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Mitochondrial Genetic Mutations in the Pale Grass Blue Butterfly: Possible DNA Damage via the Fukushima Nuclear Accident and Real-Time Molecular Evolution

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Abstract: The pale grass blue butterfly *Zizeeria maha* has been used to evaluate the biological effects of the Fukushima nuclear accident in 2011. Here, we examined the DNA sequences of the mitochondrial gene *cytochrome oxidase subunit I* (COI) of *Z. maha* using the field samples collected in 2011–2014 and 2021. Among 641 individuals from 44 localities in Northeastern Japan, we detected a heteroplasmic nonsynonymous nucleotide substitution in one out of three 2012 individuals from Hirono, Fukushima Prefecture, where the biological impact of radioactive pollution was the highest among the localities surveyed in 2012, suggesting DNA damage via initial exposure to short-lived radionuclides. An additional 80 individuals from Hirono in 2021 did not show any substitution, suggesting the extinction of the Hirono mutant by 2021. We also detected another heteroplasmic and homoplasmic nonsynonymous substitution in four out of five 2014 individuals from Shibata, Niigata Prefecture, where radioactive pollution was low. These substitutions were not present in the GenBank records of *Z. maha* and its sister species *Z. karsandra*, indicating that intraspecific variation may exceed interspecific variation in *Z. maha*. These results highlight not only the possible impact of the initial exposure in Fukushima but also real-time molecular evolution of butterflies in the field.

Keywords: COI; Fukushima nuclear accident; heteroplasmy; mitochondria; molecular evolution; radioactive pollution; point mutation; *Zizeeria maha*



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1. Introduction

Radioactive pollution has been widespread worldwide due to nuclear weapons, nuclear experiments, nuclear waste, and nuclear power plant accidents, including the Chernobyl and Fukushima nuclear accidents [1]. Anthropogenetic radionuclides from the Fukushima Dai-ichi Nuclear Power Plant (FDNPP) in 2011 have contaminated not only Fukushima [2] but also the global environment [3,4]. In addition to many dosimetric studies that follow the philosophy and protocols recommended by the International Commission on Radiological Protection (ICRP) [5], the possible biological effects of the Fukushima nuclear accident on living organisms have been studied and discussed in the literature [6–15]. Such studies have reported both positive and negative detection of biological aberrations in the field using various organisms, including non-primate mammals (mice [16–23], boars [24,25], raccoons [26], and cattle [27–30]); birds [31–34]; snakes [25]; fish [35,36]; coastal invertebrates [37]; insects (aphids [38,39], fruit flies [40],

and flying insects [41]); crabs [42]; plants [43,44] (fir trees [45], pine trees [46,47], and rice [48,49]); and soil microorganisms [50]. Furthermore, hematological abnormalities [51,52], a small head size [53], developmental retardation [54], and a low conception rate [55] have been reported in the Japanese macaque *Macaca fuscata* living in Fukushima, which may be relevant, at least to some extent, to another primate species living in Fukushima, *Homo sapiens*. Indeed, potentially similar effects, i.e., a low birth rate [56] and high perinatal mortality [57–59], have been reported in humans. Additionally, other potential effects have been reported [60–62].

Most of these studies provided circumstantial but not conclusive evidence that radioactive pollution due to the Fukushima nuclear accident may be the causal factor for these abnormalities detected in organisms living in polluted environments. A series of studies on the pale grass blue butterfly *Zizeeria maha* in Fukushima combining repeated field surveys (for more than three years from immediately after the accident) and intensive laboratory experiments conclusively demonstrated that the morphological and developmental abnormalities of butterflies were rooted in the environmental pollution caused by the Fukushima nuclear accident [62–72]. Both morphological and hematological abnormalities were also detected in a different butterfly species, the cabbage white butterfly *Pieris rapae*, demonstrating that the abnormalities in the pale grass blue butterfly caused by the Fukushima nuclear accident are not species-specific but may be generally observable in many lepidopteran species [73]. In rearing and crossing experiments of the pale grass blue butterfly, it was demonstrated that some abnormalities were heritable; for instance, the offspring generation from Hirono individuals caught in Fall 2011 presented 99.3% mortality and abnormality rate when reared under minimal radioactive conditions in the laboratory [63], suggesting that some genes may be directly mutated via radiation exposure [64,66,69].

Studies on the mechanisms of the biological impacts of the Fukushima nuclear accident on the pale grass blue butterfly are underway [72]. Importantly, there are at least two temporal exposure modes in the field: initial acute high-dose (dose-rate) exposure to short-lived radionuclides such as ^{131}I , ^{132}I , $^{129\text{m}}\text{Te}$, and ^{132}Te and chronic low-dose (dose-rate) exposure to long-lived radionuclides such as ^{137}Cs , ^{134}Cs , and ^{90}Sr . In the field, butterflies are highly vulnerable to low-dose exposure but are highly tolerant under laboratory conditions with an artificial diet containing radioactive cesium chloride [74]. This field–laboratory paradox can be resolved by introducing the concept of indirect field effects, which stand out only in the field through biological interactions [50,71,72,75]. More concretely, the host plant of this butterfly, *Oxalis corniculata*, responds to low radiation at the molecular level, synthesizing toxic defense chemicals for butterfly larvae [50,76–79]. In this way, low-level radiation effects are amplified through biological responses.

In addition to indirect field effects, the contributions of initial exposure to biological effects should be evaluated. In this context, transgenerational effects of initial exposure immediately after the Fukushima nuclear accident in the field have been indicated in the pale grass blue butterfly [80,81]. Radioactive cesium and probably other radionuclides accumulate in the abdomen of female butterflies, suggesting that oocytes may be affected during oogenesis [82]. At the time of the Fukushima nuclear accident, this butterfly had been overwintering, mostly at the last larval stage [83]. Soon after the Fukushima nuclear accident, most larvae must have entered the most voracious state before pupation, during which, various short-lived and long-lived radionuclides were likely consumed [83], which raises the possibility that these larvae accumulated DNA damage due to internal radiation exposure.

If actually present, the biological effects of the initial exposure are likely mediated by DNA damage, as indicated by previous studies [25,27,28,31,32]. In these studies, DNA damage was investigated mostly via comet assays and other chromosomal observations.

Intriguingly, a study on Japanese tree frogs demonstrated dose-dependent genomic DNA hypermethylation, which may be considered epigenetic DNA modifications in response to radiation exposure [84]. Moreover, this frog study reported mitochondrial DNA damage on the basis of the PCR efficiency of long and short DNA amplification [84]. This frog study is important in that it proposes the possibility of molecular changes that may be heritable and result in genomic instability at the large-scale population level in Fukushima [84].

Despite these studies, DNA sequences have rarely been examined for nucleotide substitutions in organisms living in Fukushima. One exception is a study focused on masu salmon fish from Fukushima [85]. In this study, DNA sequences of the mitochondrial *cytochrome b* (*cytb*) gene (765 bp) were determined in larval samples produced through artificial gynogenesis from female fish collected in a highly polluted area, namely, the Mano River, upstream of Hayama Lake, Fukushima Prefecture, in 2014 [85,86]. The parent females lived in the highly contaminated pond and river for approximately two or three years, indicating that they might have experienced initial exposure immediately after the Fukushima nuclear accident [85]. The DNA sequences of the larval samples were compared to those of their parents and those collected from nonpolluted fish cultures [85]. No nucleotide substitutions were detected in the nonpolluted control group among the 100 individuals examined, but two nonsynonymous substitutions were detected in 2 individuals of the experimental larval samples among the 42 individuals examined [85,86]. However, because these two nonsynonymous substitutions were located at the same site, and because they were one of the substitutions already known in the masu salmon population, it is argued that these substitutions are likely natural variants [85,86]. In addition, four synonymous nucleotide substitutions were detected in five individuals [85,86]. It is argued that these substitutions may be caused by radiation exposure but are likely natural single-nucleotide polymorphisms (SNPs) [85,86]. If these substitutions are not natural SNPs but are introduced by radiation exposure, their implications are not trivial. In that case, the detected substitutions may be just the tip of the iceberg, because harmful substitutions are quickly eliminated by natural selection. Through this process, individuals with low radiation resistance that do not have high DNA repair ability are eliminated, resulting in a significant change in the genetic diversity of organisms in Fukushima after the Fukushima nuclear accident.

Another exception is a study focused on wild boars in Fukushima chronically exposed to high-dose (dose-rate) radiation [87]. The main topic of that study is dosimetric estimates of radiation exposure in wild boars, but 24 nuclear microsatellite loci were sequenced, and their allelic richness values were compared using wild boar muscle samples (dietary meat for humans) before and after (2016–2019) the Fukushima nuclear accident [87,88]. The allelic richness values did not differ before and after the Fukushima nuclear accident [87,88]. However, the possibility of initial exposure was not tested in that study; the boar individuals caught in 2016–2019 [87] did not experience initial exposure immediately after the Fukushima nuclear accident, because the longevity of the boar is approximately two or three years.

In contrast, Murase et al. (2022) [89] examined 12 microsatellites from muscle samples of wild boars in Fukushima before and after (2014–2017) the Fukushima nuclear accident and reported that the microsatellite compositions were different before and after the accident. Notably, novel microsatellite alleles, which were not found before the Fukushima nuclear accident, were dominant after the accident, indicating a drastic change in genetic population structures [89]. The mitochondrial D-loop region was also sequenced, in which no nucleotide substitutions were discovered [89]. The change in population structures was interpreted as microsatellite instability [89]. Another possibility would be a simple result of natural selection; individuals with major microsatellites before the Fukushima nuclear acci-

dent might have been relatively vulnerable to radioactive pollution, and thus, the number of individuals with minor microsatellites might have increased after the Fukushima nuclear accident. In either case, this study [89] is important in that it clearly shows genetic compositional changes in wild boars in response to the Fukushima nuclear accident. However, the issue of initial exposure and its direct DNA damage immediately after the Fukushima nuclear accident remains elusive. It is technically and logically difficult to detect *de novo* mutations in nuclear DNAs (due to high repair efficiency) and in microsatellites (due to high variability). Furthermore, when DNA sequences are computationally examined as a collection of datasets (but not one by one at the single nucleotide level), important heteroplasmic information may be lost.

The scarcity of molecular studies at the level of DNA sequences in Fukushima research may be because relatively low radiation levels in Fukushima are generally believed to be too low to cause any genetic mutations in organisms [90]. However, the initial exposure level immediately after the Fukushima nuclear accident may have been sufficient to cause genetic mutations. For example, the ground deposition of radioactivity was approximately 5000 kBq/m² in Futatsunuma, Hirono, Fukushima Prefecture, on 15 March 2011 (T. Imanaka, personal communication). We reasoned that, to detect any mutations that are potentially caused by radiation from the Fukushima pollutants, mitochondrial genes are suitable, because, compared to nuclear DNA, mitochondrial DNA is known to be susceptible to mutations [91–98]. This susceptibility seems to originate from the fact that mitochondrial DNA is not covered with histones and that the DNA repair system in mitochondria is not efficient [91–98]. Moreover, once mitochondrial mutations occur, they may be allowed to exist heteroplasmically, because the wild-type allele in the same cell, mitochondrion, or individual can compensate for the nonfunctional mutant allele [91–98]. If such mutations occur in oocytes, mutant alleles will be inherited in subsequent generations.

Here, we focused on the mitochondrial gene *cytochrome oxidase I* (COI) in *Z. maha*. This gene is frequently used for phylogenetic analyses of lycaenid butterflies [99–101] and is used for DNA barcoding [102,103]. We amplified a DNA fragment of 564 bp via PCR, which was then subjected to DNA sequencing. Expecting heteroplasmy of mitochondrial DNA, we visually examined nucleotide spectral data for double signals at each single site and found such substitutions in a 2012 sample from Hirono and 2014 samples from Shibata.

2. Materials and Methods

2.1. Butterfly Species and Subspecies

We focused on the pale grass blue *Zizeeria maha* (Kollar, 1844). It is also called *Pseudozizeeria maha*, but we use *Zizeeria maha* because the genus *Pseudozizeeria* Beuret, 1955 [104] is likely invalid and is considered a synonym of the genus *Zizeeria* [105–109], although taxonomic evaluations should be presented in the future. A group of Japanese populations of mainland Japan is the subspecies *Z. maha argia* [106,107]. A group of Okinawan populations is the subspecies *Z. maha okinawana* [106,107]. A group of Chinese populations of mainland China and its surroundings is the nominotypical subspecies *Z. maha maha* [106,107]. A group of sister taxa of *Z. maha* is the dark grass blue *Zizeeria karsandra* (Moore, 1865) and the African grass blue *Zizeeria knysna* (Trimen, 1862) [108]. At present, the former species has only the nominotypical subspecies *Z. karsandra karsandra* [106]. The pale grass blue butterfly *Z. maha* is a small lycaenid butterfly and is a specialist of *Oxalis corniculata* [64,105–107,109]. This butterfly is suitable for environmental assessment, because it shares living space with humans [64] and because it has limited dispersibility [110].

2.2. Butterfly Samples

We first used field-collected butterfly samples for DNA sequencing that were obtained in previous field surveys in 2011–2013 [63,67] (Table 1). These samples were collected from seven localities, namely, Fukushima, Motomiya, Hirono, Iwaki, Takahagi, Mito, and Tsukuba (Figure 1a). The Hirono samples were collected on 12–16 May 2012, mainly from Futatsunuma. These field-collected samples included individuals with and without morphological abnormalities. We first analyzed 197 male samples without morphological abnormalities and then 93 male and female samples with morphological abnormalities (290 samples in total) (Table 1). In these studies, offspring (F_1) from field-caught females were obtained in the laboratory, which were subjected to DNA sequencing in the present study (Table 2). The butterfly samples with morphological abnormalities are shown in Supplementary Tables S1–S6. Image data on these morphological abnormalities have been deposited in Figshare [111].

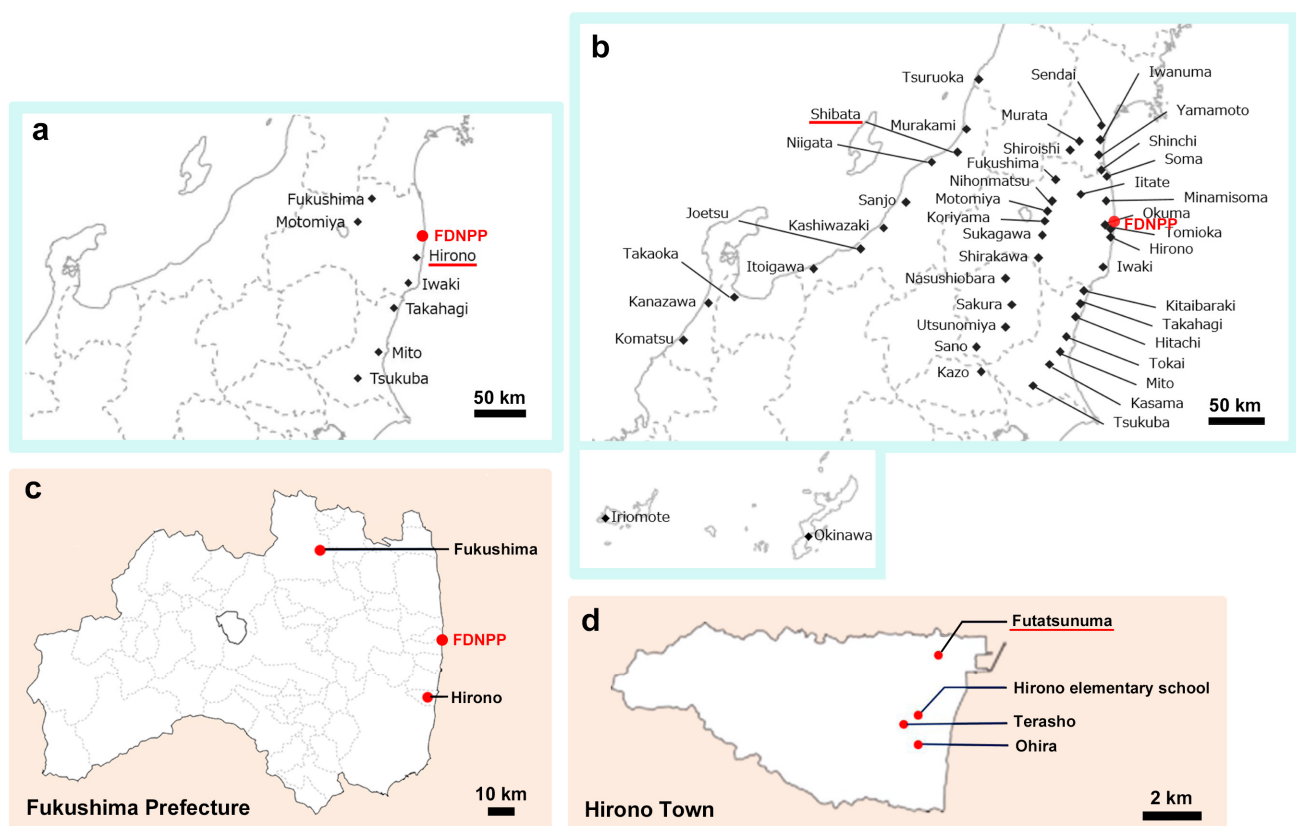


Figure 1. Sampling localities. The location of the Fukushima Dai-ichi Nuclear Power Plant (FDNPP) is shown in red. (a) Seven sampling localities in the 2011–2013 surveys in Fukushima Prefecture and Ibaraki Prefecture. Hirono is underlined, from which a nucleotide substitution was detected in the present study. (b) Forty-four sampling localities in the 2014 survey in Northeastern Japan and Okinawa Prefecture, Japan. Shibata is underlined, from which a nucleotide substitution was detected in the present study. Hirono contains two sampling sites (Futatsunuma and Terasho), although these sites are not shown on this map (but see (d)). (c) Two sampling localities within Fukushima Prefecture in the 2021 survey. (d) Four sampling sites in Hirono Town in 2021. Futatsunuma is underlined, from which a nucleotide substitution was detected in 2012 but not in 2021.

Table 1. Numbers of field-collected male and female butterfly samples (2011–2013) with or without morphological abnormalities analyzed in the present study.

Year	Tsukuba	Mito	Takahagi	Iwaki	Hirono	Motomiya	Fukushima	Total
Spring 2011	5 + 1	5 + 2	5 + 0	5 + 0	5 + 0	4 + 0	8 + 1	37 + 4
Fall 2011	2 + 0	1 + 0	2 + 0	1 + 0	1 + 0	1 + 1	3 + 0	11 + 1
Spring 2012	5 + 2	5 + 2	5 + 7	7 + 10	3 + 1	2 + 1	8 + 3	35 + 26
Fall 2012	5 + 5	5 + 3	5 + 5	4 + 9	5 + 2	6 + 4	8 + 1	38 + 29
Spring 2013	5 + 1	5 + 1	5 + 1	5 + 2	5 + 2	5 + 1	8 + 2	38 + 10
Fall 2013	5 + 2	5 + 4	5 + 1	5 + 5	5 + 2	5 + 4	8 + 5	38 + 23
Total	27 + 11	26 + 12	27 + 14	27 + 26	24 + 7	23 + 11	43 + 12	197 + 93

Numbers are shown as “(the number of individuals without morphological abnormalities) + (the number of individuals with morphological abnormalities)”. Samples without morphological abnormalities are all males, and samples with morphological abnormalities are males and females.

Table 2. Numbers of F₁ generation male butterfly samples (2011–2012) without morphological abnormalities analyzed in the present study.

Year	Tsukuba	Mito	Takahagi	Iwaki	Hirono	Motomiya	Fukushima	Total
Spring 2011	3	3	2	2	1	2	2	15
Fall 2011	0	0	0	1	0	3	0	4
Spring 2012	2	1	1	1	0	1	2	8
Total	5	4	3	4	1	6	4	27

We also used 216 butterfly samples for DNA sequencing collected from 44 localities (42 localities in Northeastern Japan and 2 localities in Okinawa) in Fall 2014 [112] (Figure 1b; Table 3). These localities included 5 localities in Miyagi Prefecture (Shiroishi, Murata, Sendai, Iwanuma, and Yamamoto); 14 localities in Fukushima Prefecture (Shinchi, Soma, Iitate, Minamisoma, Okuma, Tomioka, Hirono, Iwaki, Fukushima, Nihonmatsu, Motomiya, Koriyama, Sukagawa, and Shirakawa); 7 localities in Ibaraki Prefecture (Kitaibaraki, Takahagi, Hitachi, Tokai, Kasama, Mito, and Tsukuba); 4 localities in Tochigi Prefecture (Nasushiobara, Sakura, Utsunomiya, and Sano); 1 locality in Saitama Prefecture (Kazo); 1 locality in Yamagata Prefecture (Tsuruoka); 7 localities in Niigata Prefecture (Murakami, Shibata, Niigata, Sanjo, Kashiwazaki, Joetsu, and Itoigawa); 1 locality in Toyama Prefecture (Takaoka); and 2 localities in Ishikawa Prefecture (Kanazawa and Komatsu) (Figure 1b). Shibata samples for DNA sequencing ($n = 5$) were collected on 26–29 July 2014. Sendai samples for DNA sequencing ($n = 3$) were collected on 24–30 August 2014. These samples for DNA sequencing were all male samples simply because there were more male samples than female samples.

Table 3. Numbers of localities and field-collected male butterfly samples (2014) without morphological abnormalities analyzed in this study.

	Miyagi Pref.	Fukushima Pref.	Yamagata Pref.	Niigata Pref.	Toyama Pref.	Ishikawa Pref.	Tochigi Pref.	Ibaraki Pref.	Saitama Pref.	Okinawa Pref.	Total
Number of localities	5	14	1	7	1	2	4	7	1	2	44
Number of samples (Fall 2014)	23	70	5	35	5	10	20	34	5	9	216

Because the morphological abnormality rates in Fukushima Prefecture in 2013 and 2014 reached the level of the rates in other parts of Japan, we terminated regular field surveys at that point. However, upon the discovery of a heteroplasmic substitution in Hirono, an additional field survey was conducted in Fukushima (22 October 2021) and Hirono (14–15 October 2021) (Figure 1c; Table 4). In that survey, we collected butterflies at one site in Fukushima (Omori) and four sites in Hirono (Terasho, Futatsunuma, Hirono elementary school, and Ohira) (Figure 1d; Table 4). In this case, we used both male and female samples for DNA sequencing. A sample of the nominotypical subspecies *Z. maha maha* was obtained from a personal collection of one of the authors (J.M.O.), which originated in Southern China (Guangxi Zhuang Autonomous Region of the People's Republic of China).

Table 4. Field-collected male and female butterfly samples (2021) from Fukushima and Hirono analyzed in this study.

Year	Sex	Omori, Fukushima	Terasho, Hirono	Futatsunuma, Hirono	Elementary School, Hirono	Ohira, Hirono	Total
October 2021	Male	26	27	32	5	9	99
October 2021	Female	2	0	7	0	0	9
Total	Total	28	27	39	5	9	108

2.3. DNA Extraction and PCR

To extract DNA from adult butterfly samples, we first removed the wings and abdomen from the thorax, and the remainder of the body was 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), according to the manufacturers' protocols. After the DNA extraction procedures, the DNA concentrations were measured using a Qubit 2.0 fluorometer (Invitrogen, Waltham, MA, USA). The DNA solutions obtained in this way were used as templates for amplification.

A portion of the *COI* sequence was amplified via polymerase chain reaction (PCR) using the following primers synthesized by Exigen (Tokyo, Japan): the forward primer 5'-GTAAAACGACGGCCAGTATTTGAGCAGGAATATTAGGAACATCC-3' and the reverse primer 5'-GGAAACAGCTATGACCATGTTGGATCACCTCCTCCAGCTGGGTC-3' (Figure 2). These primers contain the M13F or M13R sequences for direct Sanger sequencing. The theoretically expected PCR product was 652 bp, including primer sequences, and the length of the amplified DNA fragment was confirmed by agarose gel electrophoresis and then by DNA sequencing. Excluding the primers, the amplified sequence was 564 bp in length. The PCR reaction mixture (50 µL in total) contained the following reagents: 2× Gflex PCR Buffer (25 µL), DNase-free RNase-free deionized water (Nippon Gene, Tokyo, Japan) (22 – x µL), forward primer (10 pmol/µL; 1 µL), reverse primer (10 pmol/µL; 1 µL), high-fidelity Tks Gflex DNA Polymerase (Takara Bio, Kusatsu, Shiga, Japan) (1 µL), and template DNA solution (x µL). A negative control in which the template DNA was replaced with DNase-free RNase-free deionized water was always performed together with the experimental PCRs. The amount of template DNA used varied from 3.66 to 17.4 ng per reaction.

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1   ttggaacatt atatatttatt tttggtatattt gagcaggaat attaggaaca tccttaagaa
61  ttttaattcg aatagaatta ggtactccag gat*cattaat tggatgatgat caaatttata
121 atactattgt aacagctcat gctttttatta taattttttt catagttaatg cctattataa
181 ttggtggatt tggaaattga ttagtcccat taatattagg agcccctgat atagcatttc
241 cacgaataaa taatataaga ttttgattat tacctccatc tttaatatta ttaatttcta
301 gaagaattgt agaaaatgga gcaggaactg gatgaactgt gtacccccca ctttcatcaa
361 atattgctca tagaggatct tctgtagact tagctatattt ttcttttacac ttagctggta
421 tttcttcaat tttagggtgca attaatattt ttacaacaat tattaatata cgag*taagta
481 atttatcttt tgatcaaata tcattatttt tttgagctgt tggaaattaca gctttattac
541 ttttattatc attacctgtt ttagcgggtg ctattactat attattaaca gatcgaaatc
601 ttaatacctc attttttgac ccagctggag gaggtgatcc aattttatat caacatttat
661 tttgatt

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Figure 2. Cytochrome oxidase subunit I (COI) sequence of *Zizeeria maha argia* (partial code) (GenBank Accession Number: DQ837206.1). A 564 bp region of this sequence (54th to 617th) was set as the Reference Sequence (RefSeq) in this study. The PCR primer sequences used in this study are shown in red. The PCR-amplified sequences contain 564 bp, excluding the primer sequences. Triplets containing a heteroplasmic substitution (asterisks) discovered in this study are highlighted in yellow. Two sequences with substitutions from Hirono (G474A) and Shibata (C94T) were deposited in GenBank (GenBank Accession Numbers: PV168357.1 for Hirono and PV168358.1 for Shibata).

The reaction cycles were set as follows: 94 °C (60 s), followed by 40 cycles of 98 °C (30 s), 55 °C (30 s), and 68 °C (60 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), 55 °C (15 s), and 68 °C (20 s), and then 35–40 cycles of 98 °C (10 s), 53 °C (15 s), and 68 °C (20 s), and, finally, 68 °C (60 s). The step at 55 °C (15 s) in the first 10 cycles was changed to 54.5 or lower when necessary. For agarose gel electrophoresis, 10 µL of the PCR product together with 2 µL 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 DNA sequencing.

2.4. DNA Sequencing and Sequence Analyses

Direct Sanger 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 MEGA X [113] and were visually examined one base at a time for the presence of two mixed signals at a single site. Even if there was a site of double signals when the sequence was read in one direction, there might be no double signals detected at the same site when the sequence was read in another direction. In that case, that site was considered to have a single signal. When the double signals were clearly detected at a given site in both directions, they were considered genuine double signals. In that case, to eliminate the possibility of PCR or sequencing mistakes, all the PCR and sequencing procedures were repeated, and the results were confirmed to be reproduced correctly.

The ratio of two nucleotide bases at a single site was obtained from the spectral peak areas, which were measured manually. To do so, the spectral data were enlarged and printed on plain paper, and the areas were cut out and weighed using a SHIMADZU ATX224 electronic balance (Kyoto, Japan), which shows the weight value to the fourth decimal place in grams. This procedure was repeated three times for a given sample to obtain the mean value.

To translate DNA sequences, a genetic code table for insect mitochondria was used [114]. The DNA sequences obtained from Hirono and Shibata were deposited in GenBank (GenBank Accession Numbers: PV168357.1 for Hirono and PV168358.1 for Shibata). For sequence comparisons, we used a 564 bp region of the previously known *COI* sequence from *Z. maha argia* (GenBank Accession Number: DQ837206.1) as the Reference Sequence (RefSeq) in this study (Figure 2). This RefSeq was obtained from an individual captured in Tsubaki-yama, Fukaura, Aomori Prefecture, Japan, the northernmost population of this species at the time of 2010 [115]. Using RefSeq as a query, a nucleotide BLAST (Basic Local Alignment Search Tool) search was performed using default values (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) (accessed on 23 February 2025).

3. Results

3.1. Seven Localities in 2011–2013

We first focused on butterfly samples collected in the seven localities in 2011–2013 (Table 1). These butterflies belong to the subspecies *Z. maha argia*. We analyzed the *COI* DNA sequences of 197 male butterfly samples without morphological abnormalities. All sequences were perfectly matched with RefSeq, except for one sample. We discovered a heteroplasmic nucleotide substitution in one of the three individuals from Hirono collected in May 2012 (Figure 3). This individual was obtained at Futatsunuma in Hirono (Figure 1d). The original G was still the majority at this site, but A was also detected simultaneously. This G474A substitution was nonsynonymous; it changes an amino acid in the *COI* protein from valine (GTA) to methionine (ATA). The A/G ratios were 0.319 (forward) and 0.325 (reverse). The mean ratio was 0.322. With this ratio, the percentage of the mutant allele in this individual was calculated to be 24.3%. Because two other individuals from Hirono did not show any substitution, the grand average percentage of the mutant allele was 8.1% ($n = 3$), suggesting that this allele was not well assimilated in Hirono in 2012.

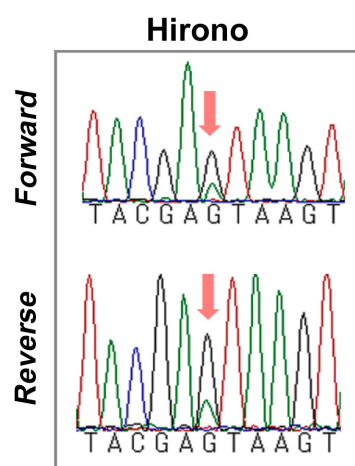


Figure 3. Heteroplasmic nucleotide substitution of a Hirono sample at position 474 (arrows). The results of both the forward (top) and reverse (bottom) sequences are shown. The original sequence is G, which is the majority, but A is also present at the same site.

We then analyzed 93 male and female butterfly samples collected in the same surveys but with various morphological abnormalities, including 1 Hirono sample collected in May 2012 (Table 1; Supplementary Tables S1–S6). Compared to RefSeq, we discovered no homoplasmic or heteroplasmic nucleotide substitutions, suggesting that the nucleotide substitutions and morphological abnormalities were not directly correlated with each other. When one Hirono sample without heteroplasmy was added, the ground average percentage of the mutant allele in May 2012 was 6.1% ($n = 4$).

3.2. Offspring F_1 Generation

To further examine whether a possible mutation in oocytes may be distributed into somatic cells in the offspring F_1 generation, we analyzed 27 F_1 generation butterfly samples. These samples were obtained in the laboratory from field-caught females in the seven localities (Table 2). We detected no nucleotide substitutions in comparison with RefSeq.

3.3. Northeastern Japan and Okinawa

To examine whether homoplasmic and heteroplasmic nucleotide substitutions are present in this butterfly population, we analyzed 207 male butterfly samples collected from 42 localities in nine prefectures in Northeastern Japan in 2014 (Table 3). We also analyzed nine butterfly samples from two localities in Okinawa Prefecture (Table 3), which belong to the subspecies *Z. maha okinawana*. We obtained perfect matches of DNA sequences from *Z. maha okinawana* individuals with RefSeq, which contained no homoplasmic or heteroplasmic substitutions, suggesting that the COI sequence of interest here is completely conserved beyond the subspecies. All the COI sequences from the northeastern samples were perfectly matched with those from RefSeq, except for four individuals from Shibata, Niigata Prefecture. Four of the five individuals of Shibata presented a nucleotide substitution, C94T (Figure 4). The original C was the minority at this site. One of them (Shibata 1) showed a complete change with no heteroplasmic indications (Figure 4). In this individual, the percentage of the mutant allele was thus 100%, indicating homoplasmy. Even in three other individuals (Shibata 2, 3, and 4), T was the majority, although the original C was also detected simultaneously. This substitution was nonsynonymous; it changes an amino acid in COI from serine (TCA) to leucine (TTA).

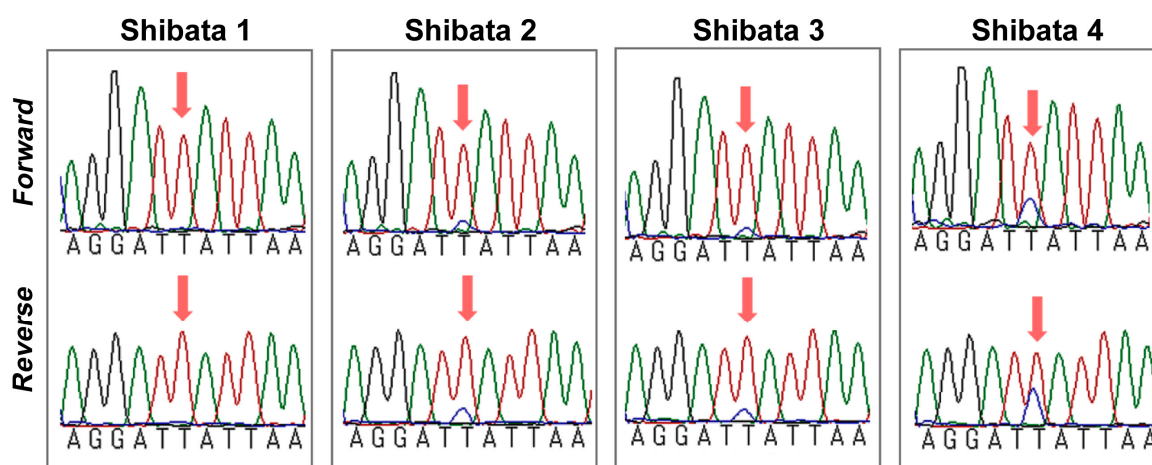


Figure 4. Homoplasmic and heteroplasmic nucleotide substitutions at position 94 (arrows) in four Shibata samples. The results of both forward (top) and reverse (bottom) sequences are shown. The original sequence is C, but T is dominant at that site. Shibata 1 shows a complete substitution, i.e., homoplasmy. Shibata 2, 3, and 4 show heteroplasmy.

In Shibata 2, the T/C ratios were 6.90 (forward) and 5.78 (reverse). The mean ratio was 6.34. With this ratio, the percentage of the mutant allele was calculated to be 86.4%. Similarly, in Shibata 3, the T/C ratios were 7.63 (forward) and 6.67 (reverse). The mean ratio was 7.15. With this ratio, the percentage of the mutant allele was calculated to be 87.7%. Finally, in Shibata 4, the T/C ratios were 2.51 (forward) and 1.91 (reverse). The mean ratio was 2.21. With this ratio, the percentage of the mutant allele was calculated to be 68.8%. Shibata 5 did not present any mutations; the percentage of the mutant allele was 0% in this case. The grand average of these five individuals for the percentage of the mutant allele was 68.6% ($n = 5$), demonstrating the relatively high level of genetic assimilation of

this mutant allele in the Shibata population of this butterfly in 2014 in comparison to the mutant allele in the Hirono population.

3.4. Additional Individuals from Fukushima and Hirono

We conducted a survey in 2021 and collected butterfly samples from Hirono (80 individuals), including 39 samples from Futatsunuma, where the G474A mutant allele was discovered, together with those from Fukushima (28 individuals) (Table 4). We analyzed these 108 male and female butterfly samples. The COI sequences of these samples perfectly matched that of RefSeq and did not contain any substitutions, including homoplasmic and heteroplasmic changes. In other words, the percentage of the G474A mutant allele detected in Hirono was 0% in 2021, suggesting the extinction of the mutant allele in the Hirono population of this butterfly.

3.5. *Zizeeria maha maha* and *Zizeeria karsandra*

To examine whether the COI sequence of interest is conserved among subspecies of *Z. maha*, we analyzed a specimen of *Z. maha maha* from Southern China. This individual showed a COI sequence identical to that of *Z. maha argia* (RefSeq) and *Z. maha okinawana*, indicating that nucleotide substitutions are rare in this COI region of interest among subspecies of *Z. maha*. To further examine nucleotide substitutions, we performed a BLAST search using the 564 bp sequence of RefSeq. There were many COI sequences of *Z. maha* (*Pseusozeeria maha*) in GenBank, many of which were identical to RefSeq (Table 5). Five substitutions were found in *Z. maha*: T416G, G170A, C161T, A530N, and A552N. They were all synonymous substitutions. Interestingly, a rare substitution, G170A, found in *Z. maha argia*, Sendai, Japan, was also found in *Z. maha maha* in Islamabad, Pakistan, suggesting their independent origin. Notably, the RefSeq was identical to the COI sequences of *Z. karsandra*, *Orthomiella sinensis*, and *Tongeia filicaudis* in China. They are different species, although they all belong to the tribe Polyommataini and the family Lycaenidae. However, *Z. karsandra* in Islamabad, Pakistan, had 21 substitutions in comparison with the RefSeq, among which, 20 substitutions were synonymous and only 1 substitution was nonsynonymous. This result suggests that *Z. karsandra* in China and Pakistan may be considered different populations.

Table 5. High-score GenBank records identified by a BLAST search using the RefSeq.

GenBank Accession Number	Species (Subspecies)	Collection Locality	Identities	Gaps	Substitutions (Nucleotides and Amino Acids)	Remarks [Ref]
DQ837206.1	<i>Pseudozizeeria maha argia</i>	Tsubakiyama, Fukaura, Aomori, Japan	564/564 (100%)	0/564 (0%)	na	RefSeq [115]
AB969805.1	<i>Pseudozizeeria maha okinawana</i>	Nishihara, Okinawa, Japan	564/564 (100%)	0/564 (0%)	none	Different subspecies [116]
KT236360.1	<i>Zizeeria karsandra karsandra</i>	Wuhu, Anhui, China	564/564 (100%)	0/564 (0%)	none	Different species in the genus <i>Zizeeria</i>
KT236339.1	<i>Orthomiella sinensis</i>	Wuhu, Anhui, China	564/564 (100%)	0/564 (0%)	none	Different genus in the family Lycaenidae
KF492057.1	<i>Pseudozizeeria maha argia</i>	Fujinomiya, Shizuoka, Japan	564/564 (100%)	0/564 (0%)	none	Same subspecies as RefSeq
ON436063.1	<i>Pseudozizeeria maha argia</i>	Shimobe, Minobu, Yamanashi, Japan	564/564 (100%)	0/564 (0%)	none	Same subspecies as RefSeq [117]
KT236376.1	<i>Tongeia filicaudis</i>	Wuhu, Anhui, China	564/564 (100%)	0/564 (0%)	none	Different genus in the family Lycaenidae

Table 5. Cont.

GenBank Accession Number	Species (Subspecies)	Collection Locality	Identities	Gaps	Substitutions (Nucleotides and Amino Acids)	Remarks [Ref]
KT236363.1	<i>Pseudozizeeria maha maha</i>	Wuhu, Anhui, China	564/564 (100%)	0/564 (0%)	none	Different subspecies
JQ344731.1	Lepidoptara sp.	China	564/564 (100%)	0/564 (0%)	none	Species identity unknown [118]
HQ990365.1	<i>Pseudozizeeria maha maha</i>	Sargodha, Punjab, Pakistan	563/564 (99.8%)	0/564 (0%)	T416G (A→A)	Synonymous substitution [119]
LC125180.1	<i>Pseudozizeeria maha argia</i>	Miyagi, Sendai, Japan (collected in 2014)	563/564 (99.8%)	0/564 (0%)	G170A (M→M)	Synonymous substitution [120]
PQ327494.1	<i>Pseudozizeeria maha maha</i>	Dharamshala, Pradesh, Himachal, India	563/564 (99.8%)	0/564 (0%)	T416G (A→A)	Synonymous substitution
KY839357.1	<i>Pseudozizeeria maha maha</i>	Islamabad, Pakistan	563/564 (99.8%)	0/564 (0%)	T416G (A→A)	Synonymous substitution [121]
KJ402272.1	<i>Pseudozizeeria maha maha</i>	India	563/564 (99.8%)	0/564 (0%)	T416G (A→A)	Synonymous substitution
KY832499.1	<i>Pseudozizeeria maha maha</i>	Islamabad, Pakistan	563/564 (99.8%)	0/564 (0%)	T416G (A→A)	Synonymous substitution [121]
KU973819.1	<i>Pseudozizeeria maha maha</i>	India	563/564 (99.8%)	0/564 (0%)	T416G (A→A)	Synonymous substitution
OR477325.1	<i>Pseudozizeeria maha maha</i>	India	563/564 (99.8%)	0/564 (0%)	T416G (A→A)	Synonymous substitution
KJ402269.1	<i>Pseudozizeeria maha maha</i>	India	563/564 (99.8%)	0/564 (0%)	T416G (A→A)	Synonymous substitution
KJ402162.1	<i>Pseudozizeeria maha maha</i>	India	563/564 (99.8%)	0/564 (0%)	T416G (A→A)	Synonymous substitution
KU973820.1	<i>Pseudozizeeria maha maha</i>	India	563/564 (99.8%)	0/564 (0%)	T416G (A→A)	Synonymous substitution
OQ147023.1	<i>Pseudozizeeria maha maha</i>	Punjab, India	563/564 (99.8%)	0/564 (0%)	T416G (A→A)	Synonymous substitution
MK719689.1	<i>Pseudozizeeria maha maha</i>	Bangladesh	563/564 (99.8%)	0/564 (0%)	T416G (A→A)	Synonymous substitution
KY843820.1	<i>Pseudozizeeria maha maha</i>	Islamabad, Pakistan	560/561 (99.8%)	0/561 (0%)	T416G (A→A)	Synonymous substitution [121]
KY845460.1	<i>Pseudozizeeria maha maha</i>	Islamabad, Pakistan	562/564 (99.6%)	0/564 (0%)	T416G (A→A) C161T (F→F)	Two synonymous substitutions [121]
KY839619.1	<i>Pseudozizeeria maha maha</i>	Islamabad, Pakistan	562/564 (99.6%)	0/564 (0%)	T416G (A→A) G170A (M→M)	Two synonymous substitutions [121]
KC158459.1	<i>Pseudozizeeria maha maha</i>	Kalam, Upper Sawat, KPK, Pakistan	510/513 (99.4%)	0/513 (0%)	T416G (A→A) A530N (T→T) A552N (S→S)	Three synonymous substitutions [121]
KY834653.1	<i>Zizeeria karsandra karsandra</i>	Islamabad, Pakistan	543/564 (96.3%)	0/564 (0%)	A89T (P→P) C161T (F→F) G170A (M→M) C206T (V→V) C224A (A→A) T239C (F→F) T353A (L→L) A386C (V→V) C389T (D→D) T419A (G→G) A473T (R→R) G478A (S→N) T504C (L→L) A524T (G→G) T543C (L→L) A545T (L→L) A551T (S→S) G566A (A→A) C600T (L→L) T602A (L→L) C608T (T→T)	20 synonymous substitutions and one nonsynonymous substitution [121]

Note: GenBank uses “*Pseudozizeeria maha*”, a synonym of “*Zizeeria maha*”. Redundant entries having the same sequence as the previous one after the 25th entry are not listed. Nucleotide numbers are assigned to substitutions according to the RefSeq. na: not applicable.

These results demonstrated the rarity of nonsynonymous substitutions in the *COI* sequence of interest, at least in *Zizeeria maha*, and that the G474A substitution in Hirono and the C94T substitution in Shibata are unique to this species. If the Hirono and Shibata substitutions are considered intraspecific variants, then the intraspecific variation in *Z. maha* may exceed the interspecific variation between *Z. maha* and *Z. karsandra* at the protein level. These results strongly argue for the recent environmental origin of the Hirono and Shibata substitutions.

4. Discussion

4.1. Significance of Homoplasmic and Heteroplasmic Nonsynonymous Substitutions

In this study, with a focus on the 564 bp sequence of the *COI* gene of the pale grass blue butterfly, we obtained nucleotide substitutions from the Hirono and Shibata populations. These substitutions were nonsynonymous (Figure 5). Because no other homoplasmic or heteroplasmic substitutions were found in our 641 samples, the possibility that the Hirono and Shibata substitutions are naturally occurring SNPs can safely be excluded. Both the Hirono and Shibata substitutions change amino acids in the *COI* protein: the Hirono G474A substitution is located at the first position of a triplet codon, and the Shibata C94T substitution is located at the second position (Figure 5). In GenBank records, only four synonymous substitutions were found in *Z. maha*, including G170A from Sendai, Miyagi Prefecture, Japan, in 2014 [120], and the RefSeq was identical to the *COI* sequences of other lycaenid species, including *Z. karsandra*, *O. sinensis*, and *T. filicaudis* (Table 5). Therefore, we consider these two substitutions discovered in this study not SNPs but genuine point mutations introduced in situ randomly, considering that the dispersibility of this species is rather low [110]. Importantly, the heteroplasmic state is considered transient in mammals [91–94,122] but relatively stable in insects; its complete assimilation may take several hundred generations in insects [123–126]. Notwithstanding, on an evolutionary time scale, this generation time is still short, and we can state that these mutations occurred relatively recently both in Hirono and Shibata. It should be noted that these mutations were not PCR mistakes. We used a high-fidelity DNA polymerase and successfully reproduced exactly the same PCR and sequencing results, including the heteroplasmic nucleotide substitutions. There was no possibility of contamination, considering that both substitutions in Hirono and Shibata are unique.

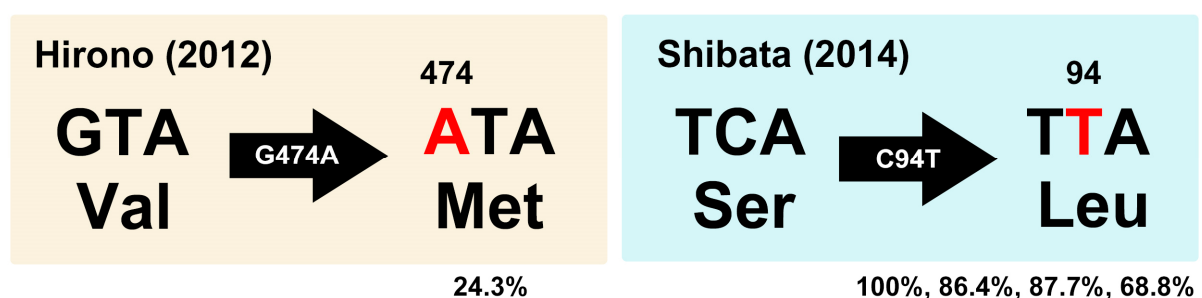


Figure 5. Summary of the homoplasmic and heteroplasmic nonsynonymous nucleotide substitutions (point mutations) discovered in this study: G474A (Hirono 2021) and C94T (Shibata 2014). Nucleotide substitutions are shown in red. Nucleotide numbers are given in reference to Figure 2 (RefSeq). The percentages of nucleotide substitution in an individual are shown at the bottom of this figure (excluding 0% individuals).

These Hirono and Shibata mutations were likely under strong natural selection pressure at the time of sample collection, because they change amino acids in the *COI* protein, which could abolish protein function unless they improve it by chance or remain neutral. The valine-to-methionine mutation in Hirono may be relatively mild because both amino

acids are nonpolar, whereas the serine-to-leucine mutation in Shibata may be deleterious because of the loss of a hydroxyl group. This biochemical interpretation is consistent with a substitution model of arthropod mitochondria, MtArt [127], in which the substitution frequency of the valine-to-methionine mutation is at a medium level and that of the serine-to-leucine mutation is low [127]. Nonetheless, the Shibata mutation may be relatively stable, judging from the relatively high assimilation level in the Shibata population. These Hirono and Shibata mutations are likely allowed to exist for some time because of heteroplasmy; the wild-type allele in a cell can compensate for the inefficient mutant allele. Our focus on a mitochondrial gene for heteroplasmic mutations was thus a suitable strategy for detecting genetic mutations via the Fukushima nuclear accident. On the other hand, these Hirono and Shibata butterflies did not present any morphological abnormalities, and we did not detect any substitutions in morphologically abnormal individuals, suggesting that these morphological abnormalities are not directly correlated with *COI* nucleotide substitutions. This is not surprising, because such phenotypes emerge as a result of multiple genes acting together and because only mutations at a limited number of nucleotide sites directly affect the morphology of butterflies.

The Shibata mutant allele appeared to be assimilated relatively well in the population; the mean percentage of the C94T substitution in an individual was 68.6% ($n = 5$). Among the five individuals from Shibata, one individual showed a 100% change (mutant allele only), whereas one other showed a 0% change (no mutant allele). The other three individuals were in between. We speculate that this mutant allele may still be present in Shibata, although an additional study is needed to demonstrate this point. In contrast, the Hirono mutant allele was likely extinct by 2021, because we could not discover it in 2021. This may be considered unfortunate, but it was fortunate that we detected the unique G474A mutation in one of three Hirono individuals collected in 2012.

We believe that this single case of Hirono stands out as evidence that the Fukushima nuclear accident caused DNA damage. Importantly, Hirono had the lowest capture rate of this butterfly species in the field among the seven localities surveyed in Fall 2011 and Spring 2012, suggesting that the Hirono population was severely affected [67]. Coincidentally, the abnormality rate of the field butterfly samples in Hirono in three years (2011–2013) was the highest in May 2012 [67] when the butterfly sample with the nucleotide substitution was captured. Moreover, Hirono had the highest growth retardation rate, abnormality rate, and mortality rate of offspring (F_1) generation among the seven localities surveyed in 2011, suggesting that the Hirono population experienced heritable DNA damage immediately after the Fukushima nuclear accident [63,64,67]. Although no substitution was detected in F_1 individuals in the present study, this is because of the scarcity of Hirono F_1 samples ($n = 1$ for Spring 2011, $n = 0$ for Fall 2011, and $n = 0$ for Spring 2012). If the Hirono substitution in Spring 2012 was introduced by nuclear pollution, we must be able to detect similar substitutions in Fall 2011, but again, it suffered from a scarcity of samples ($n = 1$ for Fall 2011). Thus, the negative result of Fall 2011 in Hirono could not be avoided.

To be sure, we cannot exclude the possibility that the Hirono mutation is spontaneous. However, the chance that such spontaneous mutations are discovered in Hirono is very low. For a single mutation evident in somatic cells to be detected, numerous point mutations should be introduced first in oocytes. It is possible that patchy but concentrated radioactive plumes from the FDNPP might have locally induced this mutation in Hirono. Mitochondrial genome sequencing may be able to identify numerous substitutions that may be induced by short-lived radionuclides from the FDNPP, but they should be distinguished from SNPs, and heteroplasmy should be taken into account.

Having mentioned our interpretation in the case of Hirono above, the same logic may be applicable to the case of Shibata. We believe that there must be a reason that the Shibata

population presented a distinct nucleotide substitution. Shibata accumulates much snow during the long winter season, which strongly reflects UV light to induce mutagenesis in overwintering larvae of this butterfly, but many similar localities accumulate snow in Japan. The hypothetical snow effect may be evaluated by the analysis of the Akita and Aomori samples in 2014 [112] in future studies. The possibility that the Shibata mutation was also caused by the Fukushima nuclear accident, possibly in collaboration with the snow effect, cannot be ruled out completely, but Shibata is located 158 km away from the FDNPP in contrast to Hirono, which is just 21 km away from the FDNPP. Moreover, the presence of a homoplasmic individual together with heteroplasmic individuals in Shibata suggests that this mutation occurred earlier (see Section 4.2 below). Agrichemicals are another possible cause of the Shibata mutation, but again, agrichemicals have been widely used throughout Japan.

Additionally, the possibility that the G170A synonymous substitution in Sendai, Japan, in 2014 among the 13 localities examined [120] may also be a product of the Fukushima nuclear accident should be examined in the future. Sendai is located 94 km away from the FDNPP but was partially polluted at hot spots; for example, the ground deposition of ^{131}I radioactivity was 1600 Bq/m^2 in Natori (near Sendai) at the time of 14 June 2011 [128] (note that the half-life of ^{131}I is 8 days). There is also the possibility that the Sendai allele may be transported from heavily contaminated areas by artificial means because of heavy traffic to Sendai, the largest city in Tohoku District. Sendai is located north of the FDNPP, and they are almost directly connected via National Route 6. Considering that we did not detect the G170A substitution in our samples (including three Sendai samples) collected in 2014, the G170A substitution in Sendai may be a local and declining allele that emerged due to the Fukushima nuclear accident.

Mitochondrial genes are susceptible to reactive oxygen species (ROS), which are produced during the oxidative phosphorylation process in mitochondria [95–98]. The absorption of ionizing radiation also results in the production of ROS in organisms, mostly via water ionization. Moreover, oocytes and their mother cells may be susceptible to ROS and ionizing radiation due to cell division in the ovary. One of the previous studies demonstrated that radioactive substances accumulate in the abdomen of adult butterflies when larvae consume polluted leaves [82]. When a point mutation occurs in a mitochondrial gene at that time, it is possible that this single mutation is amplified in mitochondria during oogenesis, as in mammals [91–98]. When this oocyte produces a complete individual after successful fertilization, a heteroplasmic state may be realized. Because the wild-type allele is present in mitochondria, this mutation may not be expressed as a result of phenotypic changes, even if the mutation nullifies the function of that gene. In other words, heteroplasmic mutations may behave like neutral changes to some extent. Notably, in *Drosophila* forward genetics, mutagenesis is carried out by feeding flies mutagens such as ethyl methane sulfonate (EMS), and the next generation of treated flies will have mutations in somatic cells [129,130]. This F_1 genetic screen has been performed in the pale grass blue butterfly, which shows morphological abnormalities similar to those of the Fukushima individuals [131]. The pale grass blue butterfly is likely vulnerable to mutagenesis when mutagens act on oogenesis and spermatogenesis.

On the basis of the present data and interpretations, the biological effect of the direct radiation exposure effect (in addition to the indirect field effects [50,71,72,75]) should be taken seriously in Fukushima. Since low-dose exposure is not enough to damage DNA directly or through the ionization of water [90], the Hirono mutation can be caused by initial exposure to short-lived radionuclides such as ^{131}I immediately after the Fukushima nuclear accident. Therefore, heteroplasmy should be taken into account when evaluating the biological effects of radiation exposure. Numerous DNA damage sites might have been

produced in Fukushima by the initial exposure, but they were instantly repaired by DNA repair enzymes and, if not, quickly eliminated by natural selection almost before being discovered by researchers. In this context, the importance of early sampling immediately after the Fukushima nuclear accident cannot be overemphasized. At this point, we have no way to obtain more Hirono samples in 2011 and 2012 without a time machine.

4.2. Real-Time Molecular Evolution of COI

The discovery of a missense point mutation in the Shibata population is unexpected and deserves attention from the perspective of molecular evolution. Both heteroplasmic and homoplasmic states were discovered in Shibata, suggesting that this mutation occurred relatively recently. However, the Shibata mutation has been assimilated relatively well. For the sake of discussion, we here assume that 700 generations are needed to complete the full assimilation of an allele in a population, according to previous studies [123–126]. Then, assuming that the pale grass blue butterfly has five generations per year in Northeastern Japan [64], the Shibata mutation must spend 140 years to achieve full assimilation. Because the Shibata mutation is a way to achieve full assimilation, this mutation might have occurred approximately one hundred years ago. This discussion ignores the natural selection pressures on mutations and, thus, is not accurate. However, this line of discussion suggests that the Shibata mutation is unlikely to have been caused by the Fukushima nuclear accident (just three years ago), but it occurred “relatively recently” on an evolutionary time scale.

Although we do not know the precise reason for its origin in Shibata (i.e., it spontaneously occurred or was induced by a pollution event), regardless of its origin, this allele may survive for a while. Four of the five individuals presented this mutation at various degrees of heteroplasmy. Indeed, one of them showed homoplasmy. Assimilation at this level may require many generations without extinction. That is, their existence may be either due to chance or due to their superior functionality over the original allele. The Shibata population may be partially isolated, although we do not notice it well, which may allow this mutant to survive, but at the same time, sibling crosses may produce genetically weak populations. Indeed, the Shibata population presented a relatively high abnormality rate (11%) in contrast to the average rate among localities in Northeastern Japan, in 2014 (3%) [112].

If this mutation is advantageous over the original nucleotide, this allele may be assimilated in a defined population in the future. At that point, this allele may be considered a SNP. Furthermore, upon a potential event of geographic isolation, for example, via an earthquake, the Shibata population may be isolated rigorously and may complete assimilation within the isolated population. At that point, because this substitution in *Z. maha* is different from that of *Z. karsandra* and those of subspecies *Z. maha maha*, *Z. maha argia*, and *Z. maha okinawana*, the Shibata population may be incorrectly considered an independent species if it is solely based on molecular data. Even worse, *Z. maha*, *Z. karsandra*, *O. sinensis*, and *T. filicaudis* together may be incorrectly considered single species solely on the basis of molecular data because their sequences are identical (Table 5). Thus, this thought experiment indicates that the Shibata substitution is an exemplifier case in which molecular phylogeny is not consistent with the real-world phylogenetic relationship in a population facing real-time evolutionary changes.

Importantly, a 648 bp region of COI has been used for DNA barcoding to identify species on the basis of DNA sequences alone [102,103]. A theory behind DNA barcoding is that there is a large gap between interspecific and intraspecific variations [102,103]. Although the utility of DNA barcoding in examining biodiversity according to a standard protocol is beyond doubt, this DNA barcoding gap has been shown to be an artifact of

insufficient sampling, with a focus on lycaenid butterflies, especially blue butterflies (the genus *Agrodiaetus*) [132]. Coincidentally, the present study also focused on one of the blue butterflies (i.e., grass blue). One of the reasons that the DNA barcoding gap cannot be stable, at least in lycaenid butterflies, could be that local populations evolve in real time at the molecular level, such as the Shibata population. That is, the intraspecific variation may easily exceed the interspecific variation. More fundamentally, such molecular evolution is not directly related to speciation but is just a coincidental company of speciation in most cases. On the other hand, the possibility that *Z. karsandra* may be composed of two distinct populations in China and Pakistan can be pointed out on the basis of DNA sequences alone in the present study (Table 5), demonstrating the power of DNA sequence comparisons in phylogenetics. Therefore, molecular data are very useful but should be used with other information to integratively define species, at least in lycaenid butterflies.

According to a molecular phylogenetic study of *Zizina* (the sister genus of *Zizeeria*), the divergence of three species of *Zizina* occurred 2.5 Myr (million years ago) on the basis of the 878 bp sequence of the mitochondrial *ND5* gene [108]. Divergence into two genera, *Zizina* and *Zizeeria*, occurred at approximately 6 Myr [108]. Similarly, *Z. maha* and its sister taxa, *Z. karsandra* and *Z. knysna*, differentiated 3.5 million years ago [108]. These statements are based on a small number of nucleotide substitutions according to the concept of the molecular clock [133,134]. These estimates in divergence time may be largely correct, but these statements are probably misleading at the same time. Does a nucleotide substitution such as the Shibata substitution occur once every few million years? This is unlikely. Most likely, numerous mutations are introduced continuously in real time, which may be observable within a human's lifetime, irrespective of geographical isolation and subsequent speciation. The concept of the molecular clock has been applied to many cases, since its proposal by Zuckerkandl and Pauling (1962) [133] to estimate the divergence time of speciation, although modern molecular clocks have been considerably modified [134]. The concept of the molecular clock is extremely useful in phylogenetics but may simultaneously give researchers an incorrect impression that nucleotide substitutions are extremely rare in the field.

The Shibata and Hirono mutations identified in the present study may be considered records of the real-time molecular evolution of this species in the field. In addition to real-time molecular evolution in Shibata and Hirono, real-time phenotypic evolution has occurred in this species, one of which is likely driven by the phenotypic plasticity revealed by environmental hazards [115]. The adaptive evolution of this species to radiation exposure in Fukushima has also been reported [135]. The Fukushima nuclear accident might have introduced numerous mutations and initiated novel natural (but artificial) selection pressures on organisms in Fukushima for molecular and phenotypic evolution. Changes in the genetic population structures of *Z. maha* after the Fukushima nuclear accident should be demonstrated in the future, as shown in wild boars [89].

5. Conclusions

The discovery of a heteroplasmic single-nucleotide substitution (point mutation) in a Hirono butterfly sample can be considered important supporting evidence for DNA damage caused by the direct exposure of butterflies in Fukushima to ionizing radiation from the FDNPP. This mutation is probably caused by initial exposure to short-lived radionuclides. The Hirono mutation suggests the high occurrence of random mutagenesis and natural (but artificial) selection via radioactive pollution in Fukushima in the field. Although the origin of the Shibata substitution is not clear, both the Shibata and Hirono substitutions were not found in the GenBank records of *Z. maha* and *Z. karsandra*, although these populations cannot be considered independent species, suggesting the recent origin

of these substitutions at the local sites. The present results can be considered a case of real-time molecular evolution, which provides an opportunity to discuss the fundamental aspects of the molecular clock, molecular evolution, and speciation.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/d17040275/s1>: Table S1: Spring 2011; Table S2: Fall 2011; Table S3: Spring 2012; Table S4: Fall 2012; Table S5: Spring 2013; Table S6: Fall 2013.

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Institutional Review Board Statement: Not applicable.

Data Availability Statement: The original contributions presented in this study are included in the article. The original butterfly specimens have been kept in the laboratory, and their images can be found in Figshare [111]. Further inquiries can be directed to the corresponding author.

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Abbreviations

The following abbreviations are used in this manuscript:

BLAST	Basic Local Alignment Search Tool
COI	Cytochrome oxidase subunit I
DNA	Deoxyribonucleic acid
EMS	Ethyl methane sulfonate
FDNPP	Fukushima Dai-ichi Nuclear Power Plant
ICRP	International Commission on Radiological Protection
Myr	Million years ago
ND5	NADH dehydrogenase 5
PCR	Polymerase chain reaction
ROS	Reactive oxygen species
SNP	Single-nucleotide polymorphism

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