Full paper

Lifecycle of *Pyrenopeziza protrusa* (Helotiales, Dermateaceae sensu lato) in *Magnolia obovata* revealed by field observation and molecular quantification

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ABSTRACT

Fungi exhibit saprophytic, parasitic, and symbiotic lifestyles, and flexibly switching between them by the environmental changes and host conditions. However, only a few studies have elucidated the detailed changes in fungal DNA or morphology, including the formation of reproductive structures along with lifestyle switching. We hypothesized that *Pyrenopeziza protrusa*, which occurs abundantly and specifically on *Magnolia obovata* as a saprophyte, is also associated with living hosts and switches its lifestyles as part of its lifecycle. To elucidate this hypothesis, we periodically sampled the fresh/fallen leaves of *M. obovata* to observe the seasonal occurrence of reproductive structures for the isolation and detection/quantification of *P. protrusa* DNA with newly developed species-specific primers. The isolation frequency and amount of *P. protrusa* DNA drastically increased in the fresh leaves just before defoliation in autumn, but remained high in fallen leaves from autumn to spring. Abundant production of conidiomata and apothecia was also observed in the fallen leaves with increasing DNA content. These results clarified a large part of the lifecycle of *P. protrusa*, suggesting that the lifestyle is switched from symbiotic to saprophytic stage by significantly increasing the amount of DNA in response to host conditions according to the seasonal variations.

Keywords: conidiomata, dermateaceous fungi, lifestyle switching, real-time PCR, saprophyte

Article history: Received 7 January 2021, Revised 30 August 2021, Accepted 31 August 2021, Available online 20 November 2021.

1. Introduction

Fungi exhibit saprophytic, parasitic, and symbiotic, including mutualistic and commensal lifestyles, based on the trophic interactions with their hosts. Fungi that can flexibly switch multiple lifestyles based on the environmental changes and host conditions have been increasingly recognized. For example, an endophytic lifestyle inhabiting living leaves has been reported in some *Xylariales*, known as wood-decaying fungi. They utilize the leaves as vehicles to enhance dispersal and as shelters to avoid competition with other saprophytes and/or environmental stress (Nelson, Vandegrift, Carroll, & Roy, 2020; Thomas, Vandegrift, & Roy, 2020). Pyrophilous fungi that only fruit on burned soil are suspected to inhabit the bryophytes or lichens, without exhibiting any symptoms, until the next fire event (Kuo et al., 2014; Matheny, Swenie, Miller, Petersen, & Hughes, 2018; Raudabaugh et al., 2020). Some plant pathogenic fungi are frequently isolated from healthy plant tissues (Osorio & Stephan, 1991; Promputtha et al., 2007; da Silva, Moreno, Correia, Santana, & de Queiroz, 2020). The ability to switch between multiple lifestyles may be more frequently observed in fungi

than other organisms. However, only a few studies have elucidated the phenomena seen during the lifecycle switching.

Inoue et al. (2019) firstly revealed the dynamic changes of *Hymenoscyphus fraxineus* in DNA quantity and formation of reproductive structures along with the defoliation of *Fraxinus mandshurica* var. *japonica*. The study by Inoue et al. (2019) was motivated because *H. fraxineus* is a serious pathogen for *Fraxinus* species in Europe (Gross, Holdenrieder, Pautasso, Queloz, & Sieber, 2014), while suggested to be symbiotic with *F. mandshurica* var. *japonica* in Asia. On the other hand, there is no detailed studies to elucidate possible lifestyle switching. Some saprophytes that have close affinity with specific hosts may exhibit other lifestyles, which are currently unrecognized. Therefore, investigating the host specificity may help in revealing the potential lifestyles of such fungi.

Pyrenopeziza protrusa is known as a saprophyte of *Magnolia* species (Hütter, 1958; Itagaki, Nakamura, & Hosoya, 2019). Because abundant apothecia formation was observed on almost all fallen leaves of *Magnolia obovata* every year at the Tsukuba Botanical Garden, we hypothesized that *P. protrusa* has a relationship with the living host. Since *P. protrusa* occurs abundantly and is easily culturable on artificial media, this fungus is an appropriate target to analyze the lifecycle with a focus on lifestyle switching.

Direct observation and isolation frequency are conventional measures for detecting and quantifying fungi in host leaves. How-

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ever, fungi with occasional occurrences may be overlooked by these measures. Therefore, in this study, a multi-view approach was employed that combined the observation of seasonal occurrence, isolation and fungal DNA detection/quantification with newly developed species-specific primers to demonstrate the detailed lifecycle along with lifestyle switching of *P. protrusa*. Furthermore, dermataceous fungi have been reported as one of the major components of root-associated fungi (Toju et al., 2013a, 2013b). *Pyrenopeziza protrusa* may also be present in the host roots due to its host selectivity. We also examined the possibility of *P. protrusa* inhabiting roots using species-specific primers.

2. Materials and methods

2.1. Sampling fresh/fallen leaves, roots, and seeds of *M. obovata*

The study site was designed in the Tsukuba Botanical Garden (Tsukuba, Ibaraki, Japan; 36°06'12" N, 140°06'40" E, 24.5 m elev.), where the healthy adult *M. obovata* trees were associated with young trees. One adult tree was randomly selected for periodic leaf sampling. For observation, isolation, and quantification of *P. protrusa* DNA, one to several fresh leaves were sampled every month or week from Apr 23 to Nov 28, 2018, and from Apr 25 to Nov 7, 2019 using a high twig shear. Additionally, several fallen leaves were randomly collected from the floor every month or week from Feb 26, 2018 to Nov 14, 2019. To distinguish fallen leaves from those of the previous year in autumn, the fallen leaves in 2018 were marked with a biodegradable tape and collected once a month in Sep and Oct 2019. To detect *P. protrusa* DNA from the roots, four young *M. obovata* trees with fine roots less than 30 cm in height were pulled out on Jul 8, 2019. In addition, to detect *P. protrusa* DNA, the seeds were removed from mature fruit collected from the floor on Nov 31, 2019. For the substrates to induce the formation of reproductive structures of *P. protrusa*, one fresh leaf of *M. virginiana* a relative species of *M. obovata*, *Quercus crispula* planted near the target *M. obovata* tree, and fresh/fallen leaves of *M. obovata* were sampled on Nov 28, 2018. Except for the fresh/fallen leaves used for isolation, the collected materials were packed in plastic bags and immediately stored at –30 °C until future use.

2.2. Observation of seasonal occurrence of reproductive structures in the field

The periodically collected fresh/fallen leaves of *M. obovata* were examined under a stereomicroscope (SZ61; Olympus, Tokyo, Japan) to check for the occurrence of the reproductive structures of *P. protrusa*. When observed, the reproductive structures were picked from the substrates and gently squashed with a cover glass using water as the mounting fluid for morphological observation. Slide preparations were observed under an Olympus BX51 microscope with Nomarski phase interference (Olympus) and photographed with a digital camera (DS-L3; Nikon, Tokyo, Japan).

2.3. Isolation and molecular identification

One each of fresh/fallen leaf collected on the sampling date was used for the isolation. Fresh and fallen leaves were surface sterilized with 70% ethanol (v/v) for 1 min, followed by 1% sodium hypochlorite (v/v) for 2 min (Okane, Nakagiri, Ito, & Lumyong, 2003), with the addition of final rinse in running tap water for 20 min. To suppress bacterial growth, the washed leaf were dried at 20 °C on a sterile paper for 10 h on sterile paper. Several 5 mm square pieces

were cut from the leaflet. Five centimeters at both ends of the leaf were eliminated from the rachis, and the remaining rachis was cut into 5 mm lengths, and then cut transversely (Supplementary Fig. S1). Three pieces of leaflet or rachis were inoculated on corn meal agar (CMA; Nissui, Tokyo, Japan) in a 9 cm Petri dish in triplicate and incubated at 20 °C. Hyphal growth in plates was observed under an Olympus SZ61 stereomicroscope, and hyphal tips extending from the pieces were transferred to potato dextrose agar (PDA; Nissui) in a 9 cm Petri dish. The isolation process was repeated for 5 d after inoculation to avoid the unintentional isolation of other rapidly growing fungi. The isolates inoculated on PDA plates were incubated at 20 °C for a week in the dark. Only slow-growing and whitish colonies expected as the colony of *P. protrusa* (referring to Itagaki et al., 2019) were selected and transferred to the PDA slants.

DNA was extracted from the obtained isolates, amplified using polymerase chain reaction (PCR), and sequenced using the ITS1F/ITS4 primer pair following the protocol described by Itagaki et al. (2019). The obtained sequences were assembled using ATGC v.7.0.3 software (Genetyx, Tokyo, Japan) and basic local alignment search tool (BLAST) searches in the National Center for Biotechnology Information (NCBI) database (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). The sequences with > 97% similarity to *P. protrusa* (LC425044) were identified as *P. protrusa*. The isolation frequency (IF) of *P. protrusa* was calculated using the following formula: IF (%) = number of isolates of *P. protrusa* / total number of isolates × 100.

2.4. Induction and observation of reproductive structures under culture on artificial media

To attempt reproductive structure formation under culture, 9 cm plates of water agar (WA), Miura's agar (1 g glucose, 1 g K₂HPO₄, 0.2 g MgSO₄·7H₂O, 0.2 g KCl, 2 g NaNO₃, 0.2 g yeast extract, 13 g agar, and 1 L distilled water) (Miura & Kudo, 1970), and PDA were prepared and inoculated with several 1 cm² sterilized (autoclaved for 20 min at 121 °C) leaf pieces of the fresh/fallen leaves of *M. obovata*, fresh leaves of *M. virginiana*, or fresh leaves of *Q. crispula*. To check for bacterial or fungal contamination before use, the plates were incubated at 20 °C for 5 d in the dark. A hyphal mass (5 mm square) cut from the edge of the developed colony of *P. protrusa* on a 9 cm PDA plate derived from a single or multiple ascospores was inoculated in the center of each plate. WA, Miura's agar, and PDA without leaf pieces were used as controls. The inoculated plates were sealed with Parafilm (Bemis, Neenah, USA) and incubated for 4 mo at 20 °C under black light (FL15BLB, peak wavelength 352 nm; Toshiba, Tokyo, Japan). The presence or absence of reproductive structures on the inoculated plates was examined using an Olympus SZ61 stereomicroscope. When reproductive structures were found, they were observed as described in section 2.2.

2.5. Observation of detailed morphology of conidiomata and germination test

To observe the detailed morphology of conidiomata, the cross sections of conidiomata fixed in resin were prepared from the fallen rachises sampled on Oct 26, 2019. The pieces of fallen rachises were fixed using formaldehyde: acetic acid: 50% ethanol (v/v) (5:5:90) and dehydrated with a graded ethanol series (50, 60, 70, 80, 90, 95, 99.5, and 100% [v/v]; 2 h per step) in a vacuum desiccator. Dehydrated samples were embedded in Technovit® 7100 (Kulzer, Wehrheim, Germany) following the manufacturer's protocol. The pieces of rachises in resin were sliced into sections of 2–3 µm thickness using a glass knife on an ultra-microtome (LEICA RM2155;

Leica, Vienna, Austria). The sections were stained with safranin, toluidine blue, and orange G (Jernstedt, Cutter, Gifford, & Lu, 1992), and observed under an Olympus BX51 microscope.

To observe the nuclei of conidia, conidia were fixed in 4% paraformaldehyde solution (w/v) (4% paraformaldehyde [w/v], 10 × phosphate-buffered saline [PBS]) for 30 min, rinsed with PBS, and finally stained with 10 µg/mL of 4',6'-diamino-3-phenylindole (DAPI) for 5 min. Slide preparations were observed under a fluorescence microscope (DM4000 B; Leica) and photographed using a digital camera (DFC300 FX; Leica).

To examine the germination ability, the conidia were inoculated on 1% WA (w/v), CMA, and PDA plates and incubated at 20 °C for 1 mo in the dark and observed under an Olympus BX51 microscope.

2.6. Observation of apothecial development

Fallen leaves sampled on Feb 2, 2018 (before the occurrence of apothecia in the field) were incubated for a week in a moist chamber at 20 °C under black light to promote the maturation of apothecia. During apothecial development, apothecia at different developmental stages were picked from the rachises and fixed in 70% ethanol (v/v). The cross section of the apothecium was prepared using a freezing microtome, as described by Itagaki et al. (2019). The sliced apothecia were then mounted with cotton blue in lactic acid (CB/LA) and observed under an Olympus BX51 microscope.

2.7. Design and validation of species-specific primers

Sixteen representative isolates, except *P. protrusa*, were collected from fresh rachises on Oct 22 2018: *Acrodontium crateriforme* (LC586228), *Alternaria alternata* (LC586226), *Annulohyphoxylon* sp. (LC586233), *Arthrinium phaeospermum* (LC586219), *Cladosporium* sp. (LC586224), *Colletotrichum* sp. (LC586222), *Diaporthe* sp. (LC586221), *Fusarium* sp. (LC586231), *Idriella* sp. (LC586229), *Ilyonectria* sp. (LC586232), *Microdochium* sp. (LC586225), *Periconia* sp. (LC586223), *Pestalotiopsis* sp. (LC586220), *Pezicula ericae* (LC586230), and *Pleosporales* sp. (LC586227). The representative isolates were kept in PDA slants and used for the design of specific primers, and their internal transcribed spacer (ITS) sequences were determined according to the method described in section 2.3. The ITS sequences of representative isolates were aligned using BioEdit (Hall, 1999) with the ITS sequences of *P. protrusa* (LC425044) and two other phylogenetically related species, *P. nervicola* (LC426342) and *P. petiolaris* (LC586234). The specific sequence regions for *P. protrusa* (30–40 bp in length) to which the primers bind were manually recognized from ITS1 and ITS2, respectively.

Using Primer-BLAST (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>), primer pairs with sequences satisfying the following criteria were selected: 1) the GC content was 60% or less; 2) both Tm values were nearly the same; 3) the primer size was 17–25 bp or less; and 4) the expected amplicon size was 300 bp or less. To avoid the occurrence of primer dimers, the appropriate annealing temperature for the primer pair was simulated using PerlPrimer (Marshall, 2004). The designed primers were manufactured by FAS-MAC (Kanagawa, Japan).

To check species specificity of the designed primer pair, PCR amplification was conducted with the following reagents for the extracted fungal DNA used to design the primers: 1 µL extracted DNA, 3.5 µL DNA-free water, 5 µL EmeraldAmp PCR MasterMix (Takara Bio, Kusatsu, Japan), 0.25 µL forward primer, and 0.25 µL reverse primer. The following protocol was used for PCR amplification: initial denaturation at 95 °C for 3 min, 35 cycles at 94 °C for 35

s, 60 °C for 30 s, and 72 °C for 1 min, with a final extension at 72 °C for 1 min (see section 3.7. for annealing temperature). PCR products were separated on a 1.5% agarose gel (w/v) in 1 × Tris-acetate ethylenediaminetetraacetic acid (EDTA) buffer (Wako, Japan) at 100 V for 15 min. The gel was stained with GelRed™ (Cosmo Bio, Tokyo, Japan), and the bands were visualized using an ultraviolet (UV) transilluminator. The band size was determined by comparison with a 100 bp DNA ladder (Dye Plus; Takara Bio). The amplified DNA was sequenced using the same primer set, and the obtained sequences were compared with those of *P. protrusa* (LC425044).

To calculate the amplification efficiency (AE) of the designed primers for the real-time PCR assay, a calibration curve was prepared as follows: a ten-fold serial dilution series of extracted DNA of *P. protrusa* with Tris-EDTA buffer, ranging from 5.4 to 5.4 × 10⁻⁸ ng/µL, were used as standard samples. The real-time PCR cocktail contained the following reagents: 1 µL of standard samples, 8.5 µL of DNA-free water, 12.5 µL of qPCR Master Mix (KAPA SYBR® FAST qPCR Kit; Kapa Biosystems, Wilmington, USA), and 1 µL of 10 pM of the designed primers. The real-time PCR assay was repeated five times using a Thermal Cycler Dice® Real Time System (Takara Bio) under the following conditions: initial denaturation at 95 °C for 3 min, 35 cycles at 94 °C for 5 s, and annealing at 51 °C for 30 s. Dissociation curve analysis of standard samples was also performed to determine the presence or absence of non-specific amplification by the designed species-specific primers. The calibration curve was prepared from the average threshold cycle (Ct) value and the initial DNA amount of the standard samples with five replicates. The AE of real-time PCR using the designed species-specific primers was calculated using the following formula: AE (%) = [1^(-1/slope) - 1] × 100. The forward and reverse primer sets were used to detect and/or quantify the amount of *P. protrusa* DNA in fresh/fallen leaves, roots, and seeds of *M. obovata*.

2.8. Detection and quantification of *P. protrusa* DNA in the collected material

One to several collected fresh/fallen leaves were surface-sterilized, as previously described in section 2.3, and dried for 5 min at 20 °C. The rachises separated from the leaflets were shred into small pieces using sterile scissors. The pieces were frozen in liquid nitrogen and ground into a powder using a sterile mortar and pestle. Powdered rachises (0.15 g) from each sample were collected in a 2 mL round-bottom tube. The surface-sterilized roots were cut into 1 cm-long pieces and ground as described above. Each 0.3 g of powdered roots were collected in a tube. The surface-sterilized seeds were separated into the endotesta with an external seed coat and the embryo covered with an inner seed coat and ground separately. Each 0.3 g powdered endotesta and embryo were collected in a tube.

DNA from each sample was extracted in 800 µL hexadecyltrimethylammonium bromide buffer (2% CTAB [w/v], 100 mM Tris pH 8, 20 mM EDTA, 1.4 M NaCl) at 65 °C for 30 min, and proteins were removed using 800 µL chloroform/isoamyl alcohol (24:1). To precipitate DNA, 250 µL of the supernatant was mixed with 25 µL of sodium acetate buffer and 250 µL of isopropyl alcohol, and then centrifuged at 1,800 × g for 30 min. The pellet was washed with 1 mL of 70% ethanol (v/v) and eluted in 75 µL TE at pH 8 (Wako, Tokyo, Japan). To increase the purity of DNA, 50 µL of extracted DNA was purified using MonoFas® DNA extraction and purification kits I (GL Sciences, Tokyo, Japan) and diluted to 200 µL using the elution buffer in the kit. The concentration and quality of the purified DNA were measured using a Qubit 4 fluorometer (Thermo

Fisher Scientific, Waltham, USA) and adjusted to 100 ng/μL or less.

The purified DNA was subjected to PCR amplification using species-specific primers under the conditions described in section 2.7. The PCR products were separated by electrophoresis, and the amplified DNA was visualized using a UV transilluminator. PCR products were sequenced using the same primer set, and the obtained sequences were compared with those of *P. protrusa* (LC425044). The detection frequency (DF) of *P. protrusa* in fresh/fallen rachis was calculated using the following formula: DF (%) = number of samples from which *P. protrusa* was detected/ total number of samples × 100.

To quantify the absolute amount of *P. protrusa* DNA in fresh/fallen rachis, the purified DNA of rachis was applied to the real-time PCR assay with species-specific primers under the conditions described in section 2.7. Dissociation curve analysis was also performed using real-time PCR to confirm the specific amplification of *P. protrusa* DNA. The real-time PCR products were separated by electrophoresis, and the amplified DNA was visualized using a UV transilluminator. The real-time PCR products were sequenced using the same primer set, and the obtained sequences were compared with those of *P. protrusa* (LC425044). The absolute amount of *P. protrusa* DNA in fresh/fallen rachises was determined by comparing the Ct value with the calibration curve.

2.9. Air sampling and quantification of airborne ascospores

Two membrane filters (MF-Millipore™, 47 mm in diam, 0.45 μm pore size; Merck Millipore, Burlington, USA) were cut in half, affixed on a WA strip (HYCON® agar strip; Merck Millipore), and set into an RCS® Air sampler (Biotest, Dreieich, Germany). Air sampling was conducted from Apr 15 to Jul 22, 2019, within a 2 m radius from the *M. obovata* adult tree, and 320 L of air was sampled from each air layer at heights of 5, 30, 100, and 300–500 cm above the ground. Whole membrane filters were recovered from the strip, and one was placed in a 1.5 mL round-bottom tube for DNA extraction, while the other one was mounted in CB/LA and observed under an Olympus BX51 microscope to detect the trapped ascospores anticipated to be *P. protrusa* (referring to Itagaki et al., 2019).

The filter in a 1.5 mL round-bottom tube was frozen overnight at –80 °C, followed by freeze-drying for 24 d, and finally ground using a Qiagen TissueLyser (Qiagen, Venlo, The Netherlands) and zirconia beads. DNA was extracted from the filter using 20 μL sodium dodecyl sulfate (SDS)-Proteinase K solution (1 M Tris pH 8, 1 M MgCl₂, 1 M KCl, 10% SDS [w/v], 1% Proteinase K [v/v], H₂O) at 40 °C for 20 min. The supernatant was heated at 95 °C for 10 min to inactivate proteinase K.

To quantify the absolute amount of *P. protrusa* DNA in the air, the supernatants were subjected to real-time PCR using species-specific primers under the conditions described in section 2.7. Dissociation curve analysis was also performed using real-time PCR to confirm the specific amplification of *P. protrusa* DNA. The real-time PCR products were separated by electrophoresis, and the amplified DNA was visualized using a UV transilluminator. The real-time PCR products were sequenced using the same primer set, and the obtained sequences were compared with those of *P. protrusa* (LC425044). The absolute amount of DNA contained in a 1 mm² membrane filter was determined by comparing the Ct value with the calibration curve.

3. Results

3.1. Sampling

The leaves periodically collected from Apr 28, 2018, to Oct 24, 2019, comprised a series of processes, from foliation to defoliation and decomposition (Supplementary Table S1). The leaflets were nearly decomposed within one year. In total, 209 leaves were collected, and 1,643 isolates were obtained, including 1,239 isolates from fresh leaves. As a result, conidiomata of *P. protrusa* were discovered for the first time, as described in the following sections.

3.2. Seasonal occurrence of reproductive structures in the field

The abundant production of conidiomata was also observed on fallen rachises in autumn, just after defoliation (mid-Oct to Nov), and gradually decreased by the next spring (Apr to May) (Fig. 1).

Stromata of *P. protrusa* were initially found on fallen rachises (occasionally on leaflets) in late winter (Feb) (Fig. 2F). The coexistence of conidiomata and immature apothecia was also observed from Feb to Mar. In the field, apothecia were found on damp leaves that were not exposed to direct sunlight. Mature apothecia were found in almost all fallen leaves collected in the spring (late-Apr to May), but gradually turned blackish and decreased in early summer (early Jul) (Fig. 1). After that, no apothecia was found on the fallen leaves.

3.3. Detailed observation of conidiomata induced under culture

Conidiomata formation was induced only on WA plates inoculated with *M. obovata* fresh/fallen leaves and hyphae derived from single/multiple ascospores of *P. protrusa* (Fig. 2H). No formation of apothecia was observed in any media without leaf pieces.

Conidiomata were spherical to irregular without ostiole, superficial, and 0.1–0.25 mm in diam; parietal tissue was moderately developed, composed of textura angularis, thick-walled, and pale brown cells (Fig. 2I). Conidiophores were short, hyaline, arising around the cavity of the conidioma (Fig. 2J). Conidiogenous cells were slender flask-shaped to acicular, 7.5–25 × 3–5 μm, hyaline, and thin walled (Fig. 2K). Conidia were phialidic, globose to slightly ellipsoid, 2.5–3 μm in diam, hyaline, gelatinous, and released by a crack of parietal tissue, becoming waxy when dried (Fig. 2L). Conidium had a single nucleus stained with DAPI (Fig. 2M). No conidial germination was observed in any of the artificial media. The morphology of conidiomata and conidia under culture did not differ from those in the field (Fig. 2G, H).

3.4. Observation of apothecial development

Before incubation in a moist chamber, stromata consisting of textura angularis fungal tissue of *P. protrusa* were observed under the epidermis of fallen rachises (Fig. 2A). After 3–4 d of incubation, the stroma increased in size and cell density (Fig. 2B) and eventually became erumpent through the epidermis (Fig. 2C). The immature globular apothecium developed, opening at the top of the mesohymenial phase (Kimbrough, 1981) (Fig. 2D). At this stage, the ectal excipulum consisted of textura globulosa fungal tissue, medullary excipulum, and juvenile asci were clearly distinguished. The opening of the apothecium at the top increased in diameter as the hypothecium expanded (Fig. 2E). After a few days of incubation, the hymenium was fully exposed, and ascospore production in the

Fig. 1 – Absolute amounts of *Pyrenopeziza protrusa* DNA in the fresh/fallen rachis. Note that the scale on the left axis is 1,000 times larger than the scale on the right axis. The number of fresh/fallen leaves used for the detection and quantification of *P. protrusa* DNA. -: No sample. []: The year of defoliation. Frequency of the reproductive structures observed in the fallen leaves. +: Observed but few. ++: Frequently observed. +++: Observed in all fallen leaves. -: Not observed. N/A: Fallen leaves not available.

Fig. 2 – Morphologies of the developmental stages of the apothecia and conidiomata of *Pyrenopeziza protrusa*. A–E are mounted with cotton blue in lactic acid (CB/LA). I, K, and L in water. A: Cross section of stroma under the epidermis of fallen rachises. B: Cross section of stroma with increased size and density. C: Cross section of stroma erumpent through the epidermis. D: Cross section of the immature apothecium developing an opening at the top. E: Cross section of apothecium with immature hymenium. F: Stromata on fallen rachis. G: Conidiomata on fallen rachis. H: Conidiomata produced on water agar with pieces of fresh *Magnolia obovata* leaf nearby. I: Parietal tissue of conidioma in squash mount. J: Cross section of conidioma fixed by resin and stained with safranin, toluidine blue, and orange. G: Conidiophores covering the whole the area inside the conidioma. K: Conidiogenous cells. L: Conidia. M: Nuclei of conidia stained with 4',6'-diamino-3-phenylindole (DAPI). N: Conidiomata (arrow) of *P. protrusa* on potato dextrose agar (PDA) formed by the isolate obtained from fresh rachis on Oct 24, 2019. Bars: F–H, 1 mm; I–J, 50 μ m; K–M, 5 μ m.

mature apothecium was observed. Apothecial development of *P. protrusa* showed a hemiangiocarpic type (Corner, 1929).

3.5. Isolation frequency (IF) of *P. protrusa*

Pyrenopeziza protrusa was isolated from the rachises, rather than from the leaflets (Table 1). The IF in fallen rachises showed a maximum value (76%) just after defoliation in 2018 (Nov 28), temporarily decreased to 35–41% in Jan to Feb, and increased again in Apr (65%). After Aug 22, the IF dropped dramatically, but *P. protrusa* was continuously isolated (2–7%). Formation of conidiomata was found only in colonies isolated from fresh rachises collected on Oct 24, 2019 (Fig. 2N).

3.6. Design and validation of the species-specific primers

The forward primer ITS_444F (5'-AAACCACTGTGGGCTTCG-GT-3') and reverse primer ITS_739R (5'-AGGGATCGCCAGTTA-CAGC-3') were developed to specifically amplify approximately 300 bp of the *P. protrusa* ITS region. Because PerlPrimer indicated that the primers tended to produce multiple dimers during the annealing reaction at 50 °C, the annealing step of PCR amplification was conducted at 60 °C to prevent the formation of dimers. The unique sequence of *P. protrusa* was successfully amplified by PCR using species-specific primers (Supplementary Fig. S2). The serially diluted *P. protrusa* DNA from standard samples was also successfully amplified by real-time PCR using species-specific primers. The dissociation curve of the real-time PCR product showed a sin-

gle peak at 84.25–84.75 °C, demonstrating that there was no non-specific amplification (Supplementary Fig. S3). A highly linear correlation ($R^2 > 0.99$) was observed between the average Ct value and the log of the standard DNA concentration. Since the amplification frequency of real-time PCR using the developed primers was 99.5%, confirming the highly efficient quantification of *P. protrusa* DNA using these primers (Supplementary Fig. S4).

3.7. Detection and quantification of *P. protrusa* DNA in the collected plant material

The dissociation curve of the real-time PCR product showed a single peak around 84 °C, confirming that *P. protrusa* DNA in the leaf samples was successfully amplified with species-specific primers. *Pyrenopeziza protrusa* DNA in fresh rachis was detected on Aug 17, 2018, and Jul 18, 2019. The frequency in fresh rachises fluctuated between 20–100% (Fig. 3), while it was 100% in all fallen rachises. The fungal DNA was detected in all root samples, suggesting that the fungus may also inhabit the roots. *Pyrenopeziza protrusa* DNA could not be detected in the end testa or embryo of seeds.

The absolute amount of *P. protrusa* DNA in fresh rachises was low during spring to summer (Apr to Aug), but drastically increased in autumn (Sep to Oct), and then gradually decreased until leaf fall (Fig. 1). The fluctuation in the amount of DNA in fresh rachises in 2018 and 2019 showed a similar pattern. From Oct to Nov 2018, *P. protrusa* DNA amounts in fallen rachises were approximately 50–150 times higher than those in fresh samples collected on the same day. The absolute amount of *P. protrusa* DNA in fallen

Table 1 – Isolation frequency of *Pyrenopeziza protrusa* from fresh/fallen leaves.

Sampling date	Fresh leaves		Fallen leaves in 2018		Fallen leaves in 2019	
	Leaflet	Rachis	Leaflet	Rachis	Leaflet	Rachis
Oct 15, 2018	0/54	20/50 (40%)	-	-	-	-
Nov 28, 2018	-	-	6/41 (14.6%)	38/50 (76%)	-	-
Dec 24, 2018	-	-	0/54	37/52 (71.2%)	-	-
Jan 23, 2019	-	-	0/54	26/64 (41%)	-	-
Feb 18, 2019	-	-	0/38	17/48 (35.4%)	-	-
Mar 27, 2019	-	-	0/57	27/70 (38.6%)	-	-
Apr 25, 2019	-	0/1	0/50	54/83 (65%)	-	-
Jun 18, 2019	0/13	0/9	0/49	40/67 (59.7%)	-	-
Jul 22, 2019	0/23	0/49	0/30	27/49 (55.1%)	-	-
Aug 22, 2019	0/34	1/36 (2.8%)	0/45	3/40 (7.5%)	-	-
Sep 24, 2019	0/42	7/41 (17.1%)	-	1/44 (2.3%)	0/46	39/53 (73.6%)
Oct 24, 2019	0/34	21/39 (53.8%)	-	1/32 (3.1%)	1/47 (2.1%)	38/55 (69.1%)

XX/XX: Number of *P. protrusa*/Total number of the isolates. (X%): Isolation frequency of *P. protrusa* (IF) calculated by the following formula. Number of isolates of *P. protrusa*/Total number of isolates $\times$ 100. IF is not shown if no *P. protrusa* is isolated. Note that the leaflets of fallen leaves from the previous year collected on Sep 24, 2019 and Oct 24, 2019 are completely decomposed.

Fig. 3 – 100% stacked bar plot (black) showing the detection frequency of *Pyrenopeziza protrusa* DNA in fresh rachis from 2018 (left) and 2019 (right). Note that the black bar showing the number of DNA detected samples, and the white bar showing the number of DNA undetected samples.

rachises in 2018 temporarily decreased in winter (Oct to Dec), but increased again toward the maximum value in spring (late Jan to Apr), and then decreased in summer (Jul).

3.8. Quantification of *P. protrusa* DNA in air samples

The dissociation curve of the real-time PCR product showed a single peak at approximately 84 °C, confirming that *P. protrusa* DNA in the air samples was successfully amplified with species-specific primers. The absolute amount of *P. protrusa* DNA contained in each air layer sampled in 2019 was generally low. However, more than 1 ng of *P. protrusa* DNA per 1 cm² of the membrane filter was detected from the air layer 5 cm above the ground, sampled on Apr 25, 2019, and 500–300 cm above the ground on May 18, 2019 (Table 2). From both filters, several wedge-shaped spores characteristic of *P. protrusa* ascospores were observed (Supplementary Fig. S5).

4. Discussion

This study revealed that the lifecycle of *P. protrusa* consisted of four stages (Fig. 4).

1) The increase of the fungal DNA in fresh leaves just before defoliation suggests the switch of symbiotic to saprophytic lifestyle

Pyrenopeziza protrusa was abundantly isolated and/or detected in the living leaves and roots of *M. obovata* without symptoms, and has not been recognized as a pathogen of *M. obovata* (Kishi, 1998; Phytopathological Society of Japan, 2020), suggesting that the fungus is symbiotically living in the host. However, direct observation

Table 2 – Absolute amounts of *Pyrenopeziza protrusa* DNA (ng) detected from 1 cm² of membrane filter.

Sampling date in 2019	Vertical height from the ground (cm)			
	5	30	100	200–500
Apr 15	0.04	0.02	0.02	0.01
Apr 22	0.02	0.02	0.01	0.02
Apr 25	1.89	0.26	0.02	0.06
May 8	0.02	0.37	0.03	3.00
May 14	0.03	0.03	0.02	0.02
May 31	0.04	0.03	0.03	0.02
Jun 2	0.02	0.02	0.04	0.02
Jun 28	0.13	0.05	0.02	0.02
Jul 8	0.04	0.02	0.02	0.09
Jul 22	0.08	0.02	0.03	0.02

Dark gray shaded cells indicate >1 ng detected DNA. Pale gray shaded cells show 0.1–1 ng detected DNA.

of *P. protrusa* hyphae in host tissues and inoculation experiments are required to clarify whether the fungus has a potential pathogenicity or a mutualistic function that promotes host growth or survival. The low DNA level (Fig. 3) and IF of *P. protrusa* in fresh rachises from early summer (Jun to Aug; Table 1) indicated that the fungal activity might be suppressed by some external factors, such as host resistance toward pathogenic fungi (Schulz, Roemmert, Dammann, Aust, & Strack, 1999; Hiruma et al., 2016) and temperature. Because the suitable growth temperature of *P. protrusa* was 20 ± 2 °C (see Supplementary Fig. S1 of Itagaki et al. 2019), while the average temperature at the study site in 2018–2019 in summer exceeded 27 °C (Supplementary Fig. S6), temperature is suggested to be an important factor that suppresses fungal activity. Other possi-

Fig. 4 – Schematic representation of the presumed lifecycle of *Pyrenopeziza protrusa* on *Magnolia obovata* leaves.

ble factors that may affect *P. protrusa* growth were direct sunlight, drought (Bahnweg et al., 2005; Hashizume, Fukuda, & Sahashi, 2010), and competition with other fungi coexisting in the host leaf (Gazis & Chaverri, 2015; Van Bael, Estrada, & Arnold, 2017).

The increase in the fungal DNA of *P. protrusa* in autumn (Sep to Oct) in fresh rachis was thought to be caused by a decrease in the temperature preferable for the growth of *P. protrusa* (Supplementary Fig. S6). Host resistance may also decrease due to leaf senescence. The fluctuation pattern in isolation frequency and amount of fungal DNA in fresh host leaves of *P. protrusa* was similar to that of *H. fraxineus* (Inoue et al., 2019). The fungal DNA increase just before defoliation is one of the evidences that these fungi might switch their lifestyle from symbiotic to saprophytic after host cell death caused by defoliation.

2) Possibility of sexual reproduction by conidia (spermatia) occurred on fallen leaves

Detailed observation of fungal behavior in the field and under culture revealed that *P. protrusa* had conidia. The abundant production of conidiomata on fallen rachis contributed to an increase in the amount of DNA in the autumn (Oct to Nov) (Fig. 1). The conidia of *P. protrusa* did not germinate under culture, but had one living nucleus that was stained with DAPI (Fig. 2M). Since conidiomata were produced only before the formation of apothecia in the field, the conidia were thought to be associated with sexual reproduction as spermatia (Higgins, 1920; Drayton, 1932) (Fig. 1). The lack of germination suggests this idea. However, the lack of germination did not directly indicate the sterility of *P. protrusa* conidia. In fact, Fones, Mardon, and Gurr (2016) have reported that microconidia of *H. fraxineus* known as spermatia inoculated with the host roots germinate and extend hyphae into host tissues. An inoculation experiment of *P. protrusa* conidia in host roots is required to clarify whether conidia participate in infection, as well as sexual reproduction.

3) Apothecial development correlated with the increase of the fungal DNA

In early spring (Feb), *P. protrusa* formed stromata underneath the epidermis towards apothecial formation (Fig. 2A, F). The rapid DNA increase from Mar to Apr is suggested to be attributed to the fungal biomass increase due to apothecial development under the epidermis of leaf rachises (Fig. 1, Fig. 2). Most fungi that contribute to the early stages of leaf decomposition are also isolated from fresh leaves (Okane et al., 2008; Hirose, Matsuoka, & Osono, 2013). These pioneering fungi assimilate nutrients from fallen leaves, such as carbohydrates or amino acids, before the invasion of other saprophytes (Osono & Hirose, 2009; He et al., 2012). By switching their lifestyle from symbiotic to saprophytic, *P. protrusa* might increase its biomass to use fallen rachises predominantly. Even after disappearance of the apothecia on fallen rachises from summer to autumn (Aug to Oct, 2019) (Fig. 1), *P. protrusa* was isolated from the fallen rachises at low frequency, suggesting that the fungus remained in fallen rachises and continued to live saprophytically (Table 1).

4) Airborne ascospores may infect new leaves

Because abundant production of apothecia on fallen leaves piled up the forest floor was observed from Apr to May (Fig. 1), and *P. protrusa* DNA was detected in the air around the host branch in May (Table 2) and host fresh leaves in Jul (Fig. 3), one of the possible routes for the infection is by airborne ascospores, and the fungal colonization in the host might have been established by Jun. Thus, *P. protrusa* is assumed to switch its lifestyle again from saprophytic to symbiotic in spring. Another possible infection route is hyphal transmission from branches or seeds (Petrini, 1991; Hata, Futai, & Tsuda, 1998). However, since *P. protrusa* DNA was not detected in

M. obovata seeds, vertical transmission was not positively supported.

5. Conclusion

This study clarified a large part of the life cycle of *P. protrusa*, suggested to be associated with the living leaves and roots of *M. obovata*. We demonstrated that the fungus switches its lifestyle from the symbiotic to saprophytic stage and vice versa by drastically increasing the amount of fungal DNA. However, the major factors that affect this lifestyle change, including seasonal changes and host conditions, need to be further investigated in future studies.

Disclosure

The authors declare no conflicts of interest. This study was performed according to the current laws of Japan.

Acknowledgements

We would like to thank Dr. Hirokazu Tsukaya, The University of Tokyo, for allowing us to use the fluorescence microscope. Appreciation is also due to Ms. Kazune Ezaki for her kind technical advice on fluorescent staining and observation. We would also like to thank Dr. Chie Tutumi and Ms. Yumiko Hirayama, National Museum of Nature and Science, for technical advice and assistance with the preparation of resin-embedded sections. We are grateful to Tsukuba Botanical Garden for providing the sampling place.

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