapid increase of haplotype B after World War II supports our hypothesis that the recent outbreak of *A. theophrasti* in Japan was caused by the accidental introduction of a new weedy genotype. Our results imply that all species mixed in imported grains carry a risk of invading every area of Japan even if they are a native species.

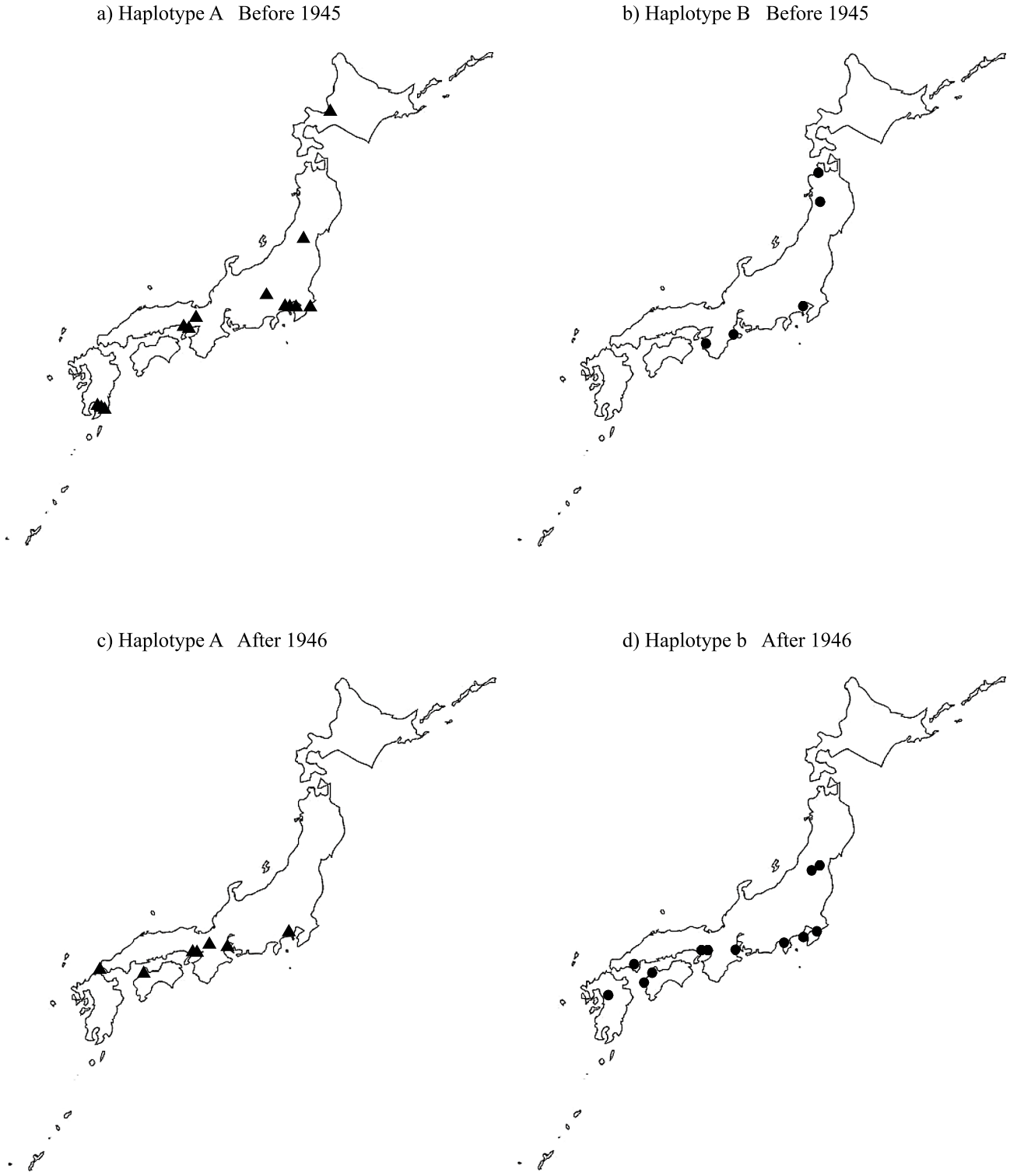

Fig. 4. Distribution maps of *Abutilon theophrasti* haplotypes based on cpDNA analysis of herbarium specimens from Japan

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