Title: Complete structure of nuclear rDNA of the obligate plant parasite *Plasmodiophora brassicae*: intraspecific polymorphisms in the exon and group I intron of large subunit rDNA

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Abstract

Plasmodiophora brassicae is a soil-borne obligate intracellular parasite in the phylum Cercozoa of the Rhizaria that causes clubroot disease of crucifer crops. To control the disease, understanding the distribution and infection routes of the pathogen is essential, and thus development of reliable molecular markers to discriminate geographic populations is required. In this study, the nuclear ribosomal RNA gene (rDNA) repeat unit of *P. brassicae* was determined, with particular emphasis on the structure of large subunit (LSU) rDNA, in which polymorphic regions were expected to be present. The complete rDNA complex was 9513 bp long, which included the small subunit, 5.8S and LSU rDNAs as well as the internal transcribed spacer and intergenic spacer regions. Among eight field populations collected from throughout Honshu Island, Japan, a 1.1 kbp region of the LSU rDNA, including the divergent 8 domain, exhibited intraspecific polymorphisms that reflected geographic isolation of the populations. Two new group I introns were found in this region in six out of the eight populations, and the sequences also reflected their geographic isolation. The polymorphic region found in this study may have potential for the development of molecular markers for discrimination of field populations/isolates of this organism.

Keywords

Cercozoa, divergent domain in LSU rDNA, group I intron, intraspecific polymorphism, nuclear rDNA, *Plasmodiophora brassicae*

Introduction

Plasmodiophora brassicae is a soil-borne plant pathogen that causes clubroot disease of crucifers. Although it was traditionally classified as a fungus, recent molecular phylogenetic analyses of the small subunit (SSU) ribosomal RNA (Castlebury and Domier 1998; Cavalier-Smith and Chao 1997), actin and polyubiquitin genes (Archibald and Keeling 2004) suggest that it belongs to the protist phylum Cercozoa in the kingdom Rhizaria (Archibald and Keeling 2004; Cavalier-Smith and Chao 2003). Within the Cercozoa, the order Plasmodiophorida has attracted particular attention because it includes not only *P. brassicae* but also several plant pathogens of worldwide importance, such as *Spongospora subterranea*, which causes potato powdery scab disease, and *Polymyxa* spp., which transmit various plant viruses (Braselton 1995; Bulman et al. 2001). Information about the genomic structure of plasmodiophorids, however, is limited due to their nature as obligate intracellular parasites.

Pathotypes of *P. brassicae* have been characterized from sets of different hosts (Buczaki et al. 1975; Hatakeyama et al. 2004; Kuginuki et al. 1999; Somé et al. 1996; Williams 1966), but this process is time-consuming, labor-intensive, and subject to varying environmental conditions. Identification of the geographic populations/isolates of *P. brassicae* is also important to understand the distribution and infection routes. Therefore, development of reliable molecular markers that enable rapid identification of the pathotypes and geographic populations is required. For this purpose, molecular characterization of the genome is essential. Recently, Bulman et al. (2007) compared the nucleotide sequences of 24 genes in the *P. brassicae* genome with those of the corresponding cDNA and found that the genome was rich in spliceosomal introns. Random amplified polymorphic DNA (RAPD) analysis on *P. brassicae* populations that differed in pathogenicity revealed that the genome was highly polymorphic among the populations, although correlations between the pathogenicity and RAPD patterns were not clear (Manzanares-Dauleux et al. 2001; Osaki et al. 2008). In *Polymyxa graminis*, on the other hand, sequence variation in the two transcribed spacer (ITS1 and ITS2) regions of the rDNA were observed among the geographical populations and also among those with different host ranges (Legréve et al. 2002). In contrast, there was no sequence variation in the ITS sequences of *Spongospora subterranea* among Australasian and European populations, except for one from Scotland (Bulman and Marshall 1998). Among *P. brassicae* populations, however, little information about sequence variation among geographic populations of particular genes is available so far.

In eukaryotes, large variation in length and sequence have been reported in the

large subunit (LSU) rDNA. Twelve divergent domains that showed sequence variability in the LSU rDNA were first identified in mouse (Hassouna et al. 1984). These domains have been employed for identification and/or detailed characterization of species/isolates in a range of organisms (e.g., Chenuil et al. 2008; Lachance et al. 2000). The intergenic spacer (IGS) region of rDNA repeats in eukaryotes also shows extensive sequence variation and has been used widely as a molecular marker in the population biology of pathogenic fungi (e.g., Chang et al. 2008; Jackson et al. 1999; Pantou et al. 2003; Pramateftaki et al. 2000). These observations led us to expect that several polymorphic regions would be present in the unexplored region of rDNA of *P. brassicae*, although no sequence information is currently available. In the present study, the complete structure of the rDNA repeat unit of *P. brassicae* was determined, with particular emphasis on polymorphisms among field populations of different geographic origin.

Results

Structure of the ribosomal RNA gene repeat unit

Clubroot galls formed on *B. rapa* var. *pekinensis* grown in an experimental field of Nagoya University were harvested and designated as population NGY. To determine the complete sequence of the rDNA repeat unit of this population, spores were purified from several galls, and then genomic DNA was extracted. Seven DNA fragments were amplified with the primer pairs Pbr1/Pbr2r, Pb121/Pbr4r, NS7/ITS4, Pbr4/NDL22, NDL22f/IGS1r, V282/Pbr1r, and IGSa-10f/Pb121r (Supplemental Table 1 and Fig. 1a), cloned, sequenced from both ends of each PCR product, and assembled. The total length of the rDNA repeat unit was 9513 bp. The lengths of the SSU rDNA, internal transcribed spacer 1 (ITS1)-5.8S rDNA-ITS2 region, LSU rDNA, and IGS region were 3105, 465, 3611, and 2332 bp, respectively, based on sequence alignment and comparisons to those of other eukaryotes. Three introns in the SSU rDNA that were described in Castlebury and Domier (1998) were named Pbr.S516, Pbr.S943, and Pbr.S1506, with reference to the corresponding positions in *Escherichia coli* rDNA. To examine the presence of introns in the LSU rDNA, four PCR fragments that covered 3145 bp of the LSU rRNA were amplified with the primer pairs LRP4/NDL22, NDL22f/28s1r, 28s1/28s3r, and 28s3/P282r using cDNA that was reverse-transcribed from RNA as template and sequenced (Supplemental Table 1 and Fig. 1b). Comparative analysis between sequences of the rRNA and rDNA revealed that no intron was present

in the LSU rDNA of this population. The IGS region located between 8168–8429 nt contained five complete copies of a 14 bp palindrome (Fig. 2).

Polymorphisms among geographical populations

Because a preliminary database search revealed that little sequence variation was present in the 3.6 kbp genomic region consisting of SSU rDNA and ITS, including the three introns in the SSU rDNA, among the geographical populations across continents (those from Japan, Korea, Australia, and the UK; GenBank accession numbers AB094977, AF353997, AF231027, and Y12831, respectively; data not shown), we focused on the LSU rDNA and IGS regions thereafter and tried to obtain sequence information on these regions from several geographic populations from Japan. Although the amplicon for the IGS region was not obtained from any of the populations with the primer combinations used to amplify this region of the NGY, several parts of the LSU rDNA were successfully amplified and aligned. Among these, a PCR product amplified with the 28s1/28s3r primer set were found to be approximately 300 bp longer in some populations than in NGY and showed extensive sequence polymorphisms. Therefore, this region of the eight field populations collected from throughout Honshu Island (Fig. 3) was amplified and cloned. Sequence analysis on three to four clones raised from each of the populations revealed that six (Akita, Fukushima, Shiraoka, Matsuzaka, Hagi, and Hiroshima) out of the eight populations possessed two different introns at positions after 1440 or 1472 nt of the LSU rDNA of NGY, which were named Pbr.L1064 and Pbr.L1094, respectively, with reference to the corresponding position of *E. coli* rDNA (Fig. 1c). The Pbr.L1094 introns were found in all six populations, whereas the Pbr.L1064 intron was found only in two out of the three clones of the Matsuzaka population. Neither of the introns was found in the Yokosuka and NGY populations. Further alignment analysis on the 1.1 kbp exon sequences of this region revealed that extensive sequence polymorphisms were concentrated in the divergent 8 (D8) domain (Hassouna et al. 1984), which starts from 2135 to 2326 nt of the LSU rDNA of the NGY (Fig. 1c): nucleotide substitutions among the eight populations occurred in 65 positions within the D8 domain that is 192 bp in length, while that in the remaining 965 bp 5'-upstream of the D8 domain was observed only in 69 positions (Fig. 4a). Secondary structural analysis on the domain indicated that the region formed a typical Y-shaped structure of the D8 domain, with D8a and D8b helices (Michot and Bachellerie 1987) (Supplemental Figure 1), and that most of the sequence polymorphisms occurred in the D8b helix (Fig. 4b).

Based on the fact that the D8 domain harbors intraspecific polymorphisms, three to four clones of the 1.1 kbp exon sequences of LSU rDNA, including the domain (Fig. 1c) from each of the eight field populations, were subjected to phylogenetic analysis (Fig. 5). All of the geographic populations of *P. brassicae* formed a robust cluster, showing close relationships with members of the phylum Cercozoa, including *Cercomonas* sp., *Paracercomonas marina, Cercozoa* sp., *Protaspis grandis*, and *Bigelowiella natans*. Even though this analysis was performed on the exon sequence, the eight *P. brassicae* populations were differentiated into two subclusters according to the presence/absence of the introns in the LSU rDNA, with high bootstrap values: the NGY and Yokosuka populations that harbor no intron, and those harboring either the Pbr.L1064 or Pbr.L1094. Generally, geographic isolation of the populations was well reflected in the tree topology, except for the Matsuzaka population, in which the three clones were split into two distinct clusters. In this population, one clone that harbored the Pbr.L1094 (a common intron present in six out of the eight populations) formed a cluster with the Shiraoka, Hagi, and Hiroshima populations, and the other two that harbored the Pbr.L1064 (a rare intron found only in this population) formed an independent cluster related to the Akita and Fukushima populations. It is also noteworthy that sequence polymorphisms were observed not only among the geographic populations but also within the individual populations.

Characterization and polymorphisms of the introns in large subunit rDNA

The Pbr.L1064 and Pbr.L1094 in the LSU rDNA were characterized as group I introns according to primary and secondary structural analyses, as follows: i) the occurrence of group I introns in the same positions is known in other organisms (Jackson et al. 2002); ii) the last exon and intron bases were uracil and guanine, respectively (Cech 1988); and iii) all of the paired elements P1–P10 (Burke et al. 1987; Cech et al. 1994; Michel and Westhof 1990) were found through the secondary structure predictions (Fig. 6a, b). Pbr.L1064 and Pbr.L1094 were further classified into subgroup IC1, which possesses a bulged nucleotide A in the P7 helix and the P2.1 and P5abc helices (Michel and Westhof 1990).

The LSU rDNA intron sequences of three clones from each of the six populations were subjected to phylogenetic analysis (Fig. 7). The type of introns and geographic isolation of the populations were also reflected in the tree topology to the same extent as in the phylogeny of the exon sequences of the partial LSU rDNA, i.e., the Pbr.L1064 found only in two clones of the Matsuzaka population formed an

191 independent cluster, whereas the Pbr.L1094 formed two small clusters of the Akita and
192 Fukushima populations, and one large cluster of the other populations, including a clone
193 of the Pbr.L1094 from the Matsuzaka population.
194
195 **Discussion**
196
197 ***Structure of nuclear rDNA repeat unit of* Plasmodiophora brassicae**
198
199 The complete nucleotide sequence of the *P. brassicae* nuclear rDNA repeat unit that
200 consisted of the three coding regions SSU, 5.8S, and LSU rDNA, interrupted by the
201 three non-coding regions ITS1, ITS2, and IGS, was determined in one of the field
202 populations in the present study. In the Rhizaria, sequence information on the complete
203 rDNA repeat unit including the LSU rDNA and IGS region is quite limited, although
204 that on the SSU rDNA and ITS regions of plasmodiophorids is abundant in the database.
205 Moreira et al. (2007) demonstrated that not only SSU rDNA sequences but also LSU
206 rDNA sequences are effective taxonomic markers for eukaryote phylogeny, especially
207 for unculturable organisms to which application of multi-gene analysis is difficult. Our
208 phylogenetic analysis based on the exon sequences of LSU rDNA confirmed that *P.*
209 *brassicae* belongs to the Cercozoa in the Rhizaria, as proposed by Archibald and
210 Keeling (2004) and Cavalier-Smith and Chao (2003), in which the sequences of actin
211 and polyubiquitin and that of the SSU rDNA were used as taxonomic markers.
212 The complete sequence of the rDNA repeat unit obtained in the present study is
213 actually a ‘conceptual assemblage’ of seven PCR fragments that may originate from
214 different genotypes/rDNA copies within the population NGY. However, we consider
215 that the nucleotide sequence, at least all except for the IGS region, would provide
216 sufficiently realistic information about a representative sequence of one particular
217 genotype/rDNA copy of the population for the following reasons: i) the SSU rDNA,
218 ITS, and 5' end of LSU rDNA regions that were covered by four PCR fragments (Fig. 1)
219 are highly conserved within the population, and ii) the rest of the LSU rDNA, in which
220 the newly found introns and D8 domain (the polymorphic regions) are present, were
221 covered by a 2.8 kbp single PCR fragment. The IGS region, however, was covered by
222 two fragments in which the polymorphic regions of the LSU rDNA was uncovered,
223 implying that there is a possibility that the sequences of the LSU rDNA and IGS were
224 from different genotypes/rDNA copies, if the IGS harbors polymorphisms within the
225 population. Establishing single spore isolates from the population and subsequent
226 analysis on the regions is required.

227 Two new self-splicing group I introns were found in the LSU rDNA region of
228 *P. brassicae*. Insertions of several group I introns into an rDNA repeat unit have been
229 reported in fungi (Gargas et al. 1995; Nikoh and Fukatsu 2001) and algae (Turmel et al.
230 1993). The fact that *P. brassicae* possesses five types of group I intron in the rDNA
231 repeat unit, three in the SSU, and two in LSU rDNAs supports the idea that its genome
232 is rich in introns (Bulman et al. 2007). Group I introns have been found in a diverse
233 range of higher organisms, including fungi, protists, and algae, where they occur in the
234 nuclear, mitochondrial, and chloroplast genomes (Cech 1988). An important insight into
235 group I intron evolution is that the introns inserted at the same site are phylogenetically
236 closely related (monophyletic) even though they reside in distantly related organisms
237 (Nikoh and Fukatsu 2001). In fact, the newly found introns Pbr.L1094 and Pbr.L1064
238 were phylogenetically distinct, suggesting that the acquisition processes of the two
239 introns might be evolutionarily distinct even though their insertion sites are quite close.
240
241 ***Intraspecific polymorphisms among the geographic populations***
242
243 The D8 domain in eukaryotic LSU rDNA is one of the most rapidly evolving divergent
244 domains that show extensive variability in length and sequence among species
245 (Hassouna et al. 1984). Intraspecific polymorphisms in the domain, however, have been
246 reported only in the ascomycete fungus *Clavispora opuntiae* (Lachance et al. 2000) and
247 the echinoderm *Echinocardium cordatum* (Chenuil et al. 2008). The present study
248 demonstrated that the protist *P. brassicae* also harbors intraspecific polymorphisms in
249 this domain. Chenuil et al. (2008) estimated the evolutionary rate of echinoderms based
250 on the nucleotide substitutions in the D8 domain and suggested that the D8a helix
251 evolved more rapidly than other parts of the domain, because most of the nucleotide
252 substitutions were concentrated within the D8a helix. In contrast, the substitutions
253 among the *P. brassicae* populations occurred mainly in the D8b helix, suggesting that
254 the helix is likely to evolve more rapidly in this organism.
255 The newly found introns in the LSU rDNA also showed intraspecific
256 polymorphisms, reflecting geographic isolation, in contrast to those in the SSU rDNA,
257 which are highly conserved within the species. Furthermore, it is of particular interest
258 that the phylogenetic relationships among the populations revealed by the intron
259 sequences were quite consistent with those revealed by the exon sequences that include
260 the D8 domain, even though the introns were located more than 600 bp upstream of the
261 domain. In addition, the presence/absence of introns, as well as the types of intron (in
262 the case of the Matsuzaka population) were also reflected in the sequences of the

domain. These results strongly suggest that the introns and D8 domain evolved in parallel within the individual populations and that acquisition/deletion of the introns rarely occurred during evolution.

Variation in the intraspecific sequences has been found in the IGS region of phytopathogenic (Pramateftaki et al. 2000) and entomopathogenic (Pantou et al. 2003) fungi, likely due to multiple insertions of perfect and/or imperfect copies and deletions. The IGS region of *P. brassicae*, however, was successfully cloned only in the NGY, suggesting that the region in other populations may also contain several palindrome sequences and AT-rich regions as found in the NGY, and that these primary structures might interfere with PCR amplification. Although it has been generally accepted that the IGS region has strong potential as a molecular marker for the population biology of eukaryotic microorganisms, its practical application to *P. brassicae* seems difficult.

The D8 domain and introns in the LSU rDNA showed sequence variation not only among the geographic populations but also within individual populations. These sequences were obtained from genomic DNA prepared from multiple galls collected from each of the populations, and under these conditions, two possible interpretations for the observed variation are considered. One is that the field populations of *P. brassicae* were actually heterogeneous, probably even in single galls, i.e., several genotypes coexist in the field (e.g., Manzanares-Dauleux et al. 2001; Somé et al. 1996; Xue et al. 2008). Another interpretation is that the individual populations consisted of homogeneous genotypes but several different rDNA copies coexist in their genomes, as found in Foraminifera, which also belong to the Rhizaria (Holzmann et al. 1996). Sequence analysis on the region of single spore isolates of *P. brassicae* will answer this question.

Conclusion

The present study provides new information about intraspecific sequence polymorphisms in the rDNA repeat unit of *P. brassicae*. The sequences of the D8 domain and newly found group I introns in the LSU rDNA showed extensive sequence variation, reflecting geographic isolation. The IGS region was also expected to harbor polymorphisms, although the sequence could not be compared among the geographic populations in the present study. We conclude, however, that using an exon sequence as a molecular marker for discrimination of field populations/isolates is more reliable and practical than using a long non-coding region such as IGS, because a short polymorphic region is generally located between conserved regions, for which reliable PCR primers

299 could easily be designed (e.g., the D1 and D2 regions of LSU rDNA that can be
300 amplified by universal primers have been extensively applied for the identification and
301 community analysis of glomeromycotan fungi, which are obligate plant symbionts; An
302 et al. 2008; Li et al. 2009; Pivato et al. 2007). Furthermore, the polymorphic regions
303 found in the present study may be applicable to the identification of geographic
304 population/isolates of other plasmodiophorid organisms. Further study to obtain
305 sequence information on this region from a range of organisms is required.
306
307 **Methods**
308
309 ***Collection of field populations and subculture conditions***
310
311 The clubroot galls of *P. brassicae* on Chinese cabbage (*Brassica rapa* var. *pekinensis*)
312 or cabbage (*B. oleracea* var. *capitata*) were collected from an experimental field of
313 Nagoya University (NGY) (Aichi prefecture, N35˚, E137˚) in 2003, from arable land in
314 Akita City (Akita prefecture, N39˚, E140˚) in 2009, Fukushima City (Fukushima
315 prefecture, N37˚, E140˚) in 2009, Shiraoka Town (Saitama prefecture, N35˚, E139˚) in
316 2009, Yokosuka City (Kanagawa prefecture, N35˚, E139˚) in 2009, Matsuzaka City
317 (Mie prefecture, N34˚, E136˚) in 2009, Hagi City (Yamaguchi prefecture, N34˚, E131˚)
318 in 2000, and Hiroshima City (Hiroshima prefecture, N34˚, E132˚) in 1993, and stored at
319 –80˚C (Fig. 3 and Supplemental Table 2). If nucleic acids extracted from the stored
320 galls were degraded, fresh material was obtained as described below. Resting spores,
321 prepared by homogenizing the frozen galls in water, were mixed with soil, and then *B.*
322 *rapa* var. *perviridis* cv. Komatsuna was grown on the soil in 2 L square plastic pots in a
323 multi-compartment greenhouse at 26˚C for 40 d. To avoid cross-contamination,
324 pathogens from different geographic origins were not cultured in the same compartment
325 of the greenhouse. The galls formed on the roots were harvested and subjected to
326 immediate DNA/RNA extraction.
327
328 ***DNA extraction***
329
330 The frozen galls were thawed in water, washed thoroughly under running tap water,
331 surface-sterilized with 70% (v/v) ethanol for 30 s, homogenized in sterilized water, and
332 filtered through eight layers of cheesecloth. The filtrate was centrifuged at 1450×*g* for
333 15 min. The pellet was washed twice with sterilized water, resuspended in a small
334 amount of sterilized water, layered on 10 mL Percoll (GE Healthcare Bio-Sciences,

Tokyo, Japan) in a 50-mL plastic tube, and centrifuged at 1010×*g* for 10 min. The supernatant was transferred to a new tube, mixed with 40 mL sterilized water, and centrifuged at 1450×*g* for 15 min. The resultant pellet was washed three times with sterilized water and resuspended in a small amount of sterilized water. The suspension was transferred to a 2 mL tube with an O-ring sealed cap (Yasui Kikai, Osaka, Japan) and centrifuged at 13200×*g* for 1 min. Then the pellet (resting spores) was frozen at –80˚C for 1 h, freeze-dried overnight, and ground in a Multi-Beads Shocker (Yasui Kikai, Osaka, Japan) with zirconium beads (0.5 mm in diameter) at 2500 rpm for 6 × 30 s at 30 s intervals. DNA was extracted from the resting spores using the DNeasy Plant Mini Kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions and stored at –30˚C.

PCR amplification, cloning, and sequencing

PCR amplification was performed in a 25 μL reaction mixture with the Expand High-Fidelity PLUS PCR System (Roche Diagnostics, Tokyo, Japan) containing 0.5 μM primers and 1 μL or 10 μL of the template DNA solution in the PTC-225 DNA Engine Tetrad thermal cycler (GMI, Minnesota, USA). An initial denaturation step of 94˚C for 2 min was followed by 30 cycles of denaturation, annealing, and extension as follows: 94˚C for 15 s, annealing for 60 s (the temperatures for each primer are listed in Supplemental Table 1), and 72˚C for 60 s in the first 10 cycles. The same parameters were used for the next 11–30 cycles, except that the 72˚C extension steps were lengthened to 10 s cycle^{-1}. After 30 cycles, the samples were incubated for an additional 10 min at 72˚C. The PCR products were analyzed on a 1.2% agarose gel in 0.5 × TBE buffer.

The PCR primers used to amplify different regions of the gene complex are shown in Supplemental Table 1, and their relative positions in the rDNA transcription unit are indicated in Fig. 1a and Fig. 1c. All primers used to amplify the rDNA transcription unit of the population NGY were designed based on the sequence information of *P. brassicae* SSU rDNA and those of LSU rDNA of other eukaryotes in the database. The primers used to amplify the LSU rDNA regions of the other populations were designed based on the sequence of the NGY determined in this study.

The PCR products were purified by the MonoFas DNA Purification Kit (GL Science, Tokyo, Japan) and cloned into the pT7Blue T-vector (Novagen/Merch, Tokyo, Japan) according to the manufacturer's instructions. The nucleotide sequences of three to four randomly chosen clones were determined via the dideoxy-sequencing method

371 using the BigDye Terminator v3.0 or v3.1 Cycle Sequencing Kit with the ABI PRISM
372 3100 Genetic Analyzer (Applied Biosystems, Tokyo, Japan). The GenBank accession
373 numbers of the sequenced clones are listed in Supplemental Table 2.
374
375 ***LSU rRNA sequencing***
376
377 Approximately 100 mg fresh gall was frozen with liquid nitrogen in a 2 mL tube with
378 an O-ring sealed cap and ground in a Multi-Beads Shocker with a metal cone at 2500
379 rpm for 6 × 30 s at 30 s intervals, during which the sample was cooled in liquid nitrogen.
380 Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen) according to the
381 manufacturer's instructions. First-strand cDNA was synthesized using AMV reverse
382 transcriptase (Roche Diagnostics, Tokyo, Japan) with the P282r or random primers
383 according to the manufacturer's instructions. The PCR primers used in this experiment
384 are shown in Supplemental Table 1, and their relative positions are indicated in Fig. 1b.
385 Detailed conditions for PCR amplification, cloning, and sequencing are described in the
386 section 'PCR amplification, cloning and sequencing.'
387
388 ***Sequence similarity and phylogenetic analyses***
389
390 Sequence similarity of the LSU rDNA among the different field populations was
391 analyzed using the AlignX program of the Vector NTI Suite 9.0 (Invitrogen, California,
392 USA) to obtain an overview of the distribution of variable regions in the gene. For
393 phylogenetic analysis on the LSU rDNA, the 1.1 kbp exon sequences including the D8
394 domain (Fig. 1c) and those of the corresponding region from other eukaryotes obtained
395 from the GenBank were employed. For analysis of the LSU rDNA introns,
396 approximately 150 nt sequences that form the conserved secondary structures of the
397 group I intron (from P3 to P7 elements, see Fig. 6) were employed. These datasets were
398 aligned using the ClustalX program (Thompson et al. 1997), and maximum likelihood
399 analysis with the GTR + SS and GTR + γ models was applied for phylogenies of the
400 LSU rDNA and introns, respectively, using the PAUP 4.0b software (Swofford 2002).
401 The reliability of the tree topologies were evaluated both from the bootstrap values
402 calculated from the maximum likelihood analysis with 100 replications (Felsenstein
403 1985) and from posterior probabilities calculated with Bayesian analysis using the
404 MrBayes 3.1.3 software (Ronquist and Huelsenbeck 2003).
405
406 ***Intron secondary structure prediction***

The secondary structures were predicted using the models proposed in Cech (1988) and Michel and Westhof (1990), and the structural elements were defined based on the nomenclature of Johansen and Haugen (2001) and from the standard secondary structure representation for group I introns (Cech et al. 1994). The individual elements (P1–P9 stem loops) were identified by comparison with available group I intron sequences from the Comparative RNA website at http://www.rna.ccbb.utexas.edu/ (Cannone et al. 2002) and drawn using the *mfold* web server at http://mfold.bioinfo.rpi.edu/ (Zuker 2003). The final structures were drawn using the XRNA software provided by the RNA Center, University of California, Santa Cruz, at http://rna.ucsc.edu/rnacenter/xrna/xrna.html.

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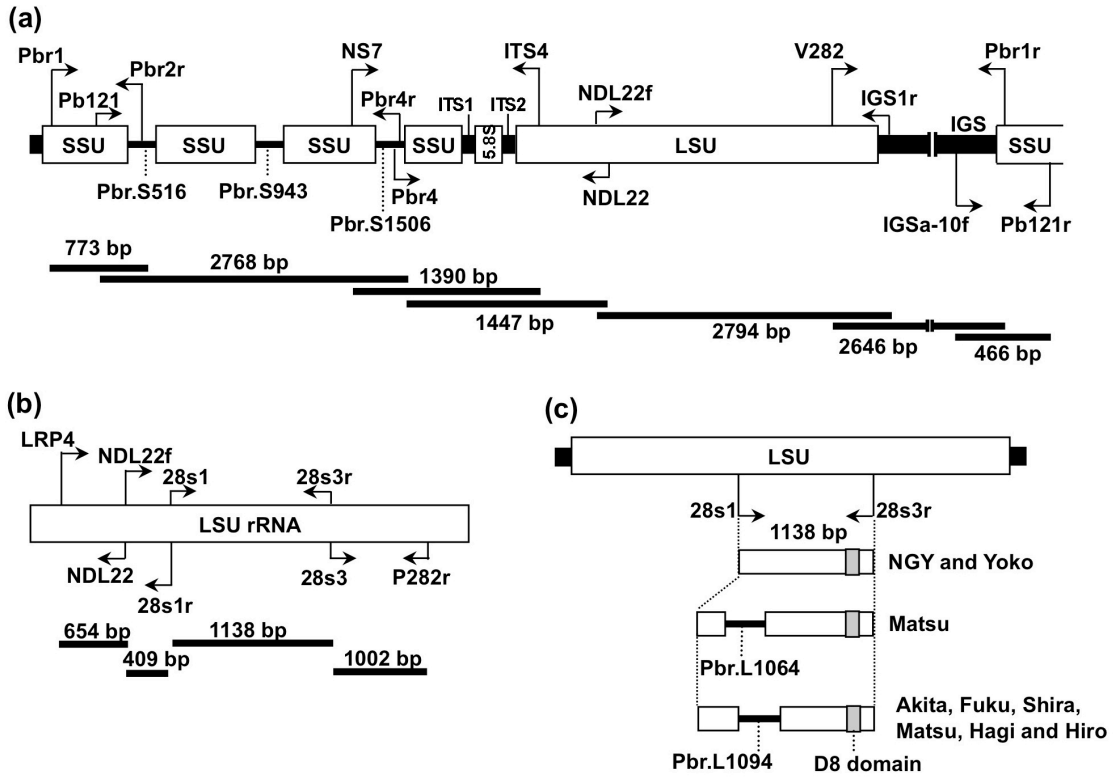

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568 Figure 1. a, Structures of the ribosomal RNA gene (rDNA) transcription unit of
569 *Plasmodiophora brassicae* population NGY and relative locations and directions of
570 PCR primers used to amplify the gene. Lengths of the PCR products are indicated. The
571 lines and open boxes indicate introns (Pbr.S516, Pbr.S943, and Pbr.S1506) and exons,
572 respectively. SSU, small subunit rDNA; 5.8S, 5.8S rDNA; LSU, large subunit rDNA;
573 ITS, internal transcribed spacer; IGS, intergenic spacer. b, Relative locations and
574 directions of the primers for reverse transcription-PCR to amplify the large subunit
575 ribosomal RNA (LSU rRNA) of population NGY. c, Locations of Pbr.L1064 and
576 Pbr.L1094 introns and divergent 8 (D8) domain in the LSU rDNA of *P. brassicae* field
577 populations. Pbr.L1064 was found only in the Matsuzaka (Matsu) population, whereas
578 Pbr.L1094 was found in Akita (Akita), Fukushima (Fuku), Shiraoka (Shira), Matsuzaka
579 (Matsu), Hagi, and Hiroshima (Hiro). The NGY and Yokosuka (Yoko) populations
580 possessed no intron in the gene. The relative locations and directions of the 28s1/28s3r
581 primer set are indicated.

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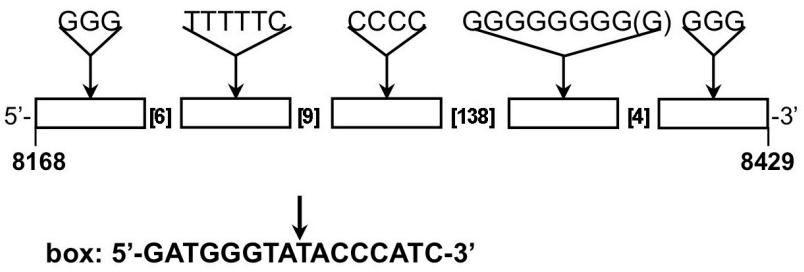

Figure 2. Schematic representation of the 14 bp palindrome sequences (box) found in the IGS region of *Plasmodiophora brassicae* population NGY. Arrows indicate the centers of palindromes interrupted by the repetitive sequences. Guanine in parentheses represents both the insertion and deletion clones observed. Numbers in bold at the 5' and 3' ends indicate nucleotide positions within the IGS region. Numbers in brackets represent the number of nucleotides between palindromes.

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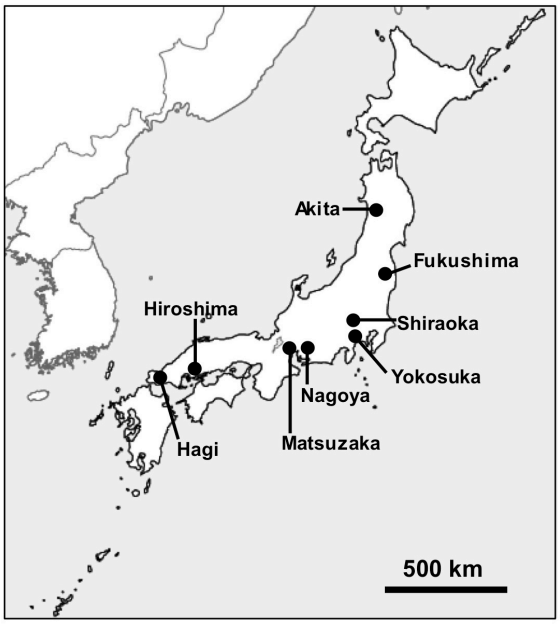

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603 Figure 3. Sampling sites of *Plasmodiphora brassicae* field populations on Honshu
604 Island, Japan.

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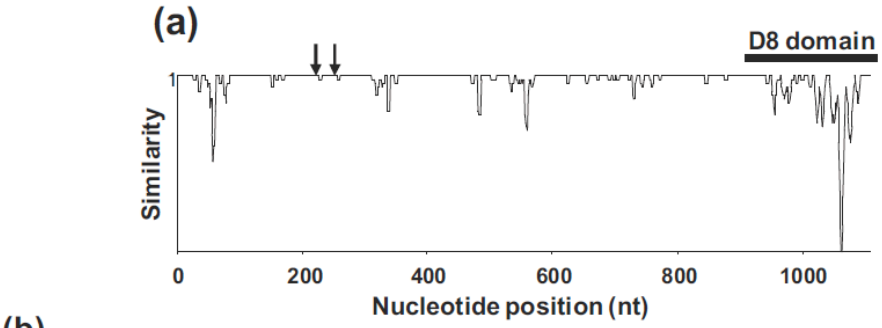

(b)

```
          1                                                          60
NGY-1     GGATTGGCTC TAAGGGTTGA GCGGATGGAG CCGTCGCTCT GAAGCTTGCG TCTAGGAGTG
Yoko-1    .......... .......... .......... ...AA....C .....CAAT. ..........
Akita-5   .......... .......... .T........ ...AT....C .....C..T. ....ATTT..
Fuku-4    .......... .......... .T........ .T.G.....C .....C..T. C....TTT..
Hagi-2    .......... .......... .T........ T..G.....C .....C..T. C....TTT..
Hiro-49   .......... .......... .T........ T..G.....C .....C..T. C....TTT..
Shira-7   .......... .......... .T........ T..G.....C .....C..T. C....TTT..
Matsu-3   .......... .......... .T........ T..G.....C .....C..T. C....TTT..
Matsu-2   .......... .......... .T........ ...G.....C ........T. C....TTT..
          61                                                         120
NGY-1     GCTTACTGTG GATGGCTCTC GGGCTGACTA CGGCGAGCTG CTCTCTGATG CAGGTGAACG
Yoko-1    .T........ .......T.. .......... T......... .......... .T........
Akita-5   ........C. .......T.. .........G ........CT TAA.....C. ..........
Fuku-4    ........C. .......T.. .........G ........CT TTAC...GCA TG........
Hagi-2    ........C. .......T.. .........G ........CT TGA....GCA T.........
Hiro-49   ........C. .......T.. .........G ........CT TGA....GCA T.........
Shira-7   ........C. .......T.. .........G ........CT TGA....GCA T.........
Matsu-3   ........C. .......T.. .........G ........CT TGA....GCA T.........
Matsu-2   ........C. .......T.. .........G ........CT TGA....GCA T.A.......
          121                                                        180
NGY-1     AGTAATGGTC ACGTGCCTGT GAGACGCAAG TTT-CACCAC TTCCTTCGCA AAGAACAACC
Yoko-1    ....TC.... ..GT.--.. C.-------- -.--G.TG.. .......... ..........
Akita-5   ..CGTC...T ..A.TA-.. .--GG....C C.ATT-T.T. ....GC.A.. ..........
Fuku-4    ...GC.A..T ..A.TA-.- ---GG....C CC.-----.T. ....GC.... ..........
Hagi-2    ....CC.A.. G.A.TA-TA AGAGG....C ...TTAT.T. ....GC.A.. ..........
Hiro-49   ....CC.A.. G.A.TA-TA AGAGG....C ...TTAT.T. ....GC.A.. ..........
Shira-7   ....CC.A.. G.A.TA-TA AGAGG....C ...TTAT.T. ....GC.A.. ..........
Matsu-3   ....CC.A.. G.A.TA-TA AGAGG....C ...TTAT.T. ....GC.A.. ..........
Matsu-2   ....CC...T ..A.T--T- ---GG....C C.------.T. ....GC.A.. ..........
```

Figure 4. Sequence polymorphisms in the LSU rDNA among the *Plasmodiophora brassicae* field populations. a, Distribution of sequence polymorphisms in the 1.1 kbp exon sequences of the LSU rDNA. Representative single clones from each of the eight populations were aligned, and the levels of similarity were scored in a 0–1 range at each alignment position using the AlignX program of Vector NTI. Arrows indicate the insertion sites of the Pbr.L1064 and Pbr.L1094 introns. b, Alignment of the D8 domain of the representative clones from each of the field populations using the AlignX program. Identical nucleotides and deletions with reference to the NGY-1 sequence are indicated by dots and dashes, respectively. The yellow, pink, and blue boxes represent nucleotide sequences that correspond to the basal, D8a, and D8b helices, respectively.

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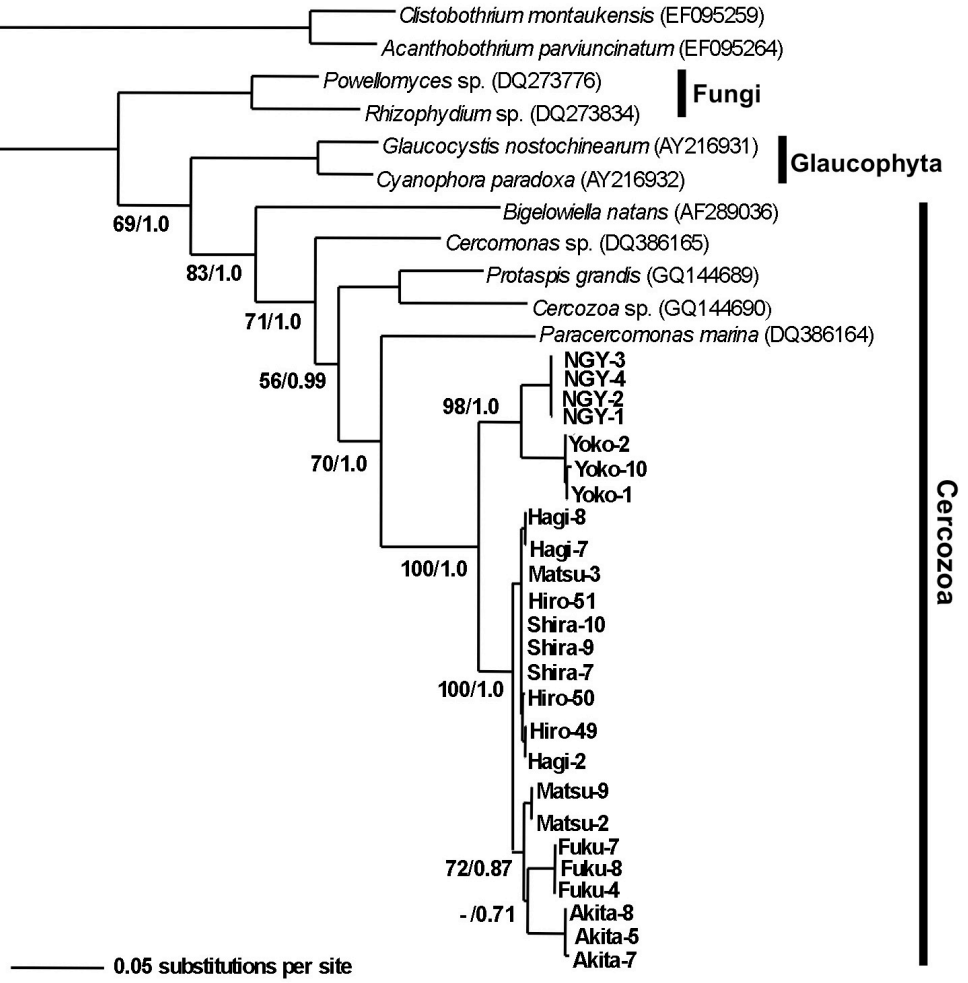

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620 Figure 5 Maximum likelihood phylogenetic tree of the 1.1 kbp exon of LSU rDNA
621 (corresponding to 1218–2322 nt of the NGY sequence) of *Plasmodiophora brassicae*
622 field populations NGY, Akita, Fukushima (Fuku), Shiraoka (Shira), Yokosuka (Yoko),
623 Matsuzaka (Matsu), Hagi, and Hiroshima (Hiro) with those of selected fungi and
624 glaucophyta in eukaryotes. The sequences from three or four randomly chosen clones
625 from each population were sequenced and subjected to analysis. Numbers at the
626 branches are maximum likelihood bootstrap values (left) and posterior probabilities
627 (right), and only those higher than 50% and 0.50, respectively, are shown. The
628 GenBank accession numbers of the sequences are shown in parentheses.
629 *Acanthobothrium parviuncinatum* and *Clistobothrium montaukensis*, classified as
630 Metazoa, were employed as the outgroup.

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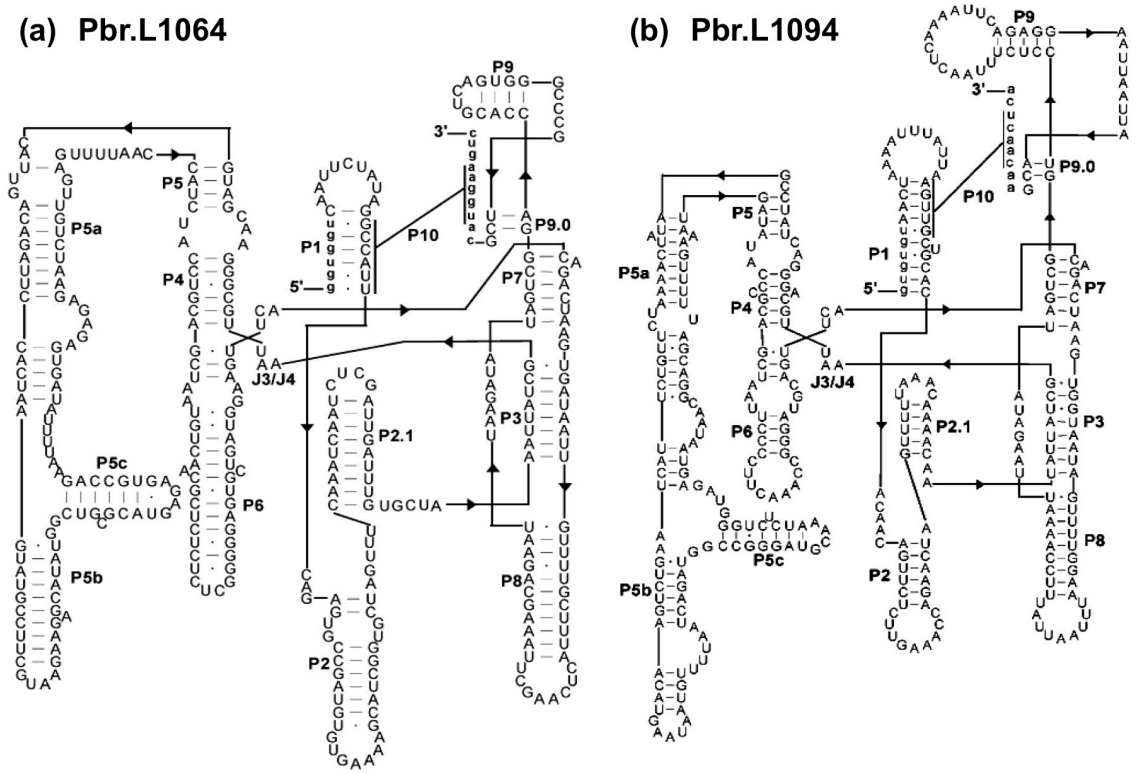

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635 Figure 6. Predicted RNA secondary structures of group I introns found in the LSU
636 rDNA of *Plasmodiophora brassicae*. The structures of Pbr.L1064 (a) and Pbr.L1094 (b)
637 introns were drawn based on sequences of the Matsuzaka (Matsu-2 clone) and Hagi
638 (Hagi-2 clone) populations, respectively. Uppercase and lowercase letters indicate
639 intron and exon sequences, respectively. The conserved paired elements of P1–P10 and
640 joining regions of J3/J4 are indicated.

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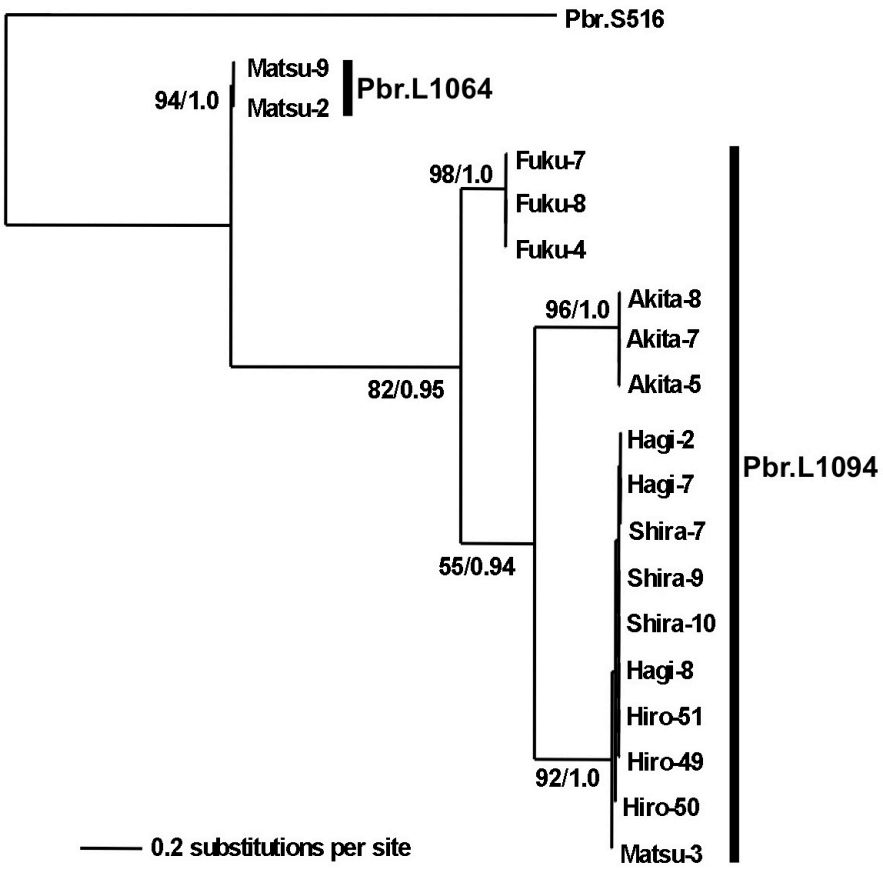


Figure 7. Maximum likelihood phylogenetic tree of the Pbr.L1064 and Pbr.L1094 introns in the LSU rDNA of the *Plasmodiophora brassicae* field populations Akita, Fukushima (Fuku), Shiraoka (Shira), Matsuzaka (Matsu), Hagi, and Hiroshima (Hiro). Numbers at the branches are maximum likelihood bootstrap values (left) and posterior probabilities (right), and only those higher than 50% and 0.50, respectively, are shown. The Pbr.S516 intron (subgroup IE) in the SSU rDNA was employed as the outgroup.

652

Supplemental table 1

PCR primers and annealing temperatures used for sequencing the ribosomal RNA gene repeat unit of *Plasmodiophora brassicae*.

Primer	Sequence (5'-3')	Annealing (˚C)[a]	Targets (source)
Pbr1	GGTTGATCCTGCCAGTAGTC	54	SSU rDNA (This study)
Pbr1r	GACTACTGGCAGGATCAACC	54	SSU rDNA (This study)
Pb121	GGATACAAAAACCAAACCTGGC	52	SSU rDNA (This study)
Pb121r	GCCAGGTTTGGTTTTTGTATCC	53	SSU rDNA (This study)
Pbr2r	CTCTATGCCCGAATCGCTTC	53	SSU rDNA (This study)
NS7	GAGGCAATAACAGGTCTGTGATGC	61	SSU rDNA (White et al. 1990)
Pbr4	GTGTCGCTTAAGATATAGTC	48	SSU rDNA (This study)
Pbr4r	GACTATATCTTAAGCGACAC	48	SSU rDNA (This study)
ITS4	TCCTCCGCTTATTGATATGC	50	LSU rDNA (White et al. 1990)
LRP4	CGTTGGAAGGCGGCATCA	54	LSU rDNA (This study)
NDL22f	CGTCTTGAAACACGGACCA	52	LSU rDNA (This study)
NDL22	TGGTCCGTGTTTCAAGACG	52	LSU rDNA (van Tuinen et al. 1998)
28s1	CCGGAGCTGAAACAGCTT	52	LSU rDNA (This study)
28s3r	AATTAAACAGTCGGATTCCCC	50	LSU rDNA (This study)
V282	GTGGGATAACGGCTGAACG	54	LSU rDNA (This study)
P282r	CGTTCAGCCGTAATCCTAC	52	LSU rDNA (This study)
IGS1r	GTTGATGTGTTGTAAGGGGTATG	53	IGS (This study)
IGSa-10f	GCATCACCTTAGCATTCGTTC	52	IGS (This study)

653 [a]Annealing temperature in PCR.

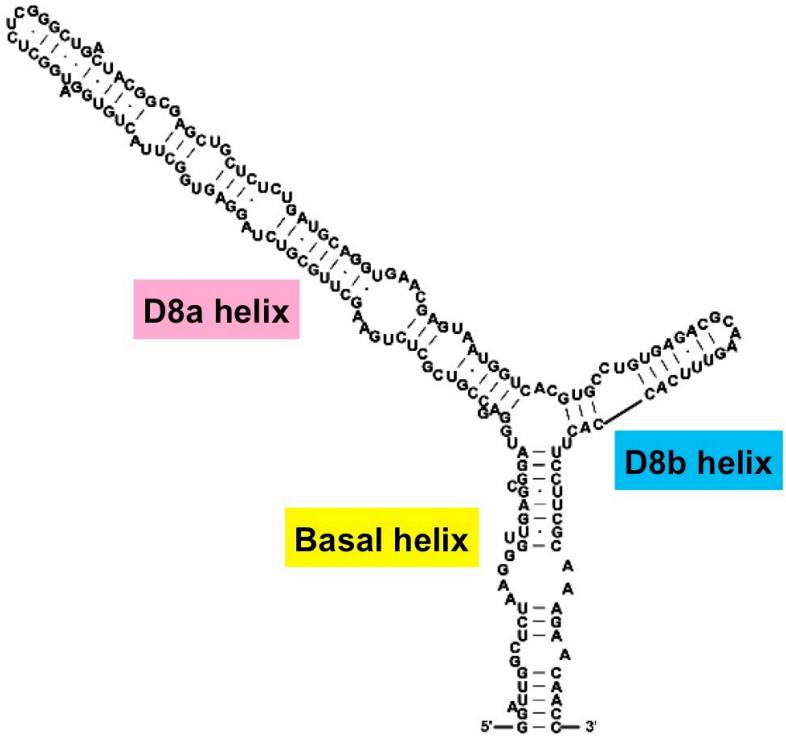
654

654

Supplemental table 2 655

GenBank accession numbers of the sequenced clones. 656

Isolates (origin)/clone names	Sequence encoding	Accession no.
NGY (Aichi pref., N35° E137°)		
Assemblage of 7 PCR fragments	Complete rRNA gene repeat unit	AB526843
NGY-1	Partial LSU rRNA gene	AB588900
NGY-2	Partial LSU rRNA gene	AB588901
NGY-3	Partial LSU rRNA gene	AB588902
NGY-4	Partial LSU rRNA gene	AB588903
Akita (Akita pref., N39° E140°)		
Akita-5	Partial LSU rRNA gene	AB588904
Akita-7	Partial LSU rRNA gene	AB588905
Akita-8	Partial LSU rRNA gene	AB588906
Fukushima (Fukushima pref., N37° E140°)		
Fuku-4	Partial LSU rRNA gene	AB588907
Fuku-7	Partial LSU rRNA gene	AB588908
Fuku-8	Partial LSU rRNA gene	AB588909
Shiraoka (Saitama pref., N35° E139°)		
Shira-7	Partial LSU rRNA gene	AB588910
Shira-9	Partial LSU rRNA gene	AB588911
Shira-10	Partial LSU rRNA gene	AB588912
Yokosuka (Kanagawa pref., N35° E139°)		
Yoko-1	Partial LSU rRNA gene	AB588913
Yoko-2	Partial LSU rRNA gene	AB588914
Yoko-10	Partial LSU rRNA gene	AB588915
Matsuzaka (Mie pref., N34° E136°)		
Matsu-2	Partial LSU rRNA gene	AB588916
Matsu-3	Partial LSU rRNA gene	AB588917
Matsu-9	Partial LSU rRNA gene	AB588918
Hagi (Yamaguchi pref., N34° E131°)		
Hagi-2	Partial LSU rRNA gene	AB526844
Hagi-7	Partial LSU rRNA gene	AB526845
Hagi-8	Partial LSU rRNA gene	AB526846
Hiroshima (Hiroshima pref., N34° E132°)		
Hiro-49	Partial LSU rRNA gene	AB526847
Hiro-50	Partial LSU rRNA gene	AB526848
Hiro-51	Partial LSU rRNA gene	AB526849



Supplemental Figure 1. Predicted RNA secondary structure of the D8 domain of *Plasmodiophora brassicae*. The basal D8a and D8b helices are indicated. The structure was drawn based on the sequence of population NGY (NGY-1 clone).