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 or China. It first appeared in Japanese literature in “Honzo-Wamyo,” a Chinese-Japanese dictionary about natural medicines compiled in 918. 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. After the record of cultivation compiled by the former Ministry of Agriculture and Commerce in 1880, the cultivation of *A. theophrasti* almost stopped. The species was not recognized as a

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

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. The contaminants detected in imported grain showed strong weedy growth, and were genetically different from crop types indigenous to Japan. 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. 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). 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. 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. 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), 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 DNase Plant Mini Kit (QIA-GEN, 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>
<thead>
<tr>
<th>Sample no.</th>
<th>Capsule color</th>
<th>cpDNA type</th>
<th>Herbarium Specimen no.</th>
<th>Collected date</th>
<th>Name of collector</th>
<th>Locality</th>
<th>Habitat</th>
<th>Notes</th>
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<td>1</td>
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<td>TNS</td>
<td>53705</td>
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<td>Yamagata, Uzen (Yamagata)</td>
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<tr>
<td>2</td>
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<td>MAK</td>
<td>73420</td>
<td>1893/10/3</td>
<td>T. Makino</td>
<td>Musasi, Tokyo (Tokyo)</td>
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<td></td>
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<tr>
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<td>2492</td>
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<td>Faurie</td>
<td>Akita (Akita)</td>
<td></td>
<td></td>
</tr>
<tr>
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<td>?</td>
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<td>Komaba (Tokyo)</td>
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<td>1926/7/30</td>
<td>Tatesina, Sinano (Nagano)</td>
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<td>?</td>
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<td>A. Sobajima</td>
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<td>44527</td>
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<td>Ryobun Town, Katori, Shimofusa (Chiba)</td>
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<td>?</td>
<td>1936/8/4</td>
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<td>71397</td>
<td>1940/9/2</td>
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<td>MAK</td>
<td>71399</td>
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<td>TI</td>
<td>?</td>
<td>1942/9/24</td>
<td>M. Hara</td>
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<td>TNS</td>
<td>81998</td>
<td>1949/8/2</td>
<td>M. Togashi</td>
<td>Zysoh, Osaka, Setsu (Osaka)</td>
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<td>20</td>
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<td>117253</td>
<td>1949/11/1</td>
<td>T. Kamino</td>
<td>Campus of Faculty of Education of Ehime campus University, Matuyama City (Ehime)</td>
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<td>21</td>
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<td>?</td>
<td>1954/9/30</td>
<td>S. Kitamura,</td>
<td>Kamigamo experimental field of Kyoto University, Kyoto (Kyoto)</td>
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<td>G. Murata</td>
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<td>22</td>
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<td>27874</td>
<td>1955/8/7</td>
<td>S. Yamamoto</td>
<td>Matsuyaminami School, Suehiro, Matsuyama City, Ehime (Ehime)</td>
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Table 1. Herbarium specimens of *A. theophrasti* collected in Japan and their cpDNA haplotype
<table>
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<th>Sample no.</th>
<th>Capsule color</th>
<th>cpDNA type</th>
<th>Herbarium Specimen no.</th>
<th>Collected date</th>
<th>Name of collector</th>
<th>Locality</th>
<th>Habitat</th>
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<td>146136</td>
<td>1960/9/4</td>
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<td>24</td>
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<td>KYO</td>
<td>?</td>
<td>1961/8/7</td>
<td>S. Okamoto</td>
<td>Experimental field of Kyoto University, Tokuyama City, Yamaguchi (Yamaguchi)</td>
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<td>25</td>
<td>Ebony B</td>
<td>KYO</td>
<td>69</td>
<td>1966/10/8</td>
<td>Y. Nomura</td>
<td>Minatoura, Ikata Town, Nishiwa, Ehime (Ehime)</td>
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<td>253863</td>
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<td>F. Kazami</td>
<td>Higashikata, Yokohama City, Kanagawa (Kanagawa)</td>
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<td>27</td>
<td>Ivory A</td>
<td>TI</td>
<td>?</td>
<td>1969/8/15</td>
<td>K. Enomoto</td>
<td>Mogi, Fukuoka (Fukuoka)</td>
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<td>28</td>
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<td>TNS</td>
<td>272693</td>
<td>1970/7/18</td>
<td>Y. Shimada</td>
<td>Ohe, Kumamoto City (Kumamoto)</td>
<td>roadside</td>
<td>rare</td>
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<td>29</td>
<td>Ebony B</td>
<td>KYO</td>
<td>6</td>
<td>1971/11/6</td>
<td>M. Naruhashi, M. Takahashi, Y. Naito</td>
<td>Southern part of Higashihoda, Miki City, Hyogo (Hyogo)</td>
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<tr>
<td>30</td>
<td>Ebony B</td>
<td>TNS</td>
<td>010090</td>
<td>1976/8/23</td>
<td>H. Konta</td>
<td>Kamimarno, Mannoharasinden, Fujinomiya City, Shizuoka, SW. foot of Mt. Fuji, Elevation ca. 300 m. (Shizuoka)</td>
<td>grassy cultivated field</td>
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<td>31</td>
<td>Ebony B</td>
<td>TI</td>
<td>1326171</td>
<td>1979/9/23</td>
<td>K. Midorikawa</td>
<td>Yamaga Pref. Yonezawa-shi, Narushima (Yamagata)</td>
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<td>11802</td>
<td>1981/9/5</td>
<td>N. Kurosaki</td>
<td>Honshu, Pref. Hyogo: Mikageyamate, Higashinada-ku, Kobe City (Hyogo)</td>
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<tr>
<td>33</td>
<td>Ebony A</td>
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<td>11802</td>
<td>1981/9/5</td>
<td>N. Kurosaki</td>
<td>Mikageyamate, Higashinada, Kobe City, Hyogo (Hyogo)</td>
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<tr>
<td>34</td>
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<td>12277</td>
<td>1983/9/6</td>
<td>N. Fukuoka</td>
<td>Pref. Hyogo: Ikuno, Soei Junior College, Higashinada-ku, Kobe City (Hyogo)</td>
<td>in herb-grown place in light shade</td>
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<td>1091</td>
<td>1986/7/11</td>
<td>S. Fujii</td>
<td>Higashitarumi, Tojo Town, Kato, Hyogo (Hyogo)</td>
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<tr>
<td>36</td>
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<td>13334</td>
<td>1990/10/21</td>
<td>H. Ohota</td>
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<td>37</td>
<td>Ebony B</td>
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<td>13334</td>
<td>1990/10/21</td>
<td>H. Ohota</td>
<td>Dogasan, Yokkaichi City (Mie)</td>
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<td>1999/9/28</td>
<td>H. Konta</td>
<td>Yumenoshima, Yumenoshima Park, Koto-ku, Tokyo, Elevation ca 10m. (Tokyo)</td>
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<td>TNS</td>
<td>20385</td>
<td>2000/9/10</td>
<td>H. Konta</td>
<td>Riverside of Tonegawa, Abiko Town, Chiba (Chiba)</td>
<td>after cultivation</td>
<td></td>
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</tbody>
</table>

Parentheses show the present prefecture of the collected locality.
Temporal Change of Chloroplast DNA Haplotype Frequencies in *Abutilon theophrasti*

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: ACCTTATACCTTTAAATA, primer 3: ATATGTGATTGAATCATTTC) were designed to amplify the 6-base insertion/deletion shown in the non-coding region located between *trnL* and *trnF*. 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 MgCl₂, 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 94°C, followed by 45 cycles of 30 s at 94°C, 30 s at 45°C, 30 s at 72°C, and a final 7-min extension at 72°C in a GeneAmp 9700 (Applied Biosystems Japan, Tokyo, Japan). The amplified cpDNA fragment was separated using ALFExpressI (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 polymorphism10. 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 now10, 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 imports12. Especially in animal production, the amount

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**Table 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 speci-
mens 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%) ($\chi^2 = 4.54, P < 0.05$) (Fig. 3). Therefore, the cpD-
NA haplotype B of *A. theophrasti* tended to increase in
number after World War II, approaching current condi-
tions in crop fields (83.8% of the *A. theophrasti*
samples mainly collected from corn fields in 1998 were cpDNA
haplotype B).  

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 hap-
loftype 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 accom-
panied 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 ad-
dition, the temporal changes in their distribution did not
show a trend. Those sporadic patterns might be charac-
teristic 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 geographi-
cal areas beyond their natural range. People have recent-
ly become increasingly concerned about the problems
caused by invasive species. In Japan, a new law, “the In-
vasive 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 poten-
tial invasiveness lies not in the species’ traits themselves,
but is a result of the process of invasion itself. Under-
standing the mechanism of the invasion process might
help us to evaluate which species has the strongest poten-
tial of becoming the next invasion threat. In order to un-
derstand 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

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

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**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.

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