

## SUMMARY OF DOCTORAL THESIS

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Title: Characteristics of *Plasmodiophora brassicae* causing clubroot disease on the cruciferous weed *Cardamine occulta* in post-harvest paddy fields in Japan

(日本の収穫後水田においてアブラナ科雑草*Cardamine occulta*に根こぶ病を引き起こす *Plasmodiophora brassicae*の性状)

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Clubroot disease causes great damages to the *Brassica* crops. The causal pathogen is *Plasmodiophora brassicae*, a telluric intracellular obligate protist whose life cycle and pathogenesis remain largely unknown. *P. brassicae* survives in soil for several decades by its well-protected resting spores. These dormant spores become active when host plants are in range. The primary infection starts when the pathogen attacks the root hairs and then the main roots, also known as secondary infection, where the pathogens settle in the cortical tissues. These cortical tissues are manipulated by the pathogens with symptoms such as hyperplasia and hypertrophy (causing the swollen symptoms) in order to serve the nutritional supplies facilitating the growth of parasites. That is how the plant development is severely delayed and the senescence process initiates. Due to the complicated life cycle, scientific studies on clubroot disease in general or *P. brassicae* in specific, are made challenging by the biological context of the host, and pathogen, and the technology required to investigate and understand that relationship. Indeed, the mainstream research on clubroot merely focuses on the *Brassica* crops; however, our clubroot research on the cruciferous weed *Cardamine occulta*, commonly grown in rice paddy fields in Japan, has revealed novel populations *P. brassicae* (a.k.a *Cardamine* group). The wild weed *C. occulta*, is an overwinter perennial crucifer whose genome and life cycle have coevolve with human activities, e.g. agricultural activities. In Japan, this wild weed is well adapted to the culture of rice cultivation. Commonly, this weed is found germinating at the edges of paddy field in the late September to October, usually at the post-harvest time. The number of individuals reach the highest at the winter time, from November to January in which the clubroot incidences on this weed were also found at the most abundant (the infestation could be up to 60-80% within an examined paddy field). After this period, the weed is dying out in March and April. Its seeds lay dormant under the submergence of the rice season, waiting for the paddy fields are abandoned again in order to restart its life cycle. This weed possesses an octoploidy genome that enriched in genetic variations and mutations. These advantageous elements help this weed adapt well into the new ecological niches, especially for anthropogenic habitats such as rice fields, nursing plant houses, construction sites, etc. Simultaneously, the genetic variations of the weeds, via the host-pathogen interactions, also enhance the genetic

diversification of the *P. brassicae* populations. In the present study, the coevolution relationship was approached basically under the scope of the pathogen *P. brassicae* which was molecularly investigated in addition to pathological traits description.

The first and foremost approach is those pathological traits including morphological and histopathological description were demonstrated in comparison with those occurred on the *Brassica* crops. In that sense, few distinctive characters were observed only in the *Cardamine* group. For the morphological characters, large galls were observed on the stem (stalk) of the weeds as a sign of heavily infected despite the fact that the above-ground symptoms cannot be observed. For microscopic examination, plasmodia were detected in vascular bundles and pith tissues and even in the foliage structure such as petioles. These characters of symptomology are the signals for the genetic investigations in order to prove the *P. brassicae* infected *C. occulta* displays as newly discovered populations.

The second approach is molecular examinations to corroborate that pathological characterization by proving the genetic identity of the novel populations. *Actin* genes and ITS regions were selected to prove the genetic distinction of the *Cardamine* group from the cosmopolitan strains (*Brassica* group).

The *actin* genes investigation, known for significant role in several cellular functions and pathogenesis in *P. brassicae*, revealed the *Cardamine* group accumulated seven point mutations (three silent and four missense) in comparison with the *actin* genes of the cosmopolitan strains.

For ITS regions, popularly known in phylogenetics, the results demonstrated in both ITS1 and ITS2 regions acquired large amount of mutations. A greater number of variations and mutations (single-nucleotide polymorphisms, oligonucleotide polymorphisms, and insertions/deletion) were found in the ITS2 than those in ITS1 and these mutations acquired were greater than any cosmopolitan *P. brassicae* infected on the *Brassica* crops. Concatenated phylogenetic analysis conducted on both *actin* and ITS sequences has confirmed the separated status of the *Cardamine* groups from the global strains. Since the ITS2 sequences were enriched with large number of mutations, further structurally analyzed was deployed to reinforce the phylogenetic assessment. The secondary structure analyses of ITS2 obtained by the *Cardamine* group revealed it was greatly distanced from the ITS2 secondary structure of the *Brassica* group. The *Cardamine* group was differently structured in base ring, size of four helices comprised of bulges and loops (based on the nucleotides bonds) as opposed to fundamental structures formed by the cosmopolitan strains. These findings suggested the *P. brassicae* populations infecting on *C. occulta* could be taxonomically ranked at the different positions from the mainstream *P. brassicae* infecting on the brassica crops.

Further molecular assessment was deployed based on the extended intergenic regions (non-coding sequences), namely intronic DNA of the ribosomal RNA (both small subunit (18S) and large subunit (28S)). The sequencing results showed *P. brassicae* populations from the *C. occulta* weed carried an authentic intronic pattern and structure. The rDNA introns of *Cardamine* group have lost multiple introns in the small and large subunit regions. Moreover, the retained introns despite a largely mutual share of conserved parts with the cosmopolitan strains contained numerous novel structures. These structural differences comprised of high

level of polymorphisms such as transversion point mutations occurring at sites involving with the intronic splicing sites or insertions/deletions at the binding sites. Two geographical *P. brassicae* populations from *C. occulta* were authentic because they carried a lengthy intron-encoded ORFs and putative mobile elements established in the large subunit regions. A few aberrant *P. brassicae* populations from the *Brassica* crops also harbored polymorphic introns that shared common mutated motifs with the weed-affecting group as well as many populations were short of intron I in the small subunit regions (this result is similar to many *P. brassicae* populations found in Korea). The diversity of ribosomal introns observed from those investigated populations also demonstrated the genetic distinction of the *P. brassicae* populations from the *C. occulta*. The pattern of gain and loss as well as different structures and containing extra sequences might open an interesting case study for intergenic regions in plant parasitic protist *P. brassicae*, this might explain multiple phenomena related to evolution, origin, life cycle, pathogenesis and adaption during the domestication of the brassica crops.

This study strongly suggests that *P. brassicae* populations causing clubroot disease on *C. occulta* are genetically distinct from those causing the disease in vegetables. It was also inferred that coevolution between *P. brassicae* and *C. occulta* has been associated with the dynamics of pathogenicity and genetic diversity of *P. brassicae*.