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Morphophylogenetic study revealed that *Erysiphe gracilis* (powdery mildew of evergreen oaks, Erysiphales) is a species complex consisting of six different species

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ABSTRACT

Erysiphe gracilis is a powdery mildew species that occurs on evergreen oak species belonging to *Quercus* subgen. *Cyclobalanopsis* in East Asia (China and Japan). In a previous report, we found that *E. gracilis* var. *gracilis* is divided into four genotypes each of them forming a separate clade with strong bootstrap support. In this study, we further investigated genotype speciation in *E. gracilis* var. *longissima* occurring on *Q. acuta* and *Q. sessilifolia*, and found that this variety is also divided into two distinct genotypes. These results suggested that *E. gracilis* represents a species complex consisting of six different species. Based on detailed morphological examinations correlating with results of molecular sequence analyses, we propose to divide *E. gracilis* into six species, encompassing three new species (*E. uncinuloides*, *E. pseudogracilis*, and *E. longiappendiculata*), one new name (*E. longifilamentosa*), and two known species (*E. gracilis* s. str. and *E. hiratae*). A key to the species concerned is provided.

1. Introduction

Molecular phylogeny revealed that morphological similarity does not always reflect close relationships in fungi because of multiple convergent evolutionary developments that occurred in these organisms to adapt to changing environments. Consequently, a comprehensive phylogenetic classification of the kingdom Fungi was proposed based on molecular phylogeny (Hibbett et al., 2007), which also applied to the generic classification of the Erysiphaceae (powdery mildew family). Thus, Braun and Takamatsu (2000) proposed a new generic concept for the Erysiphaceae on the basis of molecular phylogeny (Mori, Sato, & Takamatsu, 2000; Saenz & Taylor, 1999) and morphology of asexual morphs (Cook, Inman, & Billings, 1997). The molecular phylogeny also revealed the presence of cryptic species in traditional morphology-based species, and subsequent re-examinations of morphological traits supported the molecular results in many cases. For instance, *Erysiphe alphitoides* (Griff. & Maubl.) U. Braun & S. Takam., previously recognized as a single species, was divided into five species (Takamatsu et al., 2007), *E. carpinicola* (Hara) U. Braun & S. Takam. was divided into four species (Braun et al., 2006; Meeboon & Takamatsu, 2013), and *Parauncinula septata* (E.S. Salmon) S. Takam. & U. Braun into three species (Meeboon, Siahaan, Fujioka, & Takamatsu, 2017). There are probably many similar, not yet revealed cases of complexes composed of cryptic species in the Erysiphaceae.

Quercus species belonging to subgen. *Cyclobalanopsis* are popular evergreen oak trees which are planted as garden, street or hedge trees. *Cystotheca wrightii* Berk. & M.A. Curtis and *E. gracilis* R.Y. Zheng & G.Q. Chen commonly occur on these oak species in Japan (Nomura, 1997). *Cystotheca wrightii* forms colonies with dark brown colour on the lower side of leaves except for early infection stages and is easily distinguishable from other powdery mildew species by its unique symptoms. On the other hand, *E. gracilis* produces typical white powdery mildew symptoms. This species, described in 1981 based on a specimen on *Q. glauca* Thunb. collected from China (Zheng & Chen, 1981), is endemic to East Asia (China and Japan) and has evergreen *Quercus* species belonging to subgen. *Cyclobalanopsis* as hosts. This species is currently divided into two varieties, viz., var. *gracilis* parasitic on *Q. glauca*, *Q. myrsinifolia* Blume, and *Q. salicina* Blume, and var. *longissima* Y. Nomura parasitic on *Q. acuta* Thunb. and *Q. sessilifolia* Blume (Nomura, 1997). Siahaan, Sakamoto, Shinoda, and Takamatsu (2017)

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reported that *E. gracilis* var. *gracilis* is divided into four genotypes forming separate clades with strong bootstrap support. A comprehensive survey suggested that particular host plants are associated with these genotypes (Siahaan et al., 2017). In this study, we further investigated the genotype speciation in *E. gracilis* var. *longissima.* As a result, we propose to split *E. gracilis* var. *gracilis* in four species and var. *longissima* in two species on the basis of combined analyses of molecular and detailed morphological characteristics, complemented by a key to the species concerned.

2. Materials and methods

2.1. Sample collection

Samples of *E. gracilis* var. *longissima* were collected in Mie and Okayama Prefectures, and Tokyo Metropolis between Nov 2015 and Jun 2016, including four collections at Mt. Takao, the original locality of the holotype of this variety. When *E. gracilis* was found, GPS data were recorded, and a few leaves with powdery mildew colonies were put into a plastic bag and brought to the laboratory of Mie University. Fresh specimens with asexual morphs were subjected to microscopic observations and conidial germination tests. Samples with sexual morph were dried between old newspapers and kept in TSU-MUMH herbarium until DNA extractions and microscopic observations were performed. Further specimens were loaned from HMAS, TNS, and TUAMH.

2.2. DNA sequencing

Whole-cell DNA was extracted from mycelia or chasmothecia using the chelex method (Walsh, Metzger, & Higuchi, 1991) as described by Hirata and Takamatsu (1996). The rDNA internal transcribed spacer (ITS), 5′-end of the 28S rRNA gene including D1/D2 domains, and rDNA internal transgenic spacer (IGS) were amplified by polymerase chain reaction (PCR) using the respective primer pairs: PM5 (Takamatsu & Kano, 2001)/NLP2 (Mori et al., 2000) for 3′-half of ITS and 28S rDNA, ITS5/PM6 (Takamatsu & Kano, 2001) for 5′-half of ITS, and IGS-12a/NS1R (Carbone & Kohn, 1999) for IGS. The protocol used in this study was as follows: PCR mixtures (25 μL) contained 0.4 μM of each primer, 200 μM dNTPs, 1 × PCR buffer (supplied by the manufacturer), 1 unit of KOD FX Neo DNA polymerase (Toyobo, Tokyo), and 1 μL of DNA extract solution. The following thermal cycling conditions were performed in a thermal cycler Dice TP-600 (Takara, Tokyo): an initial denaturing step of 94 °C for 2 min; thermal cycling for 40 cycles, where each cycle consisted of 10 s at 98 °C followed by 30 s at 65 °C for annealing, and 60 s at 68 °C for extension. The amplicons were sent to Solgent Co. Ltd. (Daejeon, South Korea) for direct sequencing using primers PM5 and NLP2 for the PM5/NLP2 fragment, PM6 for the ITS5/PM6 fragment, and IGS-12a and NS1R for IGS-12a/NS1R fragment. New sequences determined in this study were deposited in the DNA Data Bank of Japan (DDBJ) under the accession numbers LC260187–LC260208 and LC271381–LC271449 (Supplementary Table S1).

2.3. Molecular phylogeny

Sequences were aligned using MUSCLE (Edgar, 2004) implemented in MEGA6 (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013). Alignments were further manually refined using the MEGA6 program and were deposited in TreeBASE (http://www.treebase.org/) under the accession number S21130. Phylogenetic trees were obtained from the data using the maximum parsimony (MP) and maximum likelihood (ML) methods. MP analysis was performed in PAUP 4.0a152 (Swofford, 2002) with heuristic search option using the tree bisection reconnection (TBR) algorithm with 100 random sequence additions to find the global optimum tree. All sites were treated as unordered and unweighted, with gaps treated as missing data. The strength of internal branches of the resulting trees was tested with bootstrap (BS) analysis using 1000 replications with the step-wise addition option set as simple (Felsenstein, 1985). Tree scores, including tree length, consistency index (CI), retention index (RI), and rescaled consistency index (RC), were also calculated. The ML analysis was done using raxmlGUI (Silvestro & Michalak, 2012), under a GTRGAMMA model. The BS supports and trees were obtained by running rapid bootstrap analysis of 1000 pseudo-replicates followed by a search for the tree with the highest likelihood.

2.4. Morphological examination

In order to examine the traits of the sexual morphs, chasmothecia were stripped off from the leaf surfaces with a clean needle and mounted on a microscope slide in 3% NaOH using a standard light microscope (Axio Imager; Carl Zeiss, Göttingen, Germany) and differential interference contrast optical instruments and devices. To examine the asexual morph, hyphae, conidiophores, and conidia of fresh collections were stripped off from the leaf surfaces with clear adhesive tape, mounted on a microscope slide with the fungal mycelium uppermost, and examined in water. Herbarium samples were rehydrated before examination by boiling a small piece of infected leaf with the fungal mycelium downwards in a drop of lactic acid on a slide (Shin & La, 1993). After boiling, the rehydrated mycelium was scraped off and mounted in lactic acid using a light microscope. Thirty chasmothecia, conidia, and conidiophores were measured for each specimen examined. To observe conidial germ tubes, we inoculated conidia onto the inner cell layer of onion scales using the method of Hirata (1942, 1955).

3. Results

3.1. Molecular phylogeny

A total of 31 samples (29 from *Q. acuta* and in each case one from *Q. sessilifolia* and *Q.* × *takaoyamensis*) were collected and the nucleotide sequences of the rDNA IGS region were determined for these samples. rDNA ITS and 5′-end of the 28S rRNA gene (including domains D1 and D2) sequences were also determined for some samples. The sequences obtained were divided into two distinct genotypes differing from any other sequences retrieved from *E. gracilis*, and were designated as genotypes V and VI. The sequence similarities between the genotypes were 96.8% (18 substitutions/560 nucleotides, not including gaps), 93.8–94.1% (23–24/387) and 99.3–99.5% (4–5/737) for ITS, IGS and the 28S rRNA gene, respectively. DNAs were extracted from chasmothecia and mycelia, and/or from the upper and lower sides of leaves separately for six samples, and nucleotide sequences were determined. In each case, DNAs extracted from single samples had identical sequences.

Three phylogenetic analyses were conducted in this study. In the first analysis, in order to investigate the phylogenetic relationships of the six genotypes found in *E. gracilis* s. lat. within the genus *Erysiphe*, twelve 28S rRNA gene sequences from *E. gracilis* s. lat. were aligned with 44 sequences of *Erysiphe* retrieved from DNA databases. The data matrix consisted of 56 sequences and 759 characters, of which 167 (22.0%) characters were variable and 104 (14.1%) characters were informative for parsimony analysis. A total of 943 equally parsimonious trees with 416 steps were constructed by the MP analysis. Tree topologies were almost consistent among the trees, except for branching orders of the terminal branches and

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