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Intraspecific Variability and Distribution Difference within the Ribosomal Introns of the Discrete *Plasmodiophora brassicae* Group in Japan: A Case Study for Complex Dynamics of Intron Evolution

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Abstract: Analysis of the ribosomal introns of *Plasmodiophora brassicae* populations infecting the cruciferous weed *Cardamine occulta* revealed the complex dynamics of size, intraspecific variability, and distribution. The results showed that *P. brassicae* populations from the weed have lost multiple introns in the small and large subunits of the ribosomal RNA genes. Moreover, the retained introns, despite a largely mutual share of conserved parts with the cosmopolitan strains, contained numerous novel structures. These structural differences comprise a high level of polymorphisms, such as transversion point mutations occurring at sites involving the intronic splicing sites or insertions/deletions at the binding sites. Two geographical *P. brassicae* populations from *C. occulta* carried a lengthy intron-encoded ORF and putative mobile elements established in the large subunit. A few *P. brassicae* populations from the *Brassica* crops also harbored polymorphic introns that shared common mutated motifs with the weed-affecting group. The diversity of ribosomal introns observed from those investigated populations demonstrated the genetic distinction of the *P. brassicae* populations from *C. occulta*. The genetic variations might play a key role in the adaptability of the weed-infecting populations and are more likely related to the process of pathogenesis for the cosmopolitan *P. brassicae* infecting the *Brassica* crops.

Keywords: intronic distribution; population diversity; ribosomal DNA; clubroot disease; *Plasmodiophora brassicae*; *Cardamine occulta*

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1. Introduction

Plasmodiophora brassicae, a plasmodiophorid (phytomyxean, rhizarian) biotrophic pathogen, is a causal pathogen of clubroot disease in cruciferous species. Several studies on this obligate intracellular protist have mainly focused on the cruciferous crops because of their economic importance for agriculture, whereas other wild cruciferous species have often been overlooked. Our group has previously reported the clubroot disease that has been identified frequently in the cruciferous weed *Cardamine occulta*, previously identified as *Cardamine flexuosa* [1]. This overwinter weed is abundantly found in several human-made habitats, particularly in well-irrigated paddy fields where this wild weed flourishes in the postharvest periods and has since become established as part of the weed community in the rice cultivation of Japan. The clubroot incidents occurring in this weed have been described from the pathological traits diagnosed in fields to the multiple stages of the secondary infection observed under microscopy [2]. In addition to phenotypical identification, the genetic diversity among *P. brassicae* from *C. occulta* and the cruciferous crops was also elaborated using random amplified polymorphic DNA (RAPD) PCR, and

distinct genotypes of *P. brassicae* populations from *C. occulta* as opposed to *P. brassicae* were suggested [3]. We extend the genetic investigations on the intronic sequences established in the small and large subunits (SSU and LSU) of the ribosomal RNA gene (rDNA), since these intronic sequences are involved in operating the processing of the rRNA precursors [4,5] and have been used as a molecular genotyping tool to explore the population diversity of microorganisms [6].

The long debate over the significant role of rDNA regions in geographical differentiation was initiated by Niwa et al. [7]. Geographical *P. brassicae* populations in Japan were reported to carry a highly polymorphic LSU that contains the novel lengthy introns. However, this study was refuted by Schwelm et al. [8] who confirmed that the pathogen *P. brassicae* has no polymorphism in the LSU regions and made the correction that those novel introns found in Japanese populations were not originated from *P. brassicae* but were misidentified by a chimeric PCR product from the glissomonad *Neoheteromita globose* instead. This story was succeeded by Laila et al. [9] who reaffirmed the sequence variations detected in the rDNA of geographical isolates in Korea and that the SSU intron I was deleterious in several isolates. Nevertheless, Schwelm et al. [10] were persistent on the notion of no rDNA polymorphism in the nuclear ribosomal DNA for the plant pathogen *P. brassicae* on the global scale.

In our study, we still value the role of the nuclear rDNA of *P. brassicae* but instead of exonic sequences, we focus on the intronic sequences, especially for the *Cardamine* group. The rDNA intronic sequences of this group is immensely diverse, which could be attributed to the lifestyle and genetic make-up of the host weed. As indicated above, the weed host *C. occulta* shares a completely different lifestyle in contrast to the crop hosts. We would consider the *P. brassicae*-infected *C. occulta* weeds as "natural populations" compared to the *P. brassicae*-infected brassica crops, i.e., natural vs. domesticated. As obligate biotrophic pathogens, *P. brassicae* are genetically compelled to shift to a new host. The genetic engineering of the *Cardamine* group might be the vital source for the genetic variations in the global *P. brassicae* populations. This might explain the clubroot pathogenicity and pathotypic-associated genes because these characteristics may stem from such variations. Therefore, this research might provide another perspective on researching population-level diversity in the *P. brassicae* via the evolution of introns, bridging variations to the pathogenicity and etiology of the clubroot disease.

2. Materials and Methods

2.1. Clubroot Samples Collected

The clubroot samples were collected from diseased plants between the period of 1993 and 2014 from multiple locations across Japan (Table S1). The collected clubroot galls were washed thoroughly and stored at −40 °C.

2.2. DNA Extraction

The clubroot samples were washed thoroughly with tap water and then distilled water. Tissue samples were ground with a pestle and mortar in distilled water to extract the resting spores, described previously by Osaki et al. [3]. Centrifugation was repeated at 420× *g* for spore suspension until the supernatant became completely transparent. DNA was purified from resting spores based on the methods described by Ito et al. [11].

2.3. Primer Designation and Sequencing Analysis

All primers were designed using Primer3 tool (v. 0.4.0) (Available online: http://primer3.ut.ee/ (accessed on 3 September 2018). Primers of 18S and 28S exonic and intronic regions were designed based on KX011115 in the GenBank database (Table S2). Due to the intron loss and gain pattern (the first intron in the 18S region of the crop group and several introns in the *Cadamine* group), extra primers were designed to target the exonic regions or related landmark regions (ITS regions) in order to ensure the desired intronic regions belonging to *P. brassicae* (as many free-living or parasitic protists such as *Woronina*

pythii or *Spongospora subterranea* f. sp. *subterranea* have similar ribosomal sequences). These extra primers were able to amplify sequences including exonic parts or landmarks like ITS regions, which are exclusively identifiable for *P. brassicae*. All sequences were aligned and analyzed by MEGA X [12].

2.4. Sequence Annotation

The MFannot tool (http://megasun.bch.umontreal.ca/cgi-bin/mfannot/mfannotInterface.pl (accessed on 9 September 2020) was used to identify introns as group I or group II introns as well as open reading frames (ORFs). Annotation of ORFs was reviewed and revised by Rfam (https://rfam.org/ (accessed on 15 November 2020) and BLAST homology searches against the NCBI protein database 2.1.

3. Results

3.1. The Pattern of Loss/Gain Introns Distributed within the P. brassicae Populations in Japan

Based on the intronic distribution established in the rDNA of the *P. brassicae* AT isolate (KX011115) (Figure 1), the pattern of the rDNA intronic distribution among the *P. brassicae* Japanese populations is described in Table 1. Of all five introns, the gain and loss pattern only occurred specifically in the 18S intron I of the crop group, whereas the *Cardamine* group appeared to suffer massive intron losses in both the SSU and LSU (Table S1). The *Cardamine* group only possessed intron I either in the 18S or 28S region, except for the Nara population harboring introns I in both the 18S and 28S regions. The other introns of the *Cardamine* group (18S intron II, III and 28S intron II) were completely absent (Table 1).

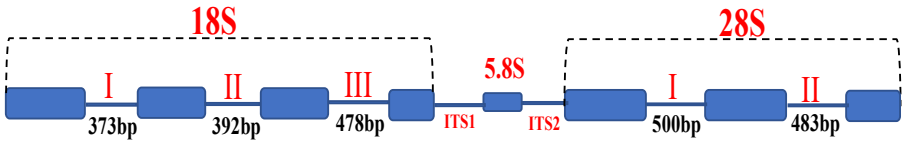

Figure 1. Sizes and distribution of the introns in the ribosomal DNA of *P. brassicae*. Shaded boxes represent the exonic parts, the exonic parts are connected by the lines with Roman numerals (I, II, and III) above that represent the intronic sequences. Small subunit (18S) and large subunit (28S) are separated by the ITS regions and 5.8S. Numbers under the lines indicate the sizes of individual introns in base pair (bp).

The BLAST results from the *Cadarmine* group have shown a certain degree of similarity with *Woronina pythii* or *Spongospora subterranea* f. sp. *subterranea* sequences (E-value = 0, coverage 80–92%) due to the status of ribosomal intronless sequences; however, when aligned and evaluated, the exonic parts of the *Cadarmine* group shared a more similar identity to the sequence of the *P. brassicae* AT strain.

Table 1. The absence–presence pattern of intronic sequences distributed in ribosomal RNA genes (18S and 28S) among the *P. brassicae* populations infecting *C. occulta* (the *Cardamine* group) vs. the *Brassica* crops (crop group).

Intronic Sequences		*Cardamine* Group	Crop Group
18S	I	Variable	Variable
	II	Absence	Presence
	III	Absence	Presence
28S	I	Variable	Presence
	II	Absence	Presence

3.2. Intron Analysis of Small (18S) and Large (28S) Subunit rRNA Genes

3.2.1. Small Subunit (18S) rRNA Gene

A completely deleted 18S intron I was found in many populations. However, both groups also had heterozygous 18S intron I sequences (lanes 6, 11, 13, 14, 15, 16, 17, 18, and 19) that were both intron-free and intron-bearing within a single field population (Figure 2).

The sequencing results of the 18S introns I indicated that most of the crop group populations remained conserved in their sequences and sizes. They shared the same length of 374 bp with the cosmopolitan isolate ATs. Three populations possessed a haplotype illustrated by the transition point mutation A/G in the 5′ upstream position near the flanking exon–intron boundaries, but the sequenced introns from the rest were identical to the AT sequence (Figure 3, position 24). The two populations Hagi and Itoshima differed from the crop group in the sizes and mutation acquisitions, particularly the Itoshima population. The size of the 18S intron I in the Itoshima population was significantly longer (407 bp) (Figure 3, position 374) due to three insertions at specific sites. These specific sites may be related to the intronic secondary structures (branching site, binding site, and lariat structures) in the self-spliceosomal activity. The Hagi population had fewer mutations than the Itoshima, but all of the other intronic sequences of the Hagi population (from the SSU to the LSU) were detected with a high level of polymorphisms.

The *Cardamine* group had a different pattern of sequences and acquired mutations compared to the crop group. One special inclusion in the *Cardamine* group is the *P. brassicae* populations collected from the wild weed *Rorippa palustris* (LC716109) due to its 18S intron I being homologous to those of the *Cardamine* group, but its intron was longer in sequence by carrying an encoded ORF (approximately 550 bp) (Table S1). While four populations (Yamaguchi, Kumamoto, Akiota, and Togochi) were devoid of all introns in the 18S region, two populations (Nara and Nagasaki) carried the 18S intron I, and their sequences displayed homology in terms of sizes and polymorphisms acquired.

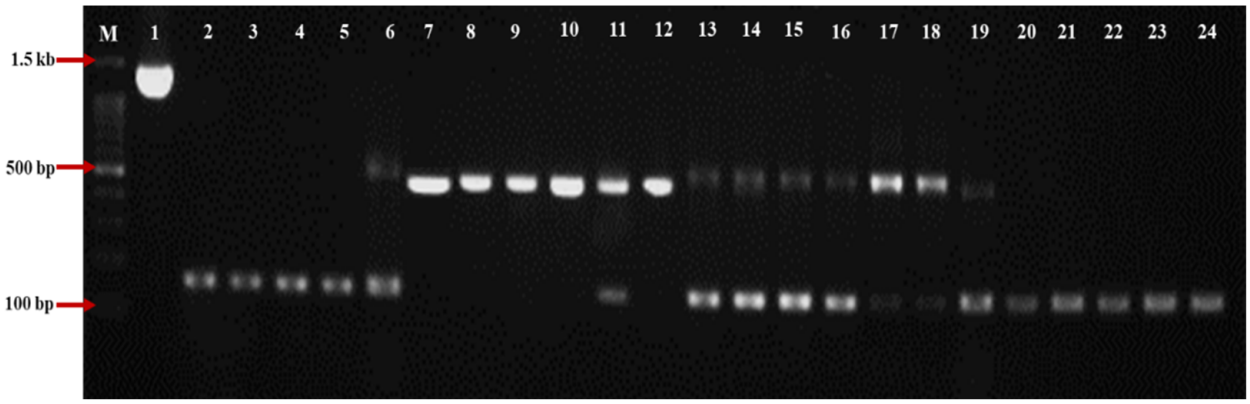

Figure 2. PCR products illustrate the 18S intron I distribution (app. 530 bp, primers listed in Table S1) of two groups, the *Cardamine* and crop groups. Lane M represents the standard ladder (arrows pointed at 1.5 kb, 500 bp, and 100 bp). Lane 1: *Rorippa palustris* (906 bp). Lanes 2–7 represent the *Cardamine* group, including Kumamoto, Yamaguchi, Akiota, Togochi, Nagasaki, and Nara, respectively. Lanes 8–24 represent the crop group, including Fukuoka, Aichi, Itoshima, Hokkaido, Ishikawa, Gunma, Nagasaki, Onga, Ibaraki, Saga, Hagi, Osaka, Fukutsu, Shimane, Kurume, Wakamatsu, and Karatsu, respectively. Populations distinguished by genotypes such as homozygous either missing the introns displaying the 102 bp of the exonic parts (2–5, 20–24) or retaining one band of the introns (7–10, 12), and heterozygous by having both bands (11, 13–19). Faint bands observed in several lanes, other than the distinct and sharp main products due to the purity of DNA.

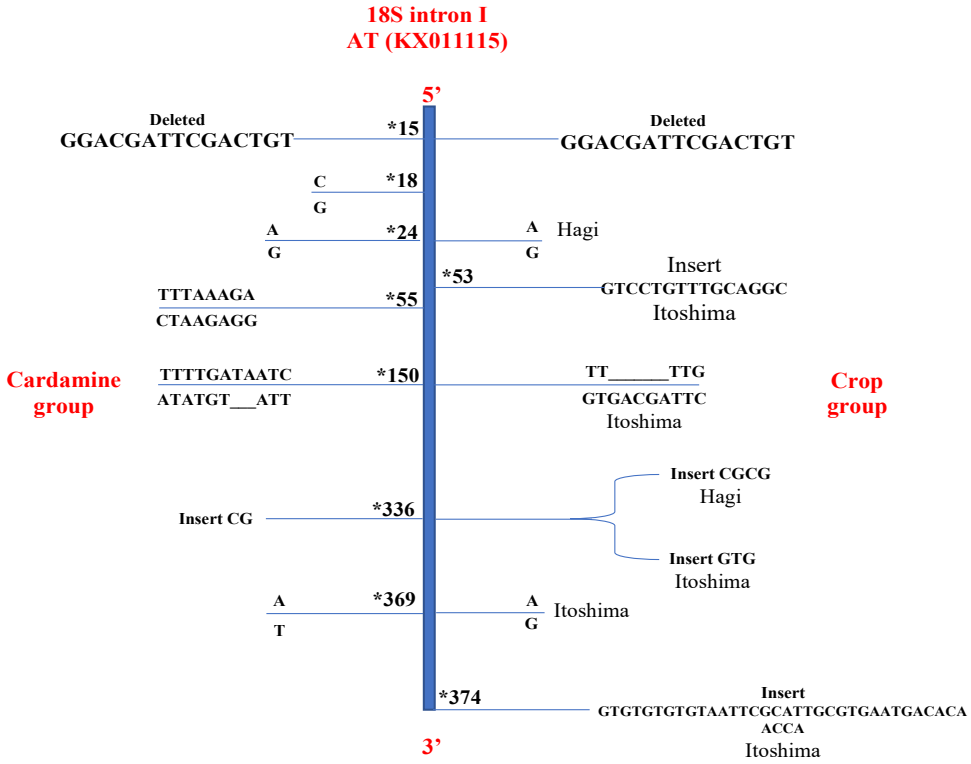

Figure 3. The scheme of intraspecific variability acquired in the 18S intron I from the examined populations of two groups, the *Cardamine* and the crop groups, designated based on the 18S intron I of the AT isolate (KX011115). Numbers with * describe the positions' nucleotides originating in KX011115. Some mutated clusters of nucleotides and single point mutations are demonstrated beneath the lines (where above the lines is the original sequence of KX011115), and an underline among the clusters denotes the deleterious nucleotides.

The *Cardamine* group had several deletions outnumbering the insertions, resulting in a shorter size (359 bp) compared to the standard size (374 bp) of the 18S intron I. These polymorphic InDels appeared at similar positions that the mutations of the Itoshima and Hagi populations occurred (Figure 3). The *Cardamine* group also had the intronic upstream deletions and nucleotide shifting, as well as transversion point mutations at the downstream where flanking regions, including the splicing sites, start. The 7-nucleotide cluster of the *Cadarmine* group positioned at the 150 site (Figure 3) was mutated (TATATGT/TTTTTGA-*Cardamine*/crop AT, mutations described in bold letters), mimicking the flanking in the downstream (positioned from nucleotide 384 to 391) of the cosmopolitan isolates. Meanwhile, at that same flanking region of the *Cardamine* group, a single polymorphism (A/T) occurred (TTTATGA/TATATGT-*Cardamine*/crop). These phenomena recurred regularly when we observed the polymorphisms in the introns of the 18S regions. For instance, the terminal flanking of the 18S intron III from the Hagi population had a single point mutation (transversion T/C, TACACG/TATACG); this 5-nucleotide cluster was found in the midstream positions (362–366, based on AT) where it was mutated (T----G/CACACG, deleted nucleotides denoted by dash lines) in the Hagi population. This extended mutated cluster (AT----GTGTGT) coincided with the terminal flanking of the 18S intron I from the Itoshima population.

3.2.2. Large Subunit (28S) rRNA Gene

The crop group remained intron-positive with two introns remaining in the LSU region. In contrast to the crop group, the *Cardamine* group had only a single intron, 28S intron I, following a similar pattern of the SSU region (Figure 4). Of the *Cardamine* group, only the Nara population had two introns (18S intron I and 28 intron I) from both the

SSU and LSU regions; other populations (Kumamoto, Yamaguchi, Nagasaki, Akiota, and Togochi) possessed only a single intron for the entire rRNA genes. The 28s intron I of the *Cardamine* group had a more divergent length and intraspecific variability than any of the introns from the 28S regions of the crop group (Figure 5).

Comparative nucleotide sequence analysis showed that the *Cardamine* group shared a partially homologous sequence of approximately 438 bp respective to the crop group, with an 87.7% sequence identity between the two groups. After the homologous part shared with the crop group, the *Cardamine* group had extra sequences added which make them longer introns than the 28S intron I of the crop group (Figure 5). Among the *Cardamine* group, the longest intron was located in the Hiroshima populations (Akiota and Togochi) owing to the 766 bp additional sequence harboring unique ORFs (identified by MFannot), while the shortest intron (561bp) was located in the Nara population. The extra sequences harbored in all of the populations (Yamaguchi, Nara, and Kumamoto) greatly varied in size but commonly showed tracts or repetitive elements similar to the extra sequences of the Hiroshima populations. For instance, the Kumamoto population had the same downstream sequence (77 bp) of the Hiroshima populations, bearing a repetitive element (CA tracts).

The 28S intron I of the crop group was more homogenous than that of the *Cardamine* group in terms of size polymorphism and InDel acquisition. Most of the tested populations had identical sequences to the AT isolate, except for a single nucleotide deleted at the 5′ upstream of the exon–introns, suggesting, similar to in the 18S, that it might be the haplotype of the *P. brassicae* affecting crops in Japan. The Hagi population, as mentioned above, accumulated a plethora of polymorphisms not only in the 28S intron I but also in the intron II.

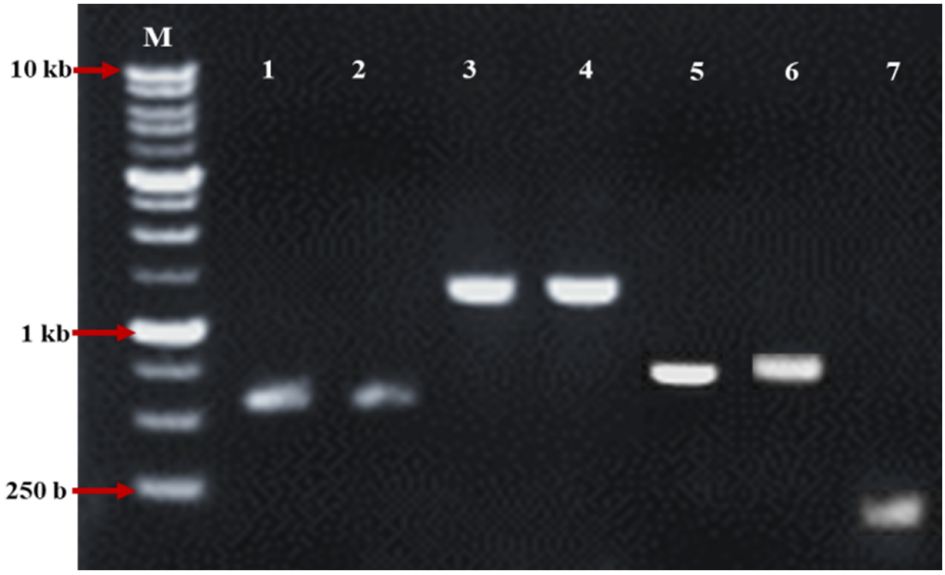

Figure 4. PCR products demonstrate the variable DNA sizes of the *Cardamine* populations. Lane M represents the standard ladders (arrows pointed at the 10 kb, 1 kb, and 250 bp). Lane 1: positive control, 501 bp 28S intron I. Lanes 2–7: Nara, Akiota, Togochi, Kumamoto, Yamaguchi, and Nagasaki, respectively.

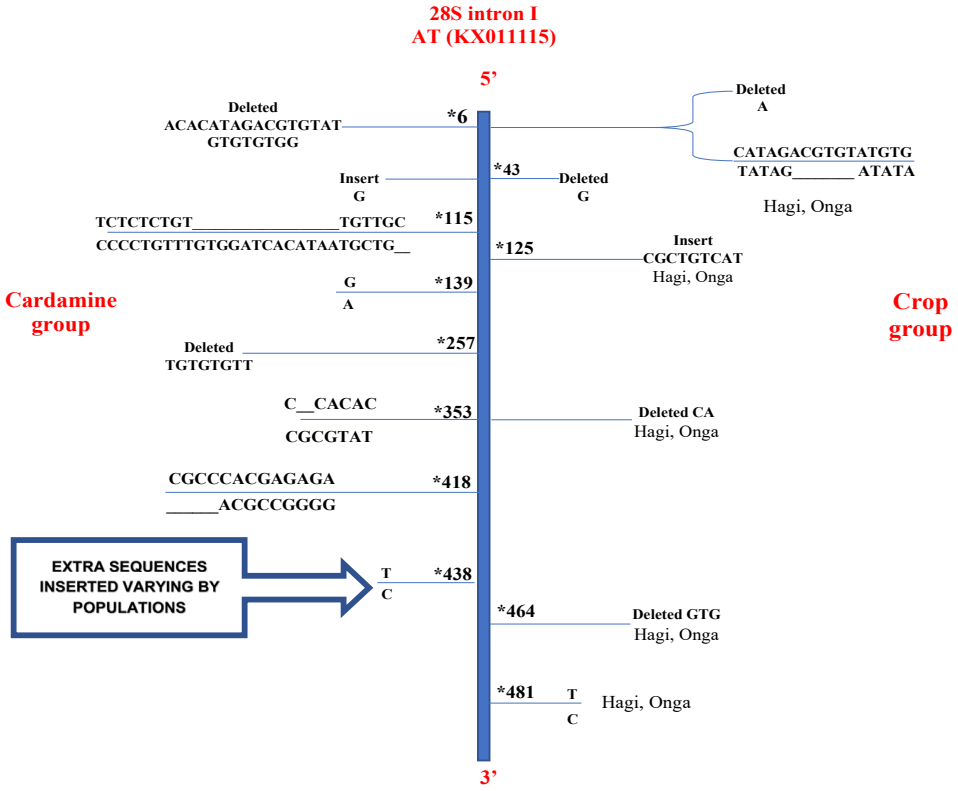

Figure 5. The scheme of intraspecific variability acquired in the 28S intron I from the examined populations of the two groups, the *Cardamine* and the crop groups, designated as the standard of the 28S intron I of the AT isolate (KX011115). Numbers with * describe the positions' nucleotides originating in KX011115. Some mutated clusters of nucleotides and single point mutations are demonstrated beneath the lines (where above the lines are the original sequences of KX011115), and an underline among the clusters denotes the deleterious nucleotides.

Another deviated population, the Onga population, also shared similar mutations acquired in both intronic sequences (28S I and II) with the Hagi population but had fewer polymorphisms. More interestingly, the Onga population had a point mutation (inserted a nucleotide G) at the exon flank (TACCACAGGGG) where the intron/exon boundary meets. This "exon flank" cluster is also repeated at the 5′ upstream of the 28S intron II (Table 2). It is noteworthy that *P. brassicae* from *Capsella bursa-pastoris* (shepherd's purse weed) had the 28S intron II sequence homologous to the Hagi and Onga populations due to similar variations (Table 2).

Table 2. The polymorphisms acquired in the 28S intron II identified in the aberrant populations from the crop group (Hagi and Onga) and from the cruciferous weeds *Capsella bursa-pastoris*. Numbers with * describe the positions' nucleotides originating in KX011115.

	Mutated Positions Identified in 28S Intron II					
AT isolate (KX011115)	30	45	200	282	342–345 *	345–347
	C	G	G	G	ATC	ATA
Capsella bursa-pastoris	G	G	A	deleted	deleted	ATA
Hagi and Onga	G	T	A	deleted	ATC	deleted

4. Discussion

In this study, we focused on the pattern of a marked increase and decrease in the introns of the rDNA in the *Cardamine* group and on the rDNA introns with characteristic polymorphisms among geographic *P. brassicae* populations.

4.1. Early or Late Theory for the Intronic Distribution in P. brassicae?

The retention and loss pattern has occurred frequently in the genetic basis of various species across the eukaryotic evolution [13]. Debate has been heated between the two contrasting theories of intron distribution, "early vs. late" [14]. The first theory, called the early intron theory, hypothesizes that the ancestral genomes contained the DNA segments that played a vital role in protein synthesis, and during the evolutionary process these segments were completely removed in prokaryote lineages but were partially retained as intragenic sequences known as introns in eukaryotic biology [15]. The point of view opposed to the early intron theory argues that introns are vestigial, originating from parasitic DNAs that have entered the host genome through the evolutionary host–parasite relationship; the introns are thus obliged to splice themselves out during protein synthesis in an effort to remain in the host genome, a survival tactic that compromised the host genome [16]. Over the years of this long debate, these hypotheses have complemented one another, resulting in a weaker version of each [16].

The rDNAs of the *P. brassicae* populations varying in the two groups (the *Brassica* crop and *Cardamine*) can be used as an example for this circumstance. For the first theory, several populations of the crop group (from this study and Laila et al. [9]) were reported to have lost 18S intron I, which is the first piece of evidence, and a more increasing body of evidence comes from the *Cardamine* group whose introns in the rDNA have been gradually eliminated in large quantities. On the other hand, the rDNA of *P. brassicae* has traces or remnants that facilitate the intronic insertions that corroborate the late intron theory. As observed in the ribosomal introns of the *P. brasicae* AT isolate (KX011115), the introns had conserved tracts (7–10 nucleotides) that flank the upstream of subsequent exons. For instance, the 18S intron III had nine nucleotides (TTCCGTAGG) that flank the last exon of the SSU; likewise in the LSU region, the 28S introns I and II had 7-nucleotide (CTCTTAA) and 10-nucleotide (TACCACAGGG) sites, respectively, that repeat the successive exons. These tracts, known as "proto-splice sites," are considered to be relics from the signaling sites to initiate the intronic insertion [17].

In addition to the recognition sites, the intronic ORFs harbored in the 28S intron I of the *Cardamine* group might perform as transposable-like elements, also known as retrotransposons, that insert themselves into the intronless sequences and genes. This phenomenon might explain the abundance of introns in the *Brassica* crop group due to the copy–paste mechanism operated by these putative retrotransposons. To understand the insights of the massive intron loss and gain occurring in the Japanese *P. brassicae* populations and answer the question of which intron models suit the case of *P. brassicae* introns, comparative genomic analysis should be deployed on the population genetic diversity of both groups. The main target is the *Cardamine* group which has been proven to have a highly diversified genetic material as well as intron distribution and variability. Indeed, only five populations were tested in this research, yet there existed three patterns of intronic distribution for the *Cardamine* group (either only one intron harbored in the SSU (Nagasaki, Japan) or the LSU (Kumamoto, Akiota, and Togochi from Hiroshima, Yamaguchi, Japan) or two for each region (Nara, Japan)). It is also worth mentioning an exceptional case that one *P. brassicae* population extracted from the crop group was confirmed intron-negative for the entire rDNA region. This case might appear as a contamination of DNA extraction, as reported by Schwelm et al. [8]. Indeed, the nature of the parasite–plant relationship renders the DNA purification difficult, and even more difficult for the wild weeds due to the contamination of other free-living protists and unidentified pathogens, and, more laboriously, the task of culturing the weeds. Nevertheless, the possibility of intron-void rDNAs in the *Cardamine* group is highly predictable based on the intron

early theory that advocates the phenomenon of intron loss for the entire genome in the eukaryotic evolution [18–20]. This phenomenon is actually not uncommon, particularly for a unicellular obligate parasite such as *P. brassicae*, if we take its close relative, *Spongospora subterranea* f. sp. *subterranea*, also known as biotrophic pathogens, as empirical evidence for being intron-poorer than *P. brassicae* as well as intron-negative in the rDNA regions [21].

4.2. The Intronic Evolution Equates to Speciation Event of P. brassicae Populations?

The goal of this study was to illustrate the speciation events from the perspective of intron evolution, especially for the *Cardamine* group, framed by the early vs. late theories. The stark contrast between the *Cardamine* group and the crop group has proven the evolutionary dynamics of introns occurring in the *P. brassicae* populations. The mechanism behind the intron dynamics might imply a correlation or association with the event of the host shifting (or switching) from the domesticated crops to the wild weeds.

Although *C. occulta* is a crucifer, its genetic material, life cycle, and conditions related to growth and reproduction are entirely disparate to those crucifers of the domesticated crops. During the process of domestication, the genomes of the *Brassica* crops have been artificially modified by selecting the desired genes [22]. By contrast, the genome of *C. occulta* weeds, considered the most dispersible of *Cardamine* species [23], accumulates a plethora of genes and genetic variations that are able to confer the adaptive advantages to a variety of niches [24,25]. The host–parasite dynamic requires the alteration of both genotypes and phenotypes alternatively between the host and pathogen [26], meaning that the pathogenic genome might be synergistic with its hosts to increase its survival opportunity [27]. In this way, the genetic variations acquired by the pathogens infecting the domesticates might not be as enriched as those acquired through infecting the wild weeds due to the less diversified genome of the domesticates.

Schwelm et al. [8] stated that the rDNA sequences of *P. brassicae* were not proper candidates for isolate or pathotype differentiation due to no genetic variation being detected in the LSU region. On the contrary, our results demonstrated that the pathogenic *P. brassicae* of the *C. occulta* weeds acquired highly genetic variations. The octoploid genome of *C. occulta* (2n = 8x = 64) is the product of a double duplication event [28] which can be construed as an advantageous factor for the evolutionary process and diversification [25,29]. This genomic plasticity of the polyploidy host has the potential to confer new DNA elements to the parasitic recipient genome and vice versa. This suggests that the underlying mechanism of intron diversification separates the two groups, and, in addition, perhaps this disparate diversification propels the mode of speciation operated in those weed-infecting *P. brassicae* lineages.

4.3. Prospective Research for Intragenic Sequences of P. brassicae

The genome of *P. brassicae* was analyzed and found to be compacted by principally decreasing intergenic spaces as well as gene losses for the purpose of being entirely reliant on intracellular resources from hosts [30]. Despite that, *P. brassicae* was still evaluated as an intron-rich parasite [21,31], and it is the rRNA genes that are particularly intron-dense. As hypothesized above with the host-shifting event, the intron dynamics regarding the numbers and patterns are segregated in the two groups, but do the tertiary structures and functions of those introns exclusively play a role for the *P. brassicae* lifestyle, such as being involved in the parasitic or survival strategies?

The ribosomal introns are considered more diverse and dominant due to their functions being essential for gene regulation, particularly for ribosomal protein genes in gene expression and diversification of the protein repertoire [32]. The intron prevalence of the crop group may reflect the upregulated genes and protein biosynthesis due to the stability of the resources provided from domesticates. Lim et al. [33] suggested that intron-present genes are more enhancing in transcription and translation than the intron-absent ones. Hence, the introns established in the crop group are plentiful to facilitate that parasitic lifestyle wherein they can reproduce and multiply with ease. In contrast to the number

of introns in the *Cardamine* group, there were merely one or two introns scattered sporadically in the entire rDNA region, but these polymorphic introns carried the putative mobile elements which have the flexibility to insert themselves into intron-less alleles or novel sites on demand, i.e., the aforementioned proto-splice sites located in the rDNA. The flexibility offered by the spliceosomal activities may be strategic for the survival and reproduction of the pathogens in the face of various environmental circumstances which are not beneficial to the hosts [34]. For example, two samples (Akiota and Togochi, Yamagata, Japan) from Hiroshima collected from the remote mountainous areas had different patterns and extra ORFs compared to the other four samples (Nara, Nagasaki, Yamaguchi, and Kumamoto, Japan) from the *Cardamine* group. Another example of the variable ribosomal intronic regions is *P. brasicae* from *Rorippa palustris* (marsh yellow cress) (LC716109). The *P. brassicae* population had a similar pattern of intronic absence found in many members of the *Cardamine* group. This *P. brassicae* population was also lacking four introns of 18S (introns II and III) and 28S (introns I and II), except in the case of the 18S intron I. The 18S intron I sequence of this population was nearly homologous to the 18S intron I sequences of the *Cardamine* group, but this 18S intron I harbored an additional sequence with the intronic ORF (approximately 550 bp) (Table S1). These examples clarify the flexibility and adaptability of intronic regions that vary according to the environmental conditions (within one group of hosts for *C. occulta*) or by shifting to a different host, i.e., the weed *R. palustris*. In addition to that, the splicing sites of these spliceosomal introns are not canonical; sites such as TC/GT-TG were commonly found in the *Cardamine* group or even in the crop group. Such non-canonical sites are distinguished from the majority (nearly 99% of commonly found introns) by having GT at the donor site and AG at the acceptor site [35]. They might have certain metabolic functions in *P. brassicae* since the non-canonical splicing sites were known for contributing to diversifying proteomic profiles and being involved in exitrons and cryptic introns, which are responsible for atypical splicing mechanisms [35].

The pathogenicity of the pathogens *P. brassicae* has been known for its capability of constant change, but research on the molecular linkages has been to no avail due to the complication of pathotyping systems as well as the mixture of several strains in a single gall [8,36]. Nevertheless, we found some common mutations at both structural sites of the introns or the same polymorphic patterns between the two groups or even within one group, suggesting that each *P. brassicae* population possesses the same introns. This may indicate at least the emergence of novel pathotypes or virulent isolates at the genetic population level. Last but not least, the power of self-splicing introns could also provide clues for the sexual reproduction of *P. brassicae*, which is still under debate [37], based on the fundamental structures or motifs inherited.

Overall, the role of introns in the life cycle of *P. brassicae* and clubroot disease progression remains indisputable, but how their dynamics and evolution are characterized in *P. brassicae* is an area requiring more research.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy12092154/s1. Table S1: The 18S and 28S ribosomal DNA sequences of *Plasmodiophora brassicae* studied in this paper; Table S2: Primers of the exonic and intronic regions, 18S and 28S ribosomal DNA sequences, and PCR condition.

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