

Article

Spatiotemporal Dynamics of Genetic Diversity in the Pale Grass Blue Butterfly After the Fukushima Nuclear Accident

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Abstract

The Fukushima nuclear accident in 2011 caused adverse phenotypic changes in wild organisms in radioactively polluted areas. However, few studies have investigated genetic changes after the accident. Here, we analyzed the nuclear DNA sequences of *internal transcribed spacer 2 (ITS2)* from the pale grass blue butterfly *Zizeeria maha* collected in 2011–2014 ($n = 389$). We detected 29 haplotypes, but the most frequent haplotype (H1) represented 86% of alleles examined. The haplotype H22 from Takahagi phylogenetically had the latest sequence, suggesting that it may be a novel mutant produced by the accident or just a minor existing haplotype. In Fukushima Prefecture, the H1 percentage oscillated; it peaked in Fall 2011 and decreased in Spring 2012 but peaked again in Fall 2012. Haplotype diversity and nucleotide diversity were low in Spring 2012 and Fall 2012 and then increased. The ratio of H1 to nonH1 was significantly different between the early and late periods of our field surveys. These results suggest that genetic diversity in Fukushima Prefecture initially decreased through a selection process in response to the Fukushima nuclear accident but was recovered by Fall 2014, probably due to immigrants and emerging mutants, which is consistent with previous morphological abnormality data.



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Keywords: butterfly; Fukushima nuclear accident; genetic diversity; genetic mutation; haplotype diversity; *ITS2*; radioactive pollution; real-time evolution; *Zizeeria maha*

1. Introduction

In the 21st century, artificial radionuclides are widespread on the surface of our planet [1]. The major sources of nuclear pollution are two nuclear weapon attacks (Hiroshima and Nagasaki in August 1945), numerous nuclear weapon experiments, and many nuclear power plant accidents (e.g., Three Mile Island in March 1979, Chernobyl in April 1986, and Fukushima in March 2011) [1]. One of the worst and most recent nuclear power plant accidents in the history of humankind is the Fukushima nuclear accident [2–4]. Facing this massive nuclear pollution event, one of the most critical concerns from the standpoint of environmental pollution is the possible biological impacts of this accident caused by radioactive materials and associated nonradioactive materials released from the Fukushima Dai-ichi Nuclear Power Plant (FDNPP). Although it is important to perform dosimetric evaluations (i.e., physical and theoretical evaluations) of environmental pollution recommended by the International Commission on Radiological Protection (ICRP) [5] and other major organizations concerning nuclear energy, such as IAEA (International Atomic Energy

Agency) and UNSCEAR (United Nations Scientific Committee on the Effects of Atomic Radiation), real-world evaluations in the field (i.e., biological, field-based, and experimental evaluations) are necessary to understand the whole picture of the biological impacts of the Fukushima nuclear accident. Accordingly, many field-based biological studies have reported the possible adverse effects of the Fukushima nuclear accident on wild organisms [6–47]. Notably, striking aphid abnormal morphology has been detected only in contaminated localities [48–50]. Tidal invertebrates along the polluted coast greatly decreased in number [51] and exhibited abnormal sexual maturation [52]. Japanese monkeys presented various abnormalities [53–57], and similar results have been reported in humans [58–64]. Most of these cases examine a specific organism only at a specific time point and locality, often a few or more years after the pollution event. To be sure, high-level initial exposure to short-lived radionuclides was possible only immediately after the accident. Hence, the adverse effects would then decline due to a decrease in radioactivity levels and due to biological adaptation to polluted environments. Nonetheless, the effects of chronic exposure to the remaining radionuclides, mostly ^{137}Cs , should not be ignored. Long-term monitoring of biological impacts is necessary to understand the real-time biological changes in the field. Considering that only a limited number of organisms have been studied from the viewpoint of the possible biological effects of the Fukushima nuclear accident, there are likely numerous cases of organisms in the field that have been severely affected but never been discovered by researchers.

Among the organisms that have been studied in Fukushima, the pale grass butterfly *Zizeeria maha* is probably one of the best-studied organisms [64–77]. It is a small lycaenid butterfly that shares living space with humans [66,78–81]. In previous studies, butterfly samples were collected immediately after the accident in May 2011, when the first adult butterflies emerged in the field [65,66]. Samples of this butterfly species were collected in seven localities in Fukushima Prefecture and Ibaraki Prefecture for three years [69]. The collected butterflies presented morphological abnormalities more frequently than those from nonpolluted localities did. The temporal abnormality rate (AR) dynamics of this butterfly species in Fukushima and Ibaraki Prefectures indicated that the adverse effects were most severe in Fall 2012 and then almost disappeared by Fall 2013 [69]. The abnormalities induced by ingesting contaminated leaves were transgenerational, but they could be alleviated by ingesting noncontaminated leaves [66,68]. On the other hand, abnormalities were inherited in the F_2 and F_3 generations from the parent generation collected in Fukushima in May 2011, suggesting that genetic mutations might have been introduced by initial radiation exposure immediately after the accident [65,66,69]. Hence, both physiological damage and genetic damage may have been introduced in this butterfly.

Despite these field-based studies, molecular studies in Fukushima research are rather rare. Importantly, a genetic mutation in the mitochondrial *cytochrome oxidase subunit I* (COI) gene was detected in the pale grass blue butterfly from Hirono Town, Fukushima Prefecture, collected in Spring 2012 [82]. This mutation was possibly introduced by the initial exposure immediately after the accident, and offspring mutants may have been produced, but they appeared to have been eliminated by 2021 [82]. There are other DNA studies on frogs [83], fish [84,85], and wild boar [86–88]. A study of microsatellite sequences of wild boar reported that microsatellites accumulated genetic damage caused by the Fukushima nuclear accident because the pre- and post-accident populations had different types of microsatellite sequences [88]. An alternative explanation for the different microsatellite sequences is that a pre-accident population was selected for the new polluted environment, resulting in the detection of new microsatellite sequences that had not been detected before but that were present as a minority. In any case, the wild boar population in Fukushima

appears to have experienced drastic genetic changes in response to the Fukushima nuclear accident at the population level.

In the present study, we focused on the rDNA region of *internal transcribed spacer 2* (*ITS2*) from the pale grass blue butterfly to understand the spatiotemporal dynamics of the *ITS2* haplotypes in the polluted area. This noncoding nuclear DNA is known to be highly variable and thus is suitable for tracking genetic diversity within a population containing closely related variants; in fact, *ITS2* has been used in phylogenetic analysis of closely related lycaenid butterflies [89–95]. Because we focused on this single region, our results may not necessarily represent the entire genome. Although genome-wide studies would show more accurate results on genetic diversity, our methodology still has a merit. As in the case of the previous *COI* study [82], we visually tracked DNA sequences of *ITS2* at the level of single nucleotide in the present study. In this way, we intended to discover “real-time” mutations that might have been introduced by the Fukushima nuclear accident *in situ*. Moreover, we intended to discover possible genetic differences between polluted and nonpolluted populations in addition to those suggested by the conventional metrics of population genetics. The results of the present study are discussed in comparison with the spatiotemporal AR dynamics already known in this species, suggesting that population-level genetic changes occurred in this insect after the Fukushima nuclear accident.

2. Materials and Methods

2.1. Butterfly Samples

In this study, we focused on the pale grass blue butterfly *Zizeeria maha*. Because this species is abundant in human living space throughout Japan except Hokkaido [78–81], it can serve as an indicator of environmental pollution [64,66]. It is often called *Pseudozizeeria maha*, but it is not recommended [78–82]. In the mainland Japan, *Zizeeria maha argia* is distributed, whereas in Okinawa, *Zizeeria maha okinawana* is distributed [78–81]. One generation is completed in approximately one month or longer. In most areas on the Honshu mainland, including Fukushima, five or six generations per year are expected in the six-month period (from late April to late October) [66]. In the present study, we presumed six generations per year in Fukushima Prefecture and other prefectures nearby just for the sake of discussion.

The butterfly samples used in this study were collected in the field during previous surveys in 2011–2014 [65,69,96]. Butterfly samples collected from ten prefectures were divided into the following two groups: five Pacific coast prefectures (Miyagi Prefecture, Fukushima Prefecture, Ibaraki Prefecture, Tochigi Prefecture, and Saitama Prefecture) and four Japan Sea coast prefectures (Yamagata Prefecture, Niigata Prefecture, Toyama Prefecture, and Ishikawa Prefecture) (Figure 1a) in addition to Okinawa Prefecture. These two coastal regions are geographically divided by the Ou Mountains. This means that the pollution levels are different between the two groups. Equally importantly, this lycaenid butterfly is distributed coastal and low-altitude regions in human residential areas. This butterfly is not usually distributed in mountain and high-altitude regions. Therefore, the distribution range of this butterfly is likely physically divided into two coastal regions by the Ou Mountains in northeastern Japan.

We used only male samples without morphological abnormalities for the present analysis simply because most collected samples were males without morphological abnormalities. Accordingly, we are not intended to discuss correspondence between morphological abnormalities and *ITS2* sequences at the individual level. This is reasonable because *ITS2* does not directly contribute to morphogenesis by any means. On the other hand, it is reasonable to discuss correspondence between *ITS2* sequences and morphological abnormalities at the population level despite the exclusive use of male specimens without morphological abnormalities.

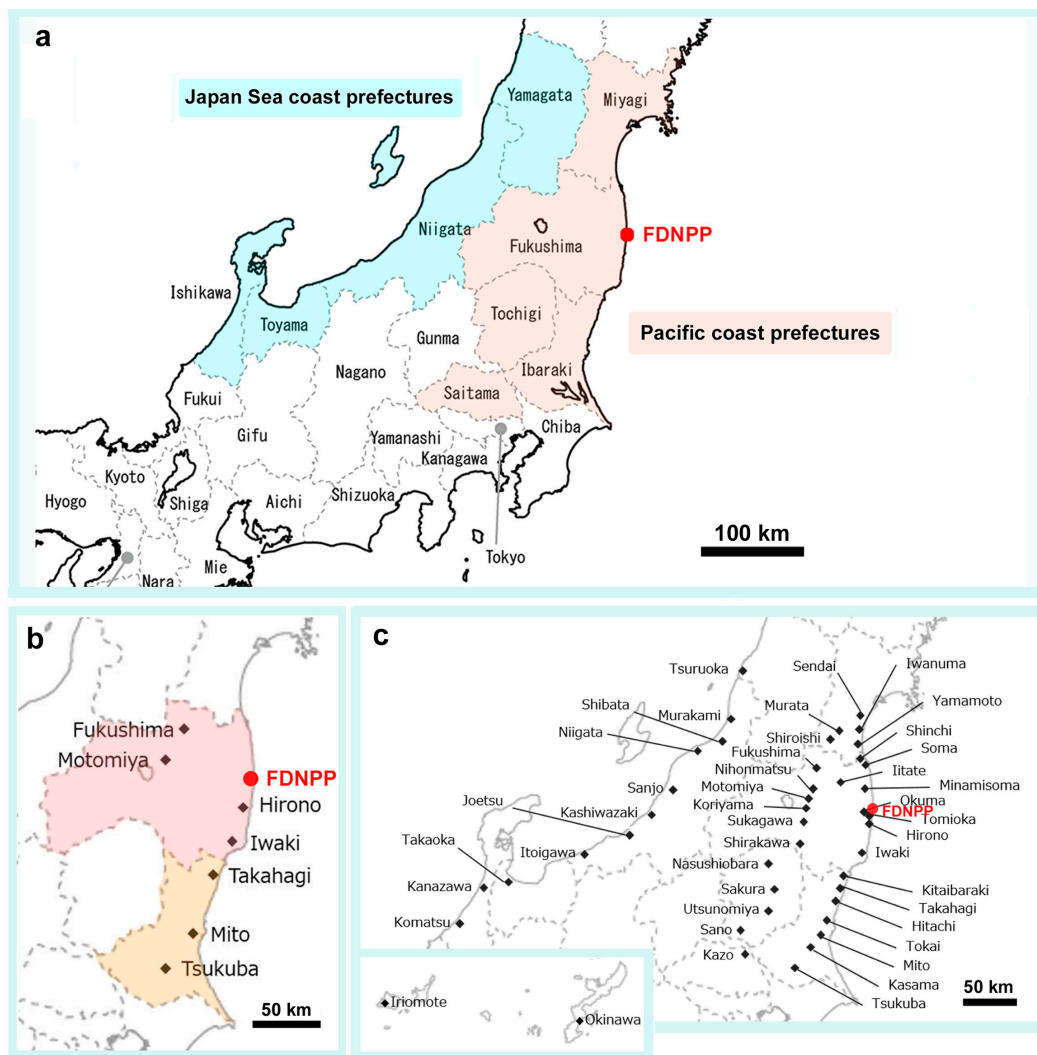


Figure 1. Field sample collection localities. The Fukushima Dai-ichi Nuclear Power Plant (FDNPP) is shown in red. A part of this figure is a modified version of the map shown in Toki et al. (2025) [82], with which samples were shared in the present study. (a) Nine prefectures in which collection surveys were conducted (excluding Okinawa Prefecture). For the sake of discussion, they are divided into five Pacific coast prefectures (Miyagi, Fukushima, Ibaraki, Tochigi, and Saitama Prefectures) and four Japan Sea coast prefectures (Yamagata, Niigata, Toyama, and Ishikawa Prefectures). (b) Seven localities (City/Town) surveyed in 2011–2013 in Fukushima and Ibaraki Prefectures. (c) Forty-four localities (City/Town/Village) in the Kanto-Tohoku district, northeastern Japan, and in Okinawa, surveyed in 2014.

In the 2011–2013 surveys, samples (total number of butterfly individuals analyzed, $n = 193$) were collected from seven localities in Fukushima and Ibaraki Prefectures as follows: Fukushima City (number of individuals analyzed, $n = 41$), Motomiya City ($n = 22$), Hirono Town ($n = 25$), Iwaki City ($n = 26$), Takahagi City ($n = 30$), Mito City ($n = 26$), and Tsukuba City ($n = 23$) (Figure 1b). Additional butterfly samples (total number of butterfly individuals analyzed, $n = 196$) were collected from 44 localities (42 localities in northeastern Japan and two localities in Okinawa) in Fall 2014 [96] (Figure 1c). These localities were as follows: five localities in Miyagi Prefecture (Shiroishi City, Murata Town, Sendai City, Iwanuma City, and Yamamoto Town; number of individual samples analyzed, $n = 22$), 14 localities in Fukushima Prefecture (Shinchi Town, Soma City, Iitate Village, Minamisoma City, Okuma Town, Tomioka Town, Hirono Town, Iwaki City, Fukushima City, Nihonmatsu City, Motomiya City, Koriyama City, Sukagawa City, and Shirakawa

City; $n = 63$), seven localities in Ibaraki Prefecture (Kitaibaraki City, Takahagi City, Hitachi City, Tokai Village, Kasama City, Mito City, and Tsukuba City; $n = 33$), four localities in Tochigi Prefecture (Nasushiobara City, Sakura City, Utsunomiya City, and Sano City; $n = 20$), one locality in Saitama Prefecture (Kazo City; $n = 5$), one locality in Yamagata Prefecture (Tsuruoka City; $n = 5$), seven localities in Niigata Prefecture (Murakami City, Shibata City, Niigata City, Sanjo City, Kashiwazaki City, Joetsu City, and Itoigawa City; $n = 27$), one locality in Toyama Prefecture (Takaoka City; $n = 5$), two localities in Ishikawa Prefecture (Kanazawa City and Komatsu City; $n = 9$), and two localities in Okinawa Prefecture (Okinawa-jima Island and Iriomote-jima Island; $n = 7$).

2.2. DNA Extraction and PCR

DNA was extracted from field-caught butterfly samples. To do so, the thorax, head, and associated parts, excluding the wings and abdomen, were homogenized and subjected to DNA extraction using a Tissue DNA Kit (Omega Bio-tek, Norcross, GA, USA), Nucleospin Tissue XS (MACHEREY-NAGEL, Dueren, Germany), or DNAzol Direct (DN131) (Molecular Research Center, Cincinnati, OH, USA). DNA extraction procedures were performed according to the manufacturers' protocols, after which we measured the DNA concentrations using a Qubit 2.0 fluorometer (Invitrogen, Waltham, MA, USA). With the extracted DNA, we subsequently performed polymerase chain reaction (PCR) analysis for *ITS2*. These DNA samples were also used in a previous study [82]. We referred to primer sequences previously used in phylogenetic analysis of lycaenid blue butterflies [89,90]. The M13F sequence (5'-GTAAAACGACGGCCAG-3') or M13R sequence (5'-CAGGAAACAGCTATGAC-3') was added at the 5' ends of the lycaenid primers for direct Sanger dideoxy DNA sequencing. These primers were synthesized by Exigen (Tokyo, Japan) as follows: the forward primer, 5'-GTAAAACGACGGCCAGACTCCTGTCTGAGGGCCGGCTG-3', and reverse primer, 5'-CAGGAAACAGCTATGACAAAAATTGAGGCAGACGCGATA-3'. The most common PCR product was 500 bp in length, including primer sequences, and the amplified DNA, excluding primer sequences, was 423 bp in length. We confirmed the amplified DNA by agarose gel electrophoresis. We then confirmed the amplified DNA by direct Sanger DNA sequencing (see below).

The PCR mixture (50.0 μ L in total) contained the following reagents: 2 $\times$ Gflex PCR Buffer (Takara Bio, Kusatsu, Shiga, Japan) (7.5 μ L), DNase-free RNase-free deionized water (Nippon Gene, Tokyo, Japan) (6.6 – x μ L), forward primer (10 pmol/ μ L; 0.3 μ L), reverse primer (10 pmol/ μ L; 0.3 μ L), high-fidelity Tks Gflex DNA Polymerase (Takara Bio) (0.3 μ L), and template DNA solution (x μ L). We always performed a negative control simultaneously with experimental PCRs. For a negative control, template DNA was replaced with DNase-free RNase-free deionized water. The amount of template DNA used varied from 2.4 ng to 3.6 ng per reaction.

The reaction cycles were set as follows: 94 °C (60 s), followed by 40 cycles of 98 °C (10 s), 60 °C (15 s), and 68 °C (15 s), and then 68 °C (60 s). Alternatively, we performed the following cycles: 94 °C (60 s), then 10 cycles of 98 °C (10 s), 60 °C (15 s), and 68 °C (15 s), and then 35 cycles of 98 °C (10 s), 58 °C (15 s), and 68 °C (15 s), and finally 68 °C (60 s). The step at 60 °C (15 s) in the first 10 cycles was occasionally changed to 59.5 °C or lower. For agarose gel electrophoresis, 2.0 μ L of the PCR product together with 8.0 μ L of water and 2.0 μ L of loading dye was applied to a well of agarose gel, and electrophoresis was performed for 25 min at 100 V. The PCR product was purified using NucleoSpin Gel and PCR Clean-up (MACHEREY-NAGEL), ExoSAP-IT *Express* PCR Product Cleanup Reagent (Applied Biosystems, Waltham, MA, USA), or Exo-CIP Rapid PCR Cleanup Kit (New England Biolabs, Ipswich, MA, USA) for direct Sanger dideoxy DNA sequencing.

2.3. DNA Sequencing and Sequence Analyses

Direct Sanger dideoxy DNA sequencing was performed using BigDye Terminator v3.1 (Thermo Fisher Scientific, Waltham, MA, USA) in GeneWiz (Saitama, Japan). A given DNA sample was read in both the forward and reverse directions. The DNA sequence spectral data were analyzed via GeneStudio (<https://sourceforge.net/projects/genestudio/>; accessed on 20 June 2025) and were visually examined one base at a time. The most frequent haplotype was termed H1 and was considered the reference sequence (RefSeq) in this study. The H1 sequence was deposited in GenBank (GenBank Accession Number: PV955657). The sequence reads were classified into the following five categories in reference to RefSeq: (1) a sequence containing no mixed signal and no insertion, (2) a sequence containing no mixed signal but containing insertions, (3) a sequence containing mixed signals for nucleotide substitutions but without insertions and deletions, (4) a sequence containing mixed signals for nucleotide substitutions with insertions and/or deletions, and (5) a sequence containing a series of mixed signals. In the case of (5), long-running high-quality signals were immediately disrupted by a series of long mixed signals, indicating that the PCR product was a mixture of two haplotypes containing indels. In the present study, we recorded sequences of (1) to (4) and excluded (5) for subsequent analyses.

Since the *ITS2* sequence is present in nuclear DNA, a single individual has at least two alleles. On the basis of this fact, we interpreted the cases above as follows: (1) an individual has two identical alleles without insertions, (2) an individual has two identical alleles with insertions, (3) an individual has two different alleles with single nucleotide substitutions, and (4) an individual has two different alleles with single nucleotide substitutions and insertion and/or deletions. When there were two mixed signals, in the cases of (3) and (4), two alleles were differentiated by the sequences of peak heights. For example, the first position of nucleotide substitution (mixed signal) was A and G, and the second position was C and T. The peak ratios were A:G = 4:1 at the first position and C:T = 4:1 at the second position. We then judged that the sample was a mixture of two alleles with a combination of A and C in one allele and a combination of G and T in another allele. Because we confirmed these sequences in both forward and reverse directions, we considered this method accurate. However, when spectral data were not resolved well for some reasons, such samples were excluded from the subsequent analyses.

ITS2 DNA sequences were aligned with one another using ClustalW (<https://www.genome.jp/tools-bin/clustalw>; accessed on 20 June 2025) with default values. Although the alignments of *ITS2* sequences are often difficult in distant taxa [91], we experienced no alignment problem because of high similarities among the sequences used in the present study. A haplotype network diagram was produced using DnaSP 5.0 [97,98] (<http://www.ub.edu/dnasp/>; accessed on 17 June 2025) and Network (<https://www.fluxus-engineering.com/sharenet.htm>; accessed on 17 June 2025). A phylogenetic tree (maximum likelihood; ML) was constructed using MEGA X [99] (<https://www.megasoftware.net>; accessed on 17 June 2025). To do so, *ITS2* DNA sequence data from *Zizina oxleyi* (GenBank Accession Number: JQ073633.1) [100] and *Zizina otis labradus* (GenBank Accession Number: JQ073629.1) [101] were included as an outgroup. We set the Kimura2-parameter model for nucleotide substitution, “all use sites” for gap sites, and 1000 repetitions for the bootstrap method.

The haplotype number (H), haplotype diversity (H_d), nucleotide diversity (π), and other indices for population genetics were obtained using DnaSP 5.0 with and/or without gaps in accordance with the localities and prefectures from which butterfly samples were obtained. We obtained the number of individuals with each haplotype and then calculated the haplotype percentage for each collection locality and collection year. Statistical analysis (χ^2

test) was performed using FreeJSTAT (<http://toukeijstat.web.fc2.com/sub5.html>; accessed on 17 June 2025). In performing the χ^2 test, p -values were adjusted by Yate's adjustment.

The discovery rate of novel haplotype per field survey was calculated using samples from the seven localities in Fukushima and Ibaraki Prefectures (Figure 1b). To do so, the number of novel haplotypes discovered was divided by the number of individuals examined at that survey. The cumulated rate of analyzed individuals and the cumulated rate of discovered haplotypes were also calculated. The maximum values of these cumulated rates were adjusted to be one.

3. Results

3.1. Diverse ITS2 Sequences

We obtained diverse ITS2 sequences from 193 individuals in the 2011–2013 samples (Table A1). Similarly, we obtained diverse ITS2 sequences from 196 individuals of the 2014 samples (Table A2). The most frequent haplotype here was named H1, which was considered the reference sequence (RefSeq) in this study (Figure 2). H1 was found at least in one individual from all the localities and prefectures examined, including Okinawa Prefecture, indicating its widespread distribution throughout Japan not only in *Zizeeria maha argia* but also in *Zizeeria maha okinawana*. In reference to H1, individuals with H1 and other haplotypes were classified into five categories (1)–(5) (Tables A1 and A2) (see Materials and Methods for categorization).

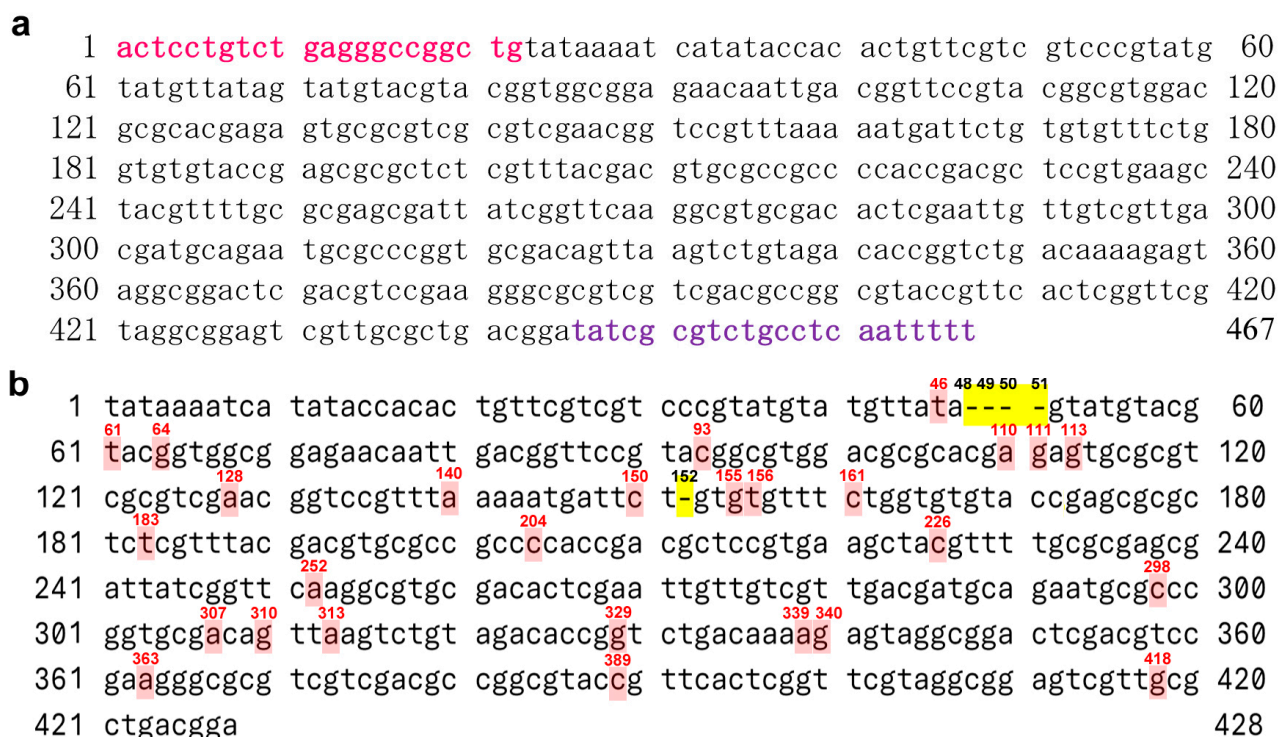


Figure 2. The most frequent haplotype (H1) of ITS2 sequences from *Zizeeria maha*. Numbers indicate nucleotide positions from the 5' end of the sequence. (a) H1 PCR product. The red and purple sequences are the PCR primer sequences. (b) H1 sequence without PCR primers. Gaps in 48–51 and 152 (highlighted in yellow) are indicated to accommodate other haplotype sequences with insertions at these sites. Nucleotide substitution sites in other haplotypes are indicated by red shading. This sequence is considered the reference sequence (RefSeq) in this study and was deposited in GenBank without gaps (GenBank Accession Number: PV955657).

Hereafter, we concentrated on samples in categories (1)–(4) for analyses; 144 individuals from the 2011–2013 samples and 129 individuals from the 2014 samples were analyzed

further. Among the 2014 samples, one individual from Kazo City, Saitama Prefecture, and one individual from Hitachi City, Ibaraki Prefecture, were classified into (3) but were excluded from the subsequent analyses because the spectral sequence data were not resolved well into two independent sequences. In reference to RefSeq (H1), in the 2011–2013 samples, we obtained 259 alleles without substitutions and 29 alleles with one or more substitutions (Table A3). Similarly, we obtained 211 alleles without substitution and 47 alleles with one or more substitutions in the 2014 samples (Table A4). In total, we obtained and examined 547 alleles.

3.2. Relationships Among ITS2 Haplotypes

Among the alleles that were examined in this study, 29 haplotypes, H1–H29 (Table 1), were detected from various localities (Tables A5–A7). The H1 haplotype had the greatest number of alleles examined, 469 (85.7%), which was by far the majority. Among the H2–H29 haplotypes, H12 had the greatest number of alleles, 20 (3.7%). Many haplotypes (H4, H7, H11, H14, H15, H16, H17, H18, H19, H20, H21, H22, H23, H24, H25, H27, and H28) had only a single allele (0.18% per haplotype), indicating that they were the minority. When examined based on prefectures, eight haplotypes were shared with two or more prefectures: H1, H2, H3, H5, H6, H9, H12, and H13 (shared haplotypes), and 21 other haplotypes were discovered only in a single prefecture: H4, H7, H8, H10, H11, and H14–H29 (private haplotypes) (Table A8), indicating that there were many unique haplotypes for each prefecture. Among prefectures, Ibaraki Prefecture (46%) and Fukushima Prefecture (44%) had the largest percentages of private haplotypes (Table A8).

To understand the relationships among these haplotypes, we constructed a haplotype network diagram (Figure 3a). In this diagram, H1 is present as the largest circle containing various prefectures. No haplotypes are comparable to H1, suggesting no clearly differentiating subpopulations. If any, H12 is relatively large and may be a small hub for minor differentiation. Indeed, many haplotypes are located around the large H1 circle, indicating that they had just a single-nucleotide substitution. Many of them were discovered only once in this study, indicating their rarity in the field.

H26 (Kashiwazaki City, Niigata Prefecture, collected in Fall 2014) deviated the most from the major haplotype H1 (Figure 3a). H26 has four nucleotide insertions and one substitution. However, H26 was not collected from the area polluted by the FDNPP. Additionally, a branch containing H14, H27, and H13 and another branch containing H22, H21, H15, and H12 seemed to deviate from the major haplotype H1. Among them, notable haplotypes collected in either Fukushima or Ibaraki Prefecture were H14 (Motomiya City, Fukushima Prefecture, collected in Spring 2013) and H22 (Takahagi City, Ibaraki Prefecture, collected in Fall 2014). These two samples were collected from two of the seven sampling localities relatively close to the FDNPP. Notably, H22 had four nucleotide substitutions from H1 without any intermediate haplotypes between H22 and H1, suggesting a unique evolutionary history of H22. Additionally, H21 (Takahagi City, Ibaraki Prefecture, collected in Fall 2013) and H15 (Fukushima City, Fukushima Prefecture, collected in Fall 2014) were notable. Because of their rarity and uniqueness, these haplotypes may be considered those that characterize the Fukushima and Ibaraki Prefectures, corresponding to the polluted area.

We also constructed a molecular phylogenetic ML tree based on the ITS2 haplotype sequences (Figure 3b). The tree has bootstrap values of 81–98%, indicating that the clades are reasonably well supported. Overall, as in the case of the haplotype network diagram, most haplotypes are not well differentiated from one another. However, the tree indicates that three haplotypes, H13 (Motomiya City, Fukushima Prefecture, collected in Spring 2013; Minamisoma City, Fukushima Prefecture, collected in Fall 2014; and Kashiwazaki City, Niigata Prefecture, collected in Fall 2014), H14 (Motomiya, Fukushima Prefecture, collected

in Spring 2013), and H27 (Kashiwazaki City, Niigata Prefecture, collected in Fall 2014), belong to an independent basal clade being sister to all other haplotypes (“Basal clade” in Figure 3b). These three haplotypes belong to either the Pacific coast group or the Japan Sea coast group, indicating that there would be no geographical clustering in this basal clade.

Interestingly, the haplotypes most recently emerged are clustered (“Latest clade” in Figure 3b), and they are H22 (Takahagi City, Ibaraki Prefecture, collected in Fall 2014), H15 (Fukushima City, Fukushima Prefecture, collected in Fall 2014), and H21 (Takahagi City, Ibaraki Prefecture, collected in Fall 2013). These haplotypes are also notable in the haplotype network diagram discussed above (Figure 3a). The localities of these three haplotypes are relatively close to the FDNPP. Therefore, the three latest haplotypes, H15, H21, and H22, from the contaminated area may indicate the following three possibilities: (1) they are new immigrants from surrounding environments; (2) they are newly expanding haplotypes that have been present as very minor haplotypes in that area; and (3) they are newly produced mutant haplotypes in response to DNA damage due to the Fukushima nuclear accident.

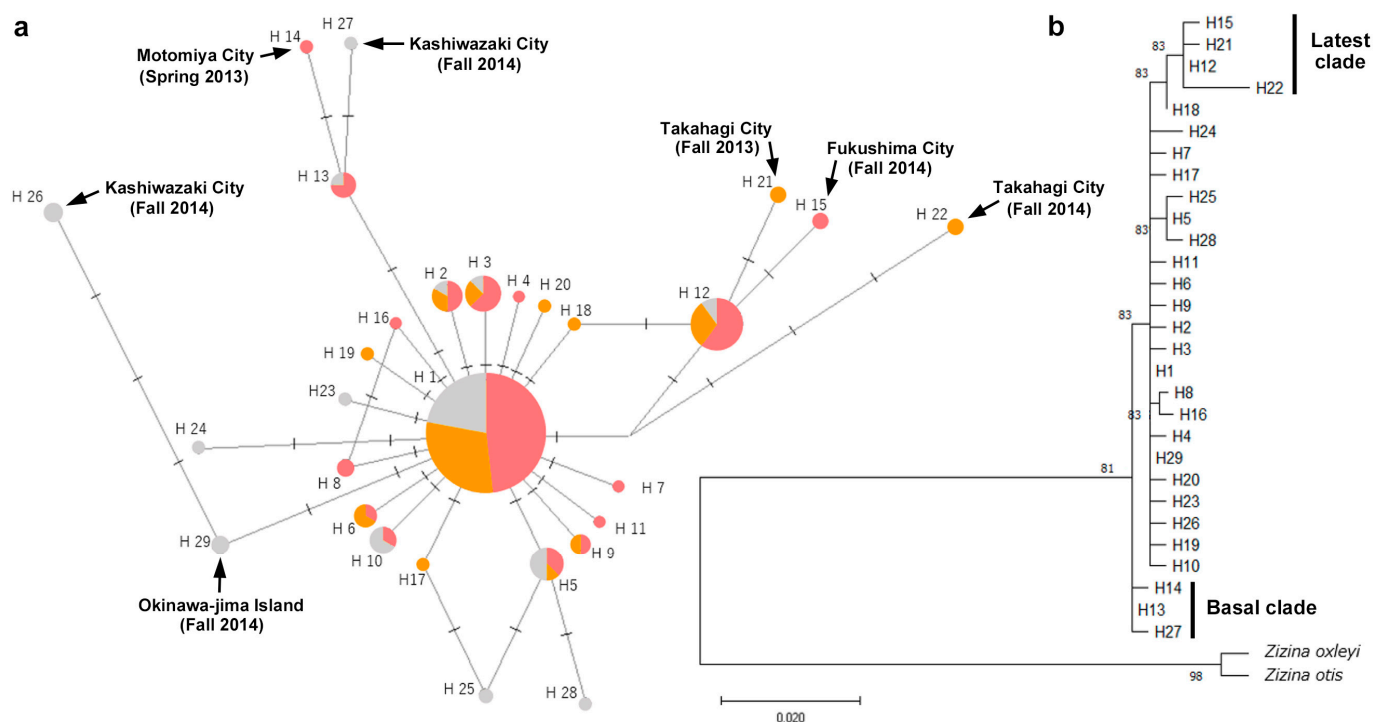


Figure 3. Relationships among various haplotypes of *ITS2*. (a) Haplotype network. The pie chart size roughly indicates the number of individuals with a given haplotype. Pink (Fukushima Prefecture), orange (Ibaraki Prefecture), and gray (nonFI; prefectures other than Fukushima and Ibaraki Prefectures) areas in a pie chart indicate the proportions of individuals occupying a given haplotype. Fukushima and Ibaraki Prefectures are color-coded as shown in Figure 1. Short crossing lines indicate the number of nucleotide substitutions and insertions. (b) Molecular phylogenetic ML tree. *ITS2* sequences of *Zizina oxleyi* and *Zizina otis* *labradus* were used as an outgroup. The basal clade and the latest clade are indicated. Bootstrap values are shown in percentage.

Table 1. ITS2 haplotype sequences detected in this study.

	Al	SI	46	48	49	50	51	61	64	93	110	111	113	128	140	150	152	155	156	161	183	204	226	252	298	307	310	313	329	339	340	363	389	418
H1	469	0	T	-	-	-	-	T	G	C	A	G	G	A	A	C	-	G	T	C	T	C	C	A	C	A	G	A	G	A	G	A	C	G
H2	6	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H3	8	1	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H4	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-
H5	8	1	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H6	3	1	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H7	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H8	2	1	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H9	2	1	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H10	3	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H11	1	1	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H12	20	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	T	-	-	-	-
H13	4	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-
H14	1	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	A	-
H15	1	3	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	T	-	-	-	-
H16	1	1	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H17	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-
H18	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-
H19	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H20	1	1	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H21	1	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	G	-	-	-	-	-	-	-	-	-	-
H22	1	4	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	A	-	-	-	-	-	-	G	-	T	-	-	-
H23	1	1	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H24	1	2	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-
H25	1	2	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-
H26	2	5	-	G	T	A	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-
H27	1	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	A
H28	1	2	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-
H29	2	2	-	-	T	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Note: Only substituted nucleotides in reference to H1 are shown in nonH1 haplotypes. See the H1 sequence in Figure 2. Al: the number of alleles detected. SI: the number of nucleotide substitutions and insertions detected.

3.3. Haplotype and Nucleotide Diversity Values

Here, we calculated haplotype diversity values (H_d) for the 2011–2014 samples, focusing on prefectural (spatial) differences and ignoring temporal ones for the sake of clarity (Figure 4a; Table A9). When gaps in sequences were included, Okinawa Prefecture presented the highest value among prefectures (0.533), and Toyama presented the lowest value (0.000). Excluding these two extremes, there seemed to be a geographical bias. Prefectures on the Pacific coast, including the Fukushima and Ibaraki Prefectures, tended to have lower values than those on the Japan Sea coast did, suggesting that there were relatively small number of haplotype variants in Fukushima and Ibaraki Prefectures. However, Saitama Prefecture presented relatively high value despite being categorized here as a Pacific coast prefecture. The same tendencies were observed when gaps were excluded (Figure 4a; Table A9), except that the haplotype diversity value of Okinawa Prefecture changed from 0.5333 to zero because of the insertions of H29, which was unique to Okinawa Prefecture.

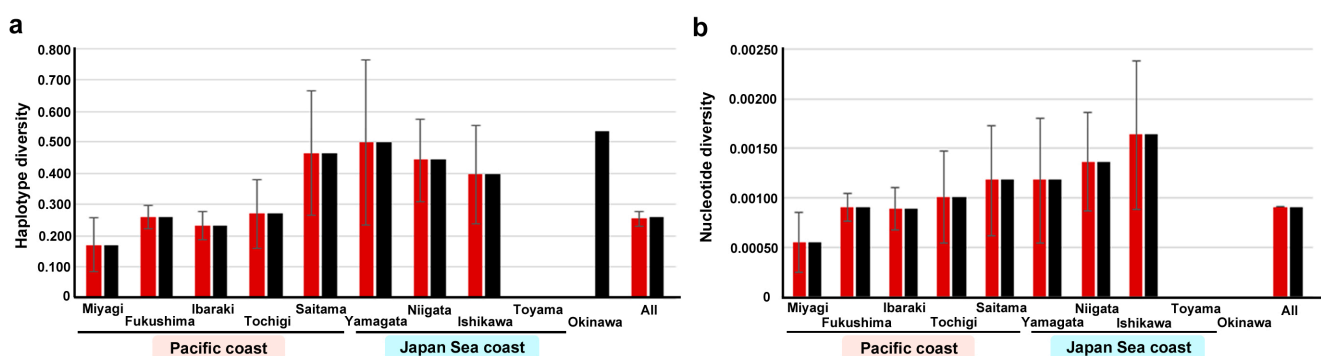


Figure 4. Haplotype and nucleotide diversity of the 2011–2014 samples. The reddish brown and black bars indicate haplotype diversity values without and with gaps, respectively. Error bars (only for data without gaps) indicate standard deviation. The Pacific coast prefectures and the Japan Sea coast prefectures are color-coded as shown in Figure 1. (a) Haplotype diversity (H_d) in ten prefectures. (b) Nucleotide diversity (π) in ten prefectures.

We also calculated nucleotide diversity values (π) (Figure 4b; Table A9). We observed that Toyama and Okinawa Prefectures presented the lowest values. Excluding these two extremes, again, there seemed to be a geographical bias. Prefectures on the Pacific coast, including Fukushima and Ibaraki Prefectures, tended to have lower values than those on the Japan Sea coast, with the highest value in Ishikawa Prefecture (0.00164), although Saitama Prefecture presented relatively high values.

The geographical bias observed in both haplotype and nucleotide diversity values might have been present even before the Fukushima nuclear accident. However, this tendency was not perfect because Saitama Prefecture presented relatively high values. An alternative possibility is that because the Pacific coast prefectures (Miyagi, Fukushima, Ibaraki, and Tochigi Prefectures), excluding Saitama Prefecture, are the most polluted prefectures, the low haplotype and nucleotide diversity values in these four prefectures may indicate the biological impacts of the Fukushima nuclear accident.

To examine genetic differences among populations, we additionally calculated pairwise F_{ST} and G_{ST} values between prefectures using sequences excluding gaps (Table A10). Most of these values were very small, and some were negative values, indicating that *ITS2* sequences were not diverse enough for these indices. If any, the largest one, 0.05364, was the G_{ST} value between Fukushima Prefecture (the Pacific coast group) and Yamagata Prefecture (the Japan Sea coast group), followed by 0.05013, the G_{ST} value between Ibaraki Prefecture (the Pacific coast group) and Yamagata Prefecture (the Japan Sea coast group). If these G_{ST} values are relevant, they suggest that Fukushima and Ibaraki populations may

be relatively differentiated from the Yamagata population, which may be consistent with the results of the haplotype diversity and nucleotide diversity (Figure 4, Table A9).

3.4. Spatiotemporal Dynamics of Haplotypes

Here, we examined spatiotemporal changes that might indicate the biological impacts of the Fukushima nuclear accident. We reasoned that the H1 percentage may be a reasonable indicator of genetic diversity. Accordingly, we obtained spatiotemporal H1 percentages in seven localities from Spring 2011 to Fall 2014 (Figure 5a). H1 dynamics varied among localities, but there seemed to be two peaks, one in Fall 2011 and the other in Fall 2012 and Spring 2013, in many localities. These trends were notable in Fukushima City and Motomiya City. Hirono Town lacked the second peak, but H1 percentage was high not only in Fall 2011 but also in Spring 2012, possibly reflecting the heavy pollution. In contrast, Takahagi City and Iwaki City behaved differently in Fall 2011, showing the lowest H1 percentage.

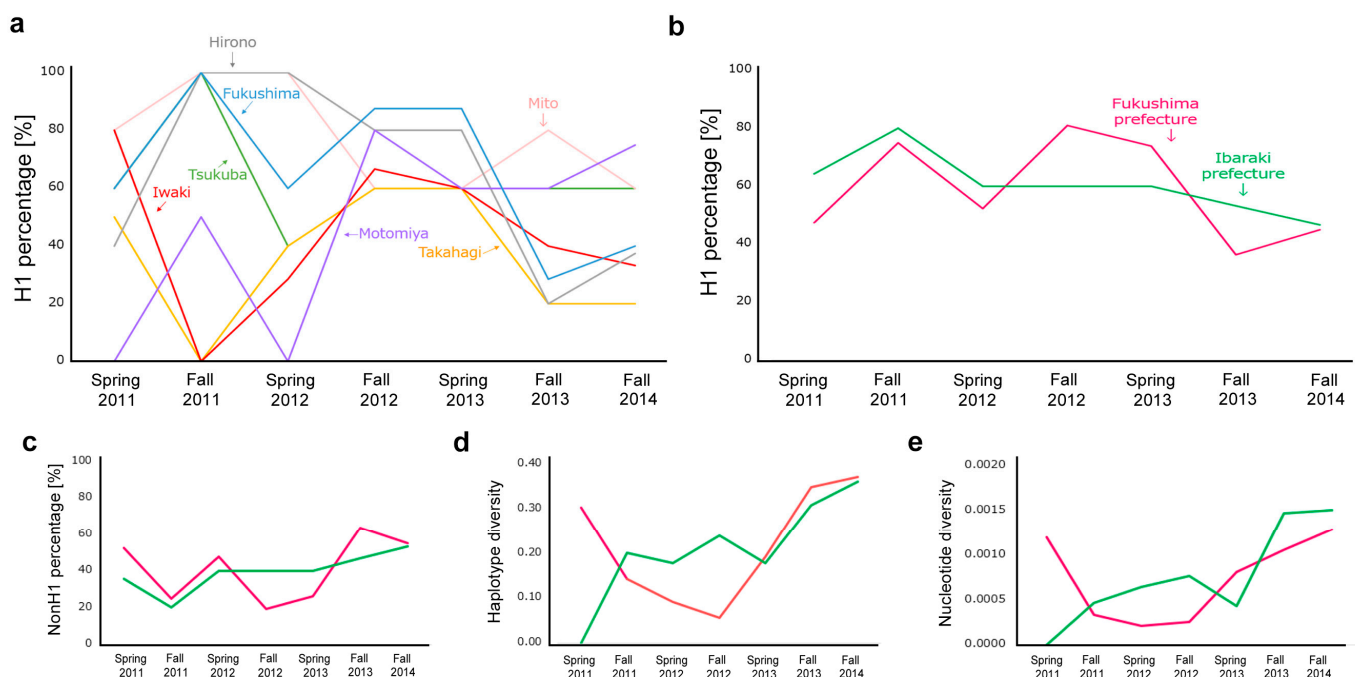


Figure 5. Spatiotemporal dynamics of genetic diversity values in Fukushima and Ibaraki Prefectures from Spring 2011 to Fall 2014. (a) H1 percentage at seven localities in Fukushima and Ibaraki Prefectures. (b) H1 percentage in Fukushima (red) and Ibaraki (green) Prefectures. (c) NonH1 percentage in Fukushima (red) and Ibaraki (green) Prefectures. (d) Haplotype diversity in Fukushima (red) and Ibaraki (green) Prefectures. (e) Nucleotide diversity in Fukushima (red) and Ibaraki (green) Prefectures.

To understand any prefectural trends, four localities in Fukushima Prefecture were combined, and three localities in Ibaraki Prefecture were also combined (Figure 5b). Both prefectures had a peak in Fall 2011, but Fukushima Prefecture had the highest peak in Fall 2012, which was not observed in Ibaraki Prefecture. The H1 percentage of Fukushima Prefecture seemed to oscillate. Both prefectures presented similar H1 percentages in Fall 2014, suggesting that the disturbance caused by the Fukushima nuclear accident, if any, had almost ceased by that time.

We also plotted nonH1 percentages for convenience (Figure 5c), indicating a relatively gradual increase in Ibaraki Prefecture and an overall oscillation in Fukushima Prefecture. This plot is compared with the plots of haplotype diversity (Figure 5d) and nucleotide diversity (Figure 5e). The nonH1 plot for Fukushima Prefecture shows a peak in Spring

2012 (Figure 5c), but haplotype and nucleotide diversity plots do not (Figure 5d,e). Except for this point, these three plots are reasonably similar to one another. The haplotype diversity values in Fukushima Prefecture decreased from Spring 2011 to Fall 2012 and then quickly increased until Fall 2014 (Figure 5d). In contrast, in Ibaraki Prefecture, haplotype diversity increased in a stepwise manner (Figure 5d). Interestingly, from Spring 2011 to Fall 2012, haplotype diversity dynamics were very different between Fukushima Prefecture and Ibaraki Prefecture. Nucleotide diversity values showed similar dynamics (Figure 5e).

3.5. H1 and NonH1 Comparisons

To further validate the temporal changes in H1 frequency, we compared the numbers of H1 and nonH1 haplotypes. In Fukushima Prefecture, one temporal group from Spring 2011 to Spring 2013 (the early period of our survey) was significantly different from another temporal group from Fall 2013 to Fall 2014 (the late period of our survey) ($\chi^2 = 7.54$, $df = 1$, $p = 0.0060$) (Figure 6a, left), indicating an overall temporal decrease in H1 individuals in Fukushima Prefecture. When Spring 2011 was eliminated from the early temporal group, the significance was more prominent ($\chi^2 = 15.0$, $df = 1$, $p = 0.00011$) (Figure 6a, right). Similar tendencies were observed in Ibaraki Prefecture; the early group was significantly different from the late group ($\chi^2 = 3.99$, $df = 1$, $p = 0.046$) (Figure 6b, left), and when Spring 2011 was omitted, the significance was more prominent ($\chi^2 = 8.16$, $df = 1$, $p = 0.0043$) (Figure 6b, right). Moreover, the H1 individuals appeared to account for the majority of the Fukushima and Ibaraki populations in the three-year period (Spring 2011–Fall 2013) in comparison with ten other prefectures in 2014, as shown in the significant statistical difference ($\chi^2 = 9.62$, $df = 1$, $p = 0.0019$) (Figure 6c). In other words, H1 individuals in the polluted areas (Fukushima and Ibaraki Prefectures) in this time period (Spring 2011–Fall 2013) constituted the majority, which was different from the haplotypes in nonpolluted and polluted areas in 2014.

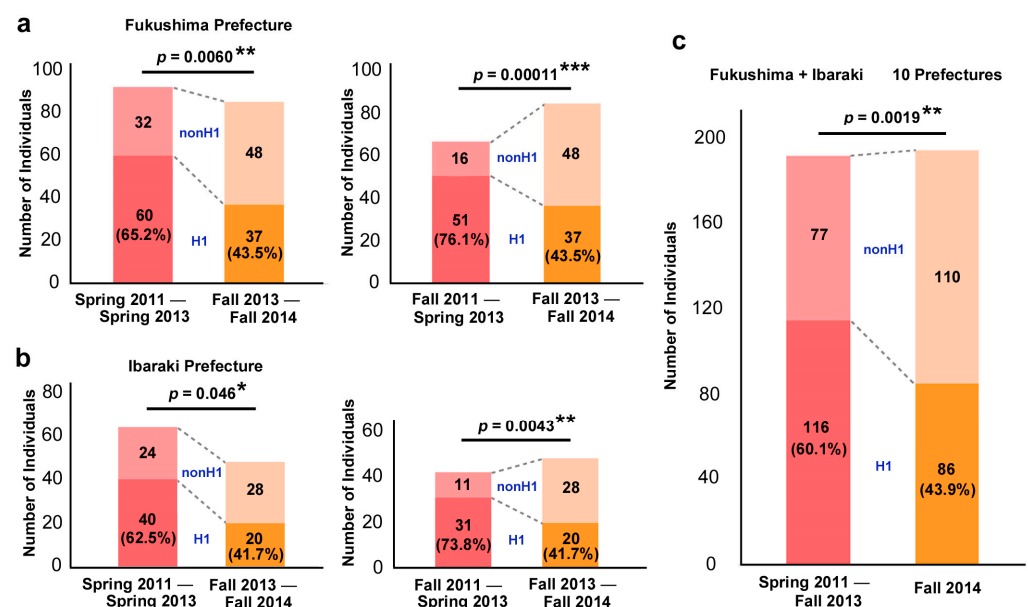


Figure 6. Comparisons of the number of individuals with the H1 haplotype and nonH1 haplotype. The bottom portion of a bar indicates H1, and the top portion of a bar indicates nonH1. The H1 percentage is shown at the bottom portion of a bar. Asterisks indicate statistical significance. *: $p < 0.05$, **: $p < 0.01$; ***: $p < 0.001$. (a) Fukushima Prefecture between the two time periods (the early and late periods of our survey). In the right panel, Spring 2011 samples were omitted. (b) Ibaraki Prefecture between the two time periods. In the right panel, Spring 2011 samples were omitted. (c) Fukushima and Ibaraki Prefectures in Spring 2011–Fall 2013 combined in comparison to ten prefectures in Fall 2014.

3.6. Discovery Rate of Novel Haplotypes

To understand when various haplotypes were detected over time, we examined the discovery rate of novel haplotypes in the seven localities in Fukushima and Ibaraki Prefectures in the entire survey period (Spring 2011–Fall 2014). (Figure 7). In these localities, there were 17 haplotypes discovered in total. The highest discovery rate was observed in Fall 2013. In this case, the number of novel haplotypes discovered in Fall 2013 (not discovered in the previous surveys) was three, and the number of individuals analyzed in Fall 2013 was 22, making the discovery rate of $3/22 = 0.136$. In other words, the possibility of discovering a novel haplotype from one sample in Fall 2013 was 13.6%. In contrast, the lowest discovery rate, 0.03, was observed in Spring 2012. This 4.5-fold increase in the discovery rate from Spring 2012 to Fall 2013 needs to be explained. The discovery rate was low until Fall 2012, and it increased from Spring 2013. This increase is consistent with that of other diversity values examined in Figure 5. It should also be noted that the discovery rate in Fall 2014 was still high, indicating that there are still numerous haplotypes yet to be discovered.

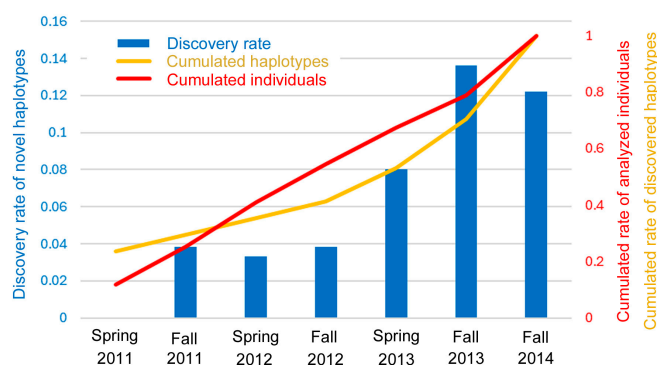


Figure 7. Discovery rate of novel haplotypes over time in the seven localities surveyed in Fukushima and Ibaraki Prefectures in 2011–2014. The discovery rate in Spring 2011 cannot be defined.

Theoretically, the discovery rate should be high at the beginning and then level off for saturation if novel haplotypes are randomly distributed. To further examine this point, we also obtained the cumulated rate of analyzed individuals and the cumulated rate of discovered haplotypes (Figure 7). These two lines do not overlap well, indicating the discovery of novel haplotype was not random but biased over time. Again, the slope of the cumulated rate of discovered haplotypes is relatively low until Fall 2012 but relatively high in Spring 2013 and afterwards, suggesting a biased distribution of various haplotypes over time.

4. Discussion

4.1. Major and Minor Haplotypes

In this study, we compared *ITS2* sequences from field samples of the pale grass blue butterfly collected from Fukushima Prefecture, Ibaraki Prefecture, and other prefectures in Japan in 2011–2014 after the Fukushima nuclear accident. Methodologically, we excluded the samples with extensive indels (the fifth category; see Materials and Methods), and we focused on just a single DNA region to be sequenced, *ITS2*. These are limitations of the present study. Nonetheless, our data demonstrated that various *ITS2* haplotypes were present in this butterfly species, although their diversity was limited; 29 haplotypes were detected (Table 1). Among these various haplotypes, H1 was by far the majority (85.7%), and many haplotypes were detected only once. We thus considered H1 to be the reference

sequence (Figure 2). H1 was widespread throughout Japan, suggesting gene flow among local populations.

Many haplotypes contained a single nucleotide substitution in reference to H1. One could argue that some of these unique substitutions were produced by DNA damage caused by initial radiation exposure immediately after the Fukushima nuclear accident. However, as seen in the haplotype network diagram (Figure 3a), some of these haplotypes with unique substitutions were also found in other prefectures with minimal radiation pollution, suggesting that most of these substitutions might have been present irrespective of the Fukushima nuclear accident. Unfortunately, we do not have a sufficient number of butterfly samples caught before the Fukushima nuclear accident in our laboratory. This is another limitation of this study. However, nine specimens of this species caught in 1979–2009 (before the accident) by respected amateur lepidopterists from various sites in Fukushima Prefecture have been donated to and stored in our laboratory [66]. They are morphologically normal [66].

As the haplotype network diagram indicates, no clear genetic differentiation of subpopulations was notable. The entire population is constructed around H1. The haplotype most distant from H1 was H26 (Kashiwazaki City, Niigata Prefecture) (Figure 3a), but it was obtained from the locality of minimal radiation pollution from the FDNPP, although Kashiwazaki City has the Kashiwazaki-Kariwa Nuclear Power Plant. Other haplotypes, such as H22, H15, H21, and H14, were unique to the Fukushima and Ibaraki Prefectures, and they may characterize the polluted area. Interestingly, the haplotype most distant from that of *Zizina* based on the phylogenetic tree was H22 (Figure 3b), which emerged most recently. H22 was obtained from Takahagi City, Ibaraki Prefecture, which is not very close but reasonably close to the FDNPP (approximately 83 km away from the FDNPP to the south). However, we do not know how recent the birth of H22 was. It is speculated that a mitochondrial heteroplasmic mutation occurred after the Fukushima nuclear accident [83]. Therefore, if any, H22 might have emerged as a consequence of genetic mutations through the initial exposure caused by the Fukushima nuclear accident.

4.2. Genetic Diversity in Space and Time

In search of possible effects of the Fukushima nuclear accident, haplotype and nucleotide diversity values were compared among prefectures, paying attention to the Pacific coast and the Japan Sea coast prefectures (Figure 4). Four Pacific coast prefectures close to the FDNPP (Miyagi, Fukushima, Ibaraki, and Tochigi Prefectures) presented relatively low haplotype diversity and nucleotide diversity values, which may be due to the Fukushima nuclear accident. Although this “geographical bias” might have been present even in the period before the Fukushima nuclear accident, Saitama Prefecture had high diversity values despite its categorization into the Pacific coast prefectures. This could be understood based on the fact that Saitama Prefecture was the least polluted and the farthest from the FDNPP among the five Pacific coast prefectures. Therefore, it is possible that the relatively low haplotype and nucleotide diversity values in the four Pacific coast prefectures reflect the biological impacts of the Fukushima nuclear accident. We additionally obtained pairwise F_{ST} and G_{ST} values. Although results were not well interpretable, if any, the Fukushima and Ibaraki populations may be relatively “differentiated” from the Yamagata population, being consistent with the geographical bias shown in the haplotype and nucleotide diversity values.

We then turned our attention to haplotype dynamics over time, using the H1 percentage as an indicator of genetic diversity. The maximal H1 percentage was observed in Fall 2011 in five localities among the seven collection localities in Fukushima and Ibaraki Prefectures (Figure 5a). Among them, Hirono Town is the most polluted and scored the highest

H1 percentage in Fall 2011 and Spring 2012. Fukushima City and Motomiya City behaved similarly. In contrast, Takahagi City, together with Iwaki City, showed the least H1 percentage in Fall 2011. This result of Takahagi City in contrast to Hirono Town and Fukushima City may indicate high allelic variation in Takahagi City, which is also consistent with Figure 3, in which Takahagi samples occupied unique positions. This high allelic variation may be attributable to a unique distance of Takahagi City from the FDNPP. Takahagi City is reasonably close but not too close to the FDNPP. Due to this fact, Takahagi populations might not have been entirely eliminated by the initial exposure, accumulating relatively small level (nonlethal level) of genetic mutations. This could increase genetic diversity. If this is the case, H22 in Figure 3 from Takahagi City may be produced by genetic mutations caused by the Fukushima nuclear accident. In contrast, most individuals in Hirono Town might have been eliminated in response to the initial exposure, as suggested by the high H1 percentage, because Hirono Town may be too close to the FDNPP (approximately 23 km away from the FDNPP to the south). This is consistent with the lowest capture rate in Hirono Town in Fall 2011 reported in a previous study [69].

4.3. Comparison with *Zizina emelina*

Our genetic diversity data of ITS2 from *Zizeeria maha* can be compared with similar data from Japanese butterflies, although there should be limitations in comparisons due to the use of different genetic markers. An endangered species, *Zizina emelina*, inhabiting in Japan, is phylogenetically closely related to the pale grass blue butterfly used in the present study. In the case of *Zizeeria emelina*, the haplotype network diagram has multiple circles of similar size and is not dominated by a single haplotype [102]. Haplotype and nucleotide diversity values from nuclear sequences in *Zizina emelina* are generally similar to those observed in the Japan Sea coast group in the present study, but our haplotype diversity values for the Pacific coast group (excluding Saitama Prefecture) are less than those of most populations of this endangered species [102], suggesting that the Pacific coast group has low genetic diversity levels that are comparable to the endangered species. In contrast, F_{ST} values are much higher in *Zizina emelina* than in *Zizeeria maha*, as expected from their different distribution patterns in Japan.

4.4. Natural Selection and Genetic Drift

In the period of 2011–2014, the H1 percentage in Fukushima Prefecture appeared to oscillate (Figure 5b). In Ibaraki Prefecture, the H1 percentage gradually declined to 2014 after the peak in Fall 2011 (Figure 5b). Similarly, in Fukushima Prefecture, haplotype and nucleotide diversity values decreased until Fall 2012 and then increased (Figure 5d,e). However, there was no oscillation. On the other hand, in Ibaraki Prefecture, it did not decrease much in Fall 2012 and then increased.

Similarly, comparisons of H1 ratios between the early and late periods of sampling revealed that H1 was relatively abundant in the early period but was less abundant in the late period (Figure 6). This was observed in both Fukushima and Ibaraki Prefectures, confirming the results shown in Figure 5. Importantly, the H1 ratio in samples combined from ten prefectures collected in 2014 was 43.9% (Figure 6c), which is similar to those of Fukushima and Ibaraki Prefectures in the late period (43.5% and 41.7%, respectively) (Figure 6a,b). These results can be interpreted as an equilibrium shift in Fukushima and Ibaraki Prefectures from the disrupted state to the steady state via gene flow. Therefore, we speculate that the H1 percentage before the Fukushima nuclear accident was likely also approximately 40% as the steady state, which then increased to 65% or 76% in Fukushima Prefecture and to 63% or 74% in Ibaraki Prefecture in response to the Fukushima nuclear accident. If this is true, the Fukushima nuclear accident severely affected this butterfly

species by eliminating nonH1 haplotypes. Then, partial adaptation of existing minor haplotypes and immigrating haplotypes likely occurred in Fukushima. This cycle appeared to be repeated a few times before the stabilization of the H1 percentage in Fukushima, as observed in oscillating dynamics.

To explain these spatiotemporal dynamics, it may be assumed that some H1 individuals are associated with the fittest genotypes to Japanese environments simply because H1 is by far the majority. In other words, it is probabilistically likely that some individuals with the H1 haplotype may be robust. Most nonH1 haplotypes may be associated with less fit genotypes, simply because they are minority, and may be less robust. Neither H1 nor nonH1 has a physiological function by any means, but based on the fact that H1 occupied 85.7% of all alleles, the probability that H1 is contained in robust genotypes is high.

To understand the possible processes of biodiversity dynamics in Fukushima, we consider four scenarios (Figure 8). In the first scenario mainly based on natural selection (Figure 8a), upon the explosion of the FDNPP, less robust nonH1 haplotypes are eliminated by the pollution. The population may be bottlenecked (in which case, the assumption of H1 association with robustness may not be necessary). This way, diversity values decrease. The different dynamics between Fukushima and Ibaraki Prefectures may be explained by the severity of pollution and its biological consequences. In Fukushima Prefecture (including Hirono Town), many haplotypes that are sensitive to radioactive pollution might have been eliminated. In Ibaraki Prefecture (including Takahagi City), however, the haplotypes that are similarly sensitive to radioactive pollution might have been partially and gradually eliminated. Moreover, Ibaraki Prefecture might have allowed the immigration and survival of other haplotypes relatively quickly due to less severe pollution in comparison to Fukushima Prefecture. In Fukushima Prefecture, because of severe pollution, such immigrants might not have been allowed to survive well so quickly.

In this selection scenario, an H1 increase indicates the process of elimination of the nonH1 haplotypes, and an H1 decrease indicates a process of immigration. However, considering that the haplotype and nucleotide diversity plots of Fukushima Prefecture showed no peak in Spring 2012 (Figure 5d,e) in contrast to the nonH1 plot (Figure 5c), such immigration may be limited. The nonH1 peak in Spring 2012 may be considered an increase in existing minor nonH1 haplotypes (potentially including novel mutants) that were allowed to survive, indicating a process of partial adaptation of Fukushima butterflies to the polluted environments. This is also consistent with the idea that immigration is limited during the Fall–Spring period due to a limited number of individuals and no adult migration in the winter season. If so, this adaptation takes just two generations, suggesting either strong selection pressure or an epigenetic mechanism for adaptation. Additionally, emerging mutants might have contributed to an increase in genetic diversity (see below).

A slightly different scenario emphasizes genetic drift (Figure 8b). In this drift scenario, both H1 and nonH1 may be equally eliminated because of equal sensitivity to radioactive pollution. A prompt immigration of H1 as a founder occurs, and this founder population contributes to an H1 increase. However, the H1 percentage must be decreased later by additional immigrants including nonH1 until the stabilization of the H1 percentage as an equilibrium of H1 and nonH1. This drift scenario must also assume the relatively severe elimination of the previous population to be effective. The re-colonizing individuals are also under the pressure of natural selection, and they must adapt to the polluted environment. Considering that the immigration process is probably not so fast (see below), the drift scenario is less preferred to the selection scenario. Genetic drift may be more influential in the case of the expansion of the range-margin populations as described in this species [103].

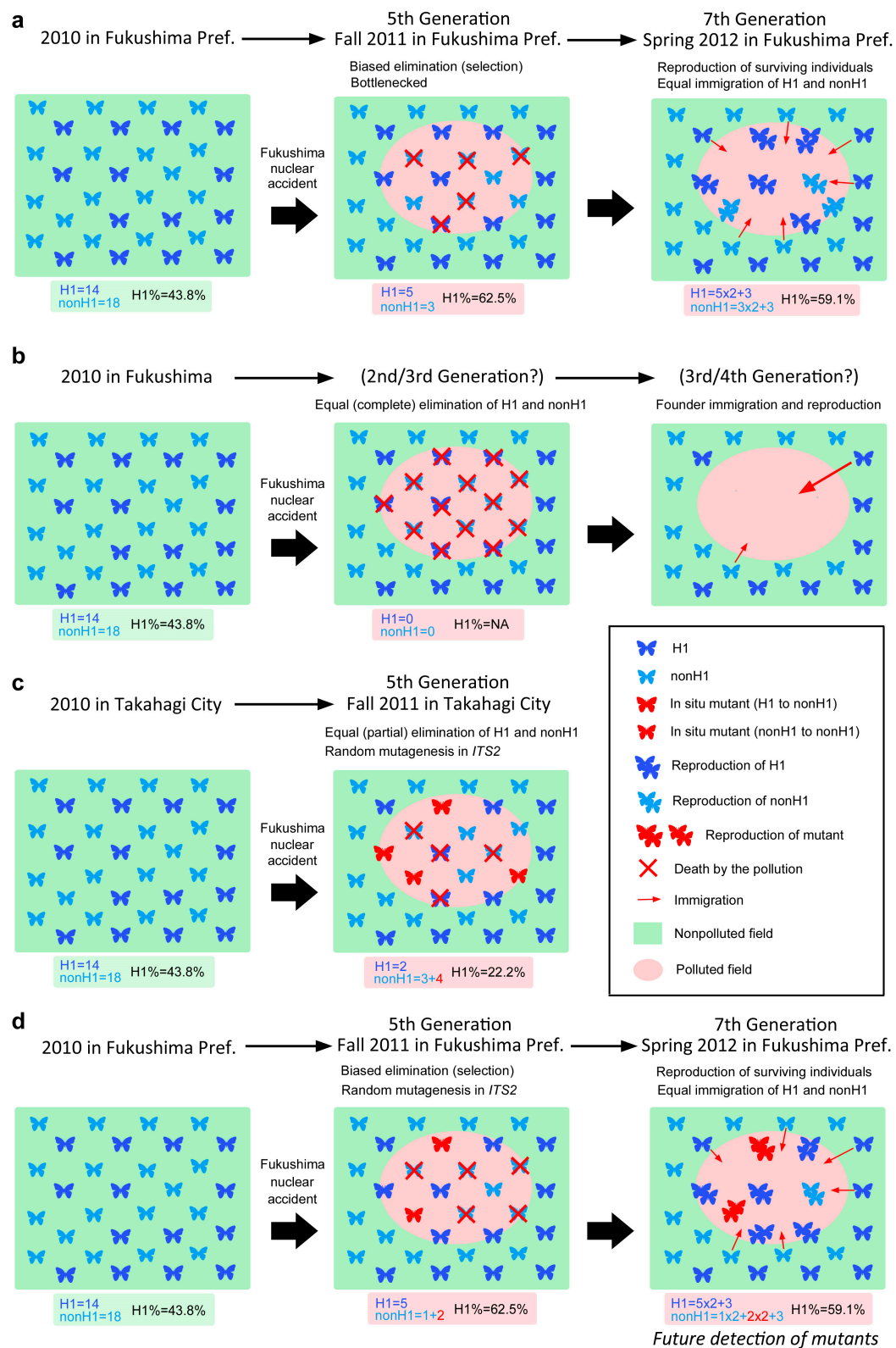


Figure 8. Possible dynamics of the H1 percentage in Fukushima Prefecture. (a) Selection scenario. In this scenario, the pollution eliminated more nonH1 than H1 by the fifth generation in Fukushima Prefecture, increasing the H1 percentage. Equal immigration into the polluted area by the seventh generation increases the H1 percentage. Alternatively, quick adaptation of nonH1 to the polluted environment decreased the H1 percentage. (b) Drift scenario. In this scenario, almost all individuals in the polluted area are eliminated, and the H1 founder immigration followed by the founder reproduction results in the high H1 percentage, as in the middle panel in (a). Immigration is biased to

H1 because H1 is the majority. Further immigration including nonH1 decreases the H1 percentage, as in the last panel in (a). (c) Mutagenesis scenario. Extensive mutagenesis decreases the H1 percentage, because H1 changes to nonH1, which is inconsistent with the increase in the H1 percentage in Fall 2011 in Fukushima Prefecture but is consistent with the decrease in the H1 percentage in Fall 2011 in Takahagi City. (d) Selection and mutagenesis scenario. NonH1 individuals are eliminated much more than H1 individuals are. Random mutagenesis is allowed to occur in a limited number of H1 and nonH1 individuals. Mutants will be discovered later when they adapt to the environment and reproduce themselves.

4.5. Genetic Mutations and Immigration

Although the selection scenario involving immigration above (Figure 8a) seems to be largely able to explain the H1 dynamics, it may not be sufficient to explain the discovery rate over time (Figure 7). The discovery rate appeared to be biased; it was relatively low in the early period of this study (until Fall 2012) and became high in the late period (Spring 2013 and afterwards). The low discovery rate in the early period may be explained by a bottlenecked state after severe selection. The high discovery rate in the late period may be explained by the immigration of novel haplotypes and/or by the successful reproduction of very minor haplotypes and/or novel mutants produced in situ. In the mutagenesis scenario, random mutations cause a decrease in the H1 percentage, because the H1 individuals change into nonH1 (Figure 8c). The emerging mutants may not be well detected in the early period, and most mutant individuals in the early period might have experienced death. This cannot explain an increase in the H1 percentage in Fall 2011 in Fukushima Prefecture, but it can explain a decrease in the H1 percentage in Fall 2011 in Takahagi City.

In the fourth scenario, selection and mutagenesis are combined. Small number of mutants can be allowed to emerge, together with selection, as long as an increase in the H1 percentage is ensured, as in Fukushima Prefecture in Fall 2011 (Figure 8d). This fourth scenario indeed incorporates selection, mutagenesis, immigration, and adaptation. Considering the potential contribution of the emerging in situ mutants to an increase in genetic diversity in the late period of our survey, the H22 haplotype from Takahagi City caught in Fall 2014 (Figure 3) may be a candidate for an in situ mutant produced by the Fukushima nuclear accident.

Based on Figure 7, the discovery rate is still high in Fall 2014. It indicates that there are more novel haplotypes to be discovered. Indeed, In the seven localities in Fukushima and Ibaraki Prefectures, only four haplotypes (H1, H2, H3, and H12) were detected twice or more among 17 haplotypes discovered. Thirteen other haplotypes were detected only once, indicating that saturation has not been reached yet. Thus, relatively many private haplotypes in Fukushima and Ibaraki Prefectures may be consistent with the mutagenesis scenario.

In Fukushima research, similar genetic studies are rare, but in the case of wild boars, new microsatellite sequences emerged after the accident [88]. Genetic mutations in situ may be responsible for this phenomenon, and it may also be explained by natural selection among various existing individuals and immigration from surrounding environments. In any case, because of technical limitations of this type of study, the use of genome-wide sequencing methods may be preferred in the future.

4.6. Abnormality Rate and Haplotype Dynamics

The abnormality rate (AR) during the period of 2011–2013 shown in a previous study [69] peaked in Fall 2011 and then decreased until Fall 2012. Thus, in Fall 2011, the butterfly population in Fukushima Prefecture experienced high ARs, high H1 percentages, and relatively low haplotype and nucleotide diversity values, indicating an ongoing selection process. In Fall 2012, the butterfly population in Fukushima Prefec-

ture experienced low ARs, high H1 percentages (after a decrease in Spring 2012), and very low haplotype and nucleotide diversity values, indicating that the selection process was nearly complete. The important results of the phenotypic studies summarized as AR dynamics [69] are now understood consistently as a selection process, together with other processes including mutagenesis, immigration, and adaptation, based on the present molecular results. Consequently, we witnessed the entire process of real-time evolution of the Fukushima butterfly population in the field initiated by the defined event (i.e., the Fukushima nuclear accident in March 2011) at both genetic and phenotypic levels.

4.7. Possible Mechanisms of Haplotype Dynamics

Direct DNA damage by ionizing radiation might have caused a decrease in nonH1 individuals that were less robust against radiation contamination because of the less efficient DNA repair system in nonH1 individuals. However, such DNA damage is likely possible only immediately after the accident through initial exposure to short-lived radionuclides; chronic radiation exposure to long-lived radionuclides such as ^{137}Cs does not seem to damage DNA [104]. This early lethal or sublethal event may explain the rapid increase in AR from Spring 2011 to Fall 2011 through the accumulation of genetic mutations [75,76,82]. The selection process after mutagenesis may also explain the decrease in haplotype diversity and nucleotide diversity to the end of 2012 in Fukushima Prefecture. It is to be noted that the DNA damage from the accident did not kill butterflies instantly. Rather, five or six generations after the accident seem to be required to reach a selection peak, i.e., in Fall 2011 in Fukushima.

However, in addition to initial exposure to short-lived radionuclides, chronic exposure to ^{137}Cs may cause physiological damage and contribute to the selection process. In other words, haplotype dynamics may be partly explained by indirect field effects [47,71–74]. Radiation stress to the host plant through stress to soil microbes initiates the activation of the plant defense system against herbivorous insects [47]. This process might have eradicated many minor haplotypes of this butterfly species.

The present study indicated a possible contribution of adaptation and migration (i.e., gene flow) to the normalization of the H1 percentage to the steady state in polluted areas. The active dispersibility of this species is low [105], but butterflies may be able to move gradually over a few years. Moreover, the significant number of individuals may be brown due to natural wind. This passive dispersibility may be important for this small butterfly species to expand its range margin [105]. Without the Fukushima nuclear accident, the immigrating and emigrating individuals would be well balanced. As the surviving butterflies existing in Fukushima and Ibaraki Prefectures must adapt to the newly polluted environment, butterflies immigrating into Fukushima and Ibaraki Prefectures must also adapt to the polluted environment. Adaptation of this species to new environments has been documented [106].

5. Conclusions

Overall, the present results suggest that many individuals of this butterfly species with nonH1 haplotypes may be relatively radiation-prone and were eliminated through natural selection, which resulted in a decrease in genetic diversity in 2011–2012 in Fukushima and Ibaraki Prefectures and other Pacific coast prefectures. Considering the spatial and temporal coincidence, the present results likely indicate biological responses to the Fukushima nuclear accident at the population level. The selection process was likely initiated by direct DNA damage through initial exposure to short-lived radionuclides, leading to genetic mutations [65,66,75,76], as well as by physiological damage through indirect field effects mediated by chronic host plant ingestion in polluted areas [71–74]. Later, the successful

reproduction and adaptation of surviving individuals might have occurred, and then the immigration of nonH1 individuals from surrounding areas to Fukushima and Ibaraki Prefectures might have restored the normal H1 percentage. Additionally, emerging mutants might have contributed to the recovery of genetic diversity. H22 from Takahagi City was the latest haplotype phylogenetically, and it might have been produced by genetic mutations caused by initial exposure, although evidence of this is not solid.

Notably, the present molecular study supports the AR dynamics of previous morphological (phenotypic) studies. Together, our series of studies recorded the real-time evolution of the Fukushima *Zizeeria maha* population for adaptation to polluted areas in the field at both molecular and phenotypic levels. Considering that the selection process may be mediated both through acute genetic damage immediately after the accident and chronic physiological damage via indirect field effects, other lepidopteran species, other herbivorous insect species, and other animal species might have been similarly affected by the Fukushima nuclear accident. In that case, the overall impact of the Fukushima nuclear accident on the ecosystem would be much greater than the impact conventionally estimated on the basis solely of dosimetric data.

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Abbreviations

The following abbreviations are used in this manuscript:

ITS2	<i>internal transcribed spacer 2</i>
COI	<i>cytochrome oxidase subunit I</i>
FDNPP	Fukushima Dai-ichi Nuclear Power Plant
ICRP	International Commission on Radiological Protection
IAEA	International Atomic Energy Agency
UNSCEAR	United Nations Scientific Committee on the Effects of Atomic Radiation
RefSeq	Reference sequence
PCR	Polymerase chain reaction
ML	Maximum likelihood
AR	Abnormality rate

Appendix A

In this study, we used the following numbers of adult butterfly individuals from the surveys of 2011–2013 (Table A1) and from the surveys of 2014 (Table A2) for successful PCR amplification of *ITS2*. As a result, we obtained and examined the following numbers of alleles in the surveys of 2011–2013 (Table A3) and in the surveys of 2014 (Table A4).

Table A1. Number of individuals used for successful PCR amplification (2011–2013 field samples) in seven localities (City/Town) in Fukushima and Ibaraki Prefectures.

Category	Tsukuba	Mito	Takahagi	Iwaki	Hirono	Motomiya	Fukushima	Total
(1)	14	20	14	13	16	11	28	116
(2)	0	0	0	0	0	0	0	0
(3)	3	2	7	7	2	3	3	27
(4)	0	0	0	0	0	1	0	1
(5)	6	4	9	6	7	7	10	49
Total	23	26	30	26	25	22	41	193

Table A2. Number of individuals used for successful PCR amplification (2014 field samples) in ten prefectures.

Category	Miyagi	Fukushima	Yamagata	Niigata	Toyama	Ishikawa	Tochigi	Ibaraki	Saitama	Okinawa	Total
(1)	14	29	1	7	4	5	10	12	2	2	86
(2)	0	1	0	1	0	0	0	0	0	0	2
(3)	3	18	1	1	0	2	4	9	3	1	42
(4)	0	0	0	1	0	0	0	0	0	0	1
(5)	5	15	3	17	1	2	6	12	0	4	65
Total	22	63	5	27	5	9	20	33	5	7	196

Table A3. Number of alleles obtained (2011–2013 field samples) in seven localities (City/Town).

Category	Tsukuba	Mito	Takahagi	Iwaki	Hirono	Motomiya	Fukushima	Total
(1)	31	42	35	33	34	25	59	259
(2)(3)(4)	3	2	7	7	2	5	3	29
Total	34	44	42	40	36	30	62	288

Table A4. Number of alleles obtained (2014 field samples) in ten prefectures.

Category	Miyagi	Fukushima	Yamagata	Niigata	Toyama	Ishikawa	Tochigi	Ibaraki	Saitama	Okinawa	Total
(1)	31	76	3	15	8	11	24	32	6	5	211
(2)(3)(4)	3	20	1	5	0	3	4	8	2	1	47
Total	34	96	4	20	8	14	28	40	8	6	258

Appendix B

The sources of the haplotypes detected in this study are shown in the following tables (Tables A5–A7).

Table A5. Sources of individuals with H1, H2, H3, H5, or H6.

Locality or Prefecture	Spring 2011	Fall 2011	Spring 2012	Fall 2012	Spring 2013	Fall 2013	Fall 2014
Tsukuba City	3, 0, 0, 0, 0	3, 0, 0, 0, 0	3, 0, 0, 0, 0	0, 0, 0, 0, 0	4, 1, 0, 0, 0	4, 0, 0, 0, 0	3, 0, 0, 0, 0
Mito City	4, 0, 0, 0, 0	1, 0, 0, 0, 0	5, 0, 0, 0, 0	4, 0, 0, 0, 0	4, 0, 0, 0, 0	4, 0, 0, 0, 0	5, 0, 1, 0, 0
Takahagi City	2, 0, 0, 0, 0	1, 0, 1, 0, 0	3, 0, 0, 0, 0	8, 1, 0, 0, 0	3, 0, 0, 0, 0	4, 0, 0, 1, 0	3, 0, 0, 0, 0
Iwaki City	4, 0, 0, 0, 0	1, 0, 0, 0, 0	3, 0, 1, 0, 0	3, 0, 0, 0, 0	4, 0, 0, 0, 0	5, 0, 0, 2, 0	2, 0, 0, 0, 0
Hirono Town	3, 1, 0, 0, 0	2, 0, 0, 0, 0	3, 0, 0, 0, 0	4, 0, 0, 0, 0	4, 0, 0, 0, 0	2, 1, 0, 0, 0	3, 0, 0, 0, 0
Motomiya City	2, 0, 1, 0, 0	1, 0, 0, 0, 0	0, 0, 0, 0, 0	4, 0, 0, 0, 0	4, 0, 0, 0, 0	3, 0, 0, 0, 0	4, 0, 0, 0, 0
Fukushima City	5, 0, 0, 0, 0	3, 0, 0, 0, 0	6, 0, 0, 0, 0	7, 0, 0, 0, 0	7, 0, 0, 0, 0	3, 1, 0, 0, 0	5, 0, 1, 0, 0
Fukushima Pref.* ¹	—	—	—	—	—	—	47, 0, 2, 0, 1
Ibaraki Pref.* ²	—	—	—	—	—	—	20, 0, 0, 0, 2
Other prefectures* ³	—	—	—	—	—	—	58, 1, 1, 0, 0

The five numbers in a cell indicate the numbers of H1, H2, H3, H5, and H6 individuals in this order. Asterisks (*) indicate that Fukushima Pref. and Ibaraki Pref. do not include the localities surveyed from 2011–2013. *1 for H1: Shinchu Town, Sukagawa City, Koriyama City, Shirakawa City, Soma City, Okuma Town, Nihonmatsu City, Minamisoma City, Iitate Village, and Tomioka Town. *2 for H1: Hitachi City, Kasama City, Kitaibaraki City, and Tokai Village. *3 for H1: Kazo City (Saitama Pref.), Sakura City (Tochigi Pref.), Sano City (Tochigi Pref.), Utsunomiya City (Tochigi Pref.), Nasu Town (Tochigi Pref.), Yamamoto Town (Miyagi Pref.), Shiraishi City (Miyagi Pref.), Iwanuma City (Miyagi Pref.), Murata Town (Miyagi Pref.), Sendai City (Miyagi Pref.), Tsuruoka City (Yamagata Pref.), Sanjo City (Niigata Pref.), Kashiwazaki City (Niigata Pref.), Niigata City (Niigata Pref.), Murakami City (Niigata Pref.), Itoigawa City (Niigata Pref.), Komatsu City (Ishikawa Pref.), Kanazawa City (Ishikawa Pref.), Takaoka City (Toyama Pref.), Okinawa-jima Island (Okinawa Pref.), and Iriomote-jima Island (Okinawa Pref.). *3 for H2: Tsuruoka City (Yamagata Pref.). *1 for H3: Koriyama City and Nihonmatsu City. *3 for H3: Sano City (Tochigi Pref.). *1 for H5: Shirakawa City. *3 for H5: Kazo City (Saitama Pref.), Joetsu City (Niigata Pref.), Kanazawa City (Ishikawa Pref.), and Sendai City (Miyagi Pref.). *1 for H6: Shinchu Town. *2 for H6: Kitaibaraki City (two individuals).

Table A6. Sources of individuals with H8, H9, H10, H12, or H13.

Locality or Prefecture	Spring 2011	Fall 2011	Spring 2012	Fall 2012	Spring 2013	Fall 2013	Fall 2014
Tsukuba City	0, 0, 0, 0, 0	0, 0, 0, 0, 0	0, 0, 0, 1, 0	0, 0, 0, 0, 0	0, 0, 0, 0, 0	0, 0, 0, 1, 0	0, 0, 0, 0, 0
Mito City	0, 0, 0, 0, 0	0, 0, 0, 0, 0	0, 0, 0, 0, 0	0, 0, 0, 0, 0	0, 0, 0, 0, 0	0, 0, 0, 0, 0	0, 0, 0, 0, 0
Takahagi City	0, 0, 0, 0, 0	0, 0, 0, 0, 0	0, 0, 0, 0, 0	0, 0, 0, 1, 0	0, 0, 0, 0, 0	0, 0, 0, 1, 0	0, 0, 0, 1, 0
Iwaki City	0, 0, 0, 0, 0	0, 0, 0, 0, 0	0, 0, 0, 0, 0	0, 0, 0, 1, 0	0, 0, 0, 1, 0	0, 0, 0, 1, 0	1, 0, 0, 0, 0
Hirono Town	0, 0, 0, 0, 0	0, 0, 0, 0, 0	0, 0, 0, 0, 0	0, 0, 0, 0, 0	0, 0, 0, 0, 0	0, 0, 0, 0, 0	0, 0, 0, 0, 0
Motomiya City	0, 0, 0, 1, 0	0, 0, 0, 0, 0	0, 0, 0, 0, 0	0, 0, 0, 0, 0	0, 0, 0, 1, 1	0, 0, 0, 0, 0	1, 0, 0, 0, 0
Fukushima City	0, 0, 0, 2, 0	0, 0, 0, 0, 0	0, 0, 0, 0, 0	0, 0, 0, 0, 0	0, 0, 0, 0, 0	0, 0, 0, 0, 0	0, 0, 1, 0, 0
Fukushima Pref.* ¹	—	—	—	—	—	—	0, 1, 0, 5, 1
Ibaraki Pref.* ²	—	—	—	—	—	—	0, 1, 0, 1, 0
Other prefectures* ³	—	—	—	—	—	—	0, 0, 2, 2, 1

The five numbers in a cell indicate the numbers of H8, H9, H10, H12, and H13 individuals in this order. Asterisks (*) indicate that Fukushima Pref. and Ibaraki Pref. do not include the localities surveyed from 2011–2013. *1 for H9: Okuma Town. *2 for H9: Kasama City. *3 for H10: Utsunomiya City (Tochigi Pref.) and Sendai City (Miyagi Pref.). *1 for H12: Koriyama City, Soma City, Okuma Town, and Iitate Village (two individuals). *2 for H12: Tokai Village. *3 for H12: Kanazawa City (Ishikawa Pref.) and Sakura City (Tochigi Pref.). *1 for H13: Minamisoma City (Fukushima Pref.). *3 for H13: Kashiwazaki City (Niigata Pref.).

Table A7. Sources of individuals with a haplotype detected from a single individual.

Locality or Prefecture	Spring 2011	Fall 2011	Spring 2012	Fall 2012	Spring 2013	Fall 2013	Fall 2014
Tsukuba City	—	—	—	—	—	—	—
Mito City	—	—	—	H18	H19	—	H20
Takahagi City	—	—	H17	—	—	H21	H22
Iwaki City	—	H4	—	—	—	—	—
Hirono Town	—	—	—	—	—	—	—
Motomiya City	—	—	—	—	H14	—	—
Fukushima City	—	—	—	—	—	—	H15
Fukushima Pref.* ¹	—	—	—	—	—	—	H7, H11, H16
Ibaraki Pref.* ²	—	—	—	—	—	—	H20
Other prefectures* ³	—	—	—	—	—	—	H23–H29

Asterisks (*) indicate that Fukushima Pref. and Ibaraki Pref. do not include the localities surveyed from 2011–2013. *1 for H7: Shinchu Town. *1 for H11: Tomioka Town. *1 for H16: Iitate Village. *2 for H20: Mito City. *3 for H23: Kazo City (Saitama Pref.). *3 for H24: Nasu Town (Tochigi Pref.). *3 for H25: Yamamoto Town (Miyagi Pref.). *3 for H26: Kashiwazaki City (Niigata Pref.). *3 for H27: Kashiwazaki City (Niigata Pref.). *3 for H28: Kanazawa City (Ishikawa Pref.). *3 for H29: Okinawa-jima Island.

Appendix C

Shared and private haplotypes for each locality and prefecture are shown in Table A8.

Table A8. Shared and private haplotypes in localities and prefectures.

Locality or Prefecture		H	H_{Shared}	H_{Private}	$H_{\text{Private}}\%$
Tsukuba City	H1, H2, H12	3	3	0	0
Mito City	H1, H3, H18, H19, H20	5	2	3	60
Takahagi City	H1, H2, H3, H5, H12, H17, H21, H22	8	4	3	38
Iwaki City	H1, H3, H4, H5, H8, H12	6	5	1	17
Hirono Town	H1, H2	2	2	0	0
Motomiya City	H1, H8, H12, H13, H14	5	3	2	40
Fukushima City	H1, H2, H3, H10, H12, H15	6	4	1	17
Miyagi Pref.	H1, H5, H10, H25	4	3	1	25
Fukushima Pref.	H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15, H16	16	9	7	44
Ibaraki Pref.	H1, H2, H3, H5, H6, H9, H12, H17, H18, H19, H20, H21, H22	13	7	6	46
Tochigi Pref.	H1, H3, H10, H12, H24	5	4	1	20
Saitama Pref.	H1, H5, H23	3	2	1	33
Yamagata Pref.	H1, H2	2	2	0	0
Niigata Pref.	H1, H5, H13, H26, H27	5	3	2	40
Ishikawa Pref.	H1, H5, H12, H28	4	3	1	25
Toyama Pref.	H1	1	1	0	0
Okinawa Pref.	H1, H29	2	1	1	50

Shared haplotypes are shown in color, and private haplotypes are shown in black.

Appendix D

The genetic diversity values for the prefectures are shown in Table A9. Based on this table, Figure 4 was produced. Additionally, pairwise F_{ST} and G_{ST} values are shown in Table A10.

Table A9. Genetic diversity values of *ITS2* in prefectures.

Prefecture	N	Gaps Excluded			h^{in}	Gaps Included		
		h^{ex}	$H_d^{ex} \pm \text{SD}$	$\pi^{ex} \pm \text{SD}$		H_d^{in}	$\pi^{in}(p)$	$\pi^{in}(i)$
Miyagi	34	4	0.171 $\pm$ 0.086	0.00055 $\pm$ 0.00030	4	0.1711	0.00055	0.00055
Fukushima	246	16	0.259 $\pm$ 0.036	0.00091 $\pm$ 0.00014	16	0.2536	0.00091	0.00090
Ibaraki	160	13	0.234 $\pm$ 0.045	0.00089 $\pm$ 0.00021	13	0.2336	0.00089	0.00089
Tochigi	28	5	0.270 $\pm$ 0.109	0.00101 $\pm$ 0.00046	5	0.2698	0.00101	0.00101
Saitama	8	3	0.464 $\pm$ 0.200	0.00118 $\pm$ 0.00056	3	0.4643	0.00118	0.00118
Yamagata	4	2	0.500 $\pm$ 0.265	0.00118 $\pm$ 0.00063	2	0.5000	0.00118	0.00118
Niigata	20	5	0.442 $\pm$ 0.133	0.00137 $\pm$ 0.00050	5	0.4421	0.00137	0.00135
Ishikawa	14	4	0.396 $\pm$ 0.159	0.00164 $\pm$ 0.00075	4	0.3956	0.00164	0.00164
Toyama	8	1	0	0	1	0	0	0
Okinawa	6	1	0	0	2	0.5333	0	0
All samples	546	28	0.254 $\pm$ 0.025	0.00091	29	0.2605	0.00091	0.00090

N : the number of alleles, h : the number of haplotypes, H_d : haplotype diversity, SD: standard deviation, π : nucleotide diversity, ex : exclusion of gaps, in : inclusion of gaps, p : pairwise comparison, i : individual comparison.

Table A10. Pairwise F_{ST} values (below the diagonal) and pairwise G_{ST} values (above the diagonal) of *ITS2* between prefectures using sequence data excluding gaps.

F_{ST}/G_{ST}	Miyagi	Fukushima	Ibaraki	Tochigi	Saitama	Yamagata	Niigata	Ishikawa	Toyama	Okinawa
Miyagi	-	0.00548	0.00328	−0.00689	0.00766	0.02514	0.01501	0.00114	0.02032	0.02791
Fukushima	0.01727	-	−0.00124	0.00397	0.02614	0.05364	0.01153	0.01261	0.03005	0.03917
Ibaraki	0.01714	−0.00293	-	0.00143	0.02404	0.05013	0.01262	0.01041	0.02931	0.03822
Tochigi	−0.00097	−0.00863	−0.00849	-	0.00144	0.01944	0.00225	−0.00985	0.02484	0.03060
Saitama	−0.03662	0.00528	0.00696	0	-	−0.3226	−0.01208	−0.02810	0.03704	0.03331
Yamagata	0.00485	−0.00113	−0.00368	0	0	-	0.01254	−0.00584	0.01031	0.00415
Niigata	0.01561	0.02343	0.02948	0.02047	−0.00367	0.01914	-	−0.01263	0.04347	0.04391
Ishikawa	−0.00881	−0.00213	−0.00083	0.0014	−0.04327	0.01810	0.01125	-	0.03284	0.03337
Toyama	0.01515	0.02632	0.02394	0	0	0	0.03509	0.03077	-	1
Okinawa	0.01515	0.02632	0.02394	0	0	0	0.03509	0.03077	0	-

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