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Molecular phylogeny reveals the presence of cryptic speciation within *Erysiphe japonica* (≡ *Typhulochaeta japonica*), a powdery mildew on *Quercus* spp.

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ABSTRACT

Molecular phylogenetic analyses based on 28S, ITS and IGS rDNA sequences indicate that *Erysiphe japonica* (≡ *Typhulochaeta japonica*) consists of two different genetic groups, one group on *Quercus aliena*, *Q. robur* and *Q. serrata*, and another group on *Q. crispula* var. *crispula* and *Q. crispula* var. *horikawae*. As morphological difference between the two groups are not yet marked distinctly, we suppose that the process of speciation has not yet been finished and propose a new variety, *E. japonica* var. *crispulae*, for the latter group based on the difference in host range and the distinct genetic segregation. *Quercus robur* (pedunculate oak) is a new host of *E. japonica*.

1. Introduction

Typhulochaeta S. Ito & Hara is a unique powdery mildew genus characterized by having clavate appendage-like cells arising from the upper half of chasmothecia (Ito 1915). These cells gelatinize in water and eject mucilaginous material. The first molecular phylogenetic analysis of *Typhulochaeta* was conducted by Mori et al. (2000), in which they demonstrated that *Typhulochaeta* nested in the *Erysiphe* clade (currently known as the tribe Erysipheae). Based on the molecular analysis, Braun and Cook (2012) re-allocated *Typhulochaeta* in the genus *Erysiphe* and proposed a new morphological, non-phylogenetic section, *Erysiphe* section *Typhulochaeta*.

Of the four species known in the section *Typhulochaeta* (Shin and Park 2011; Braun and Cook 2012), only *Erysiphe japonica* (S. Ito & Hara) C.T. Wei, the type species of *Typhulochaeta*, is distributed in Japan (Amano 1986). During preliminary phylogenetic analyses of *E. japonica*, we found that *E. japonica* is divided into two distinct groups dependent on host species (unpublished data). We thus conducted further phylogenetic analyses using additional specimens and DNA regions to confirm the result, and carried out detailed morphological observations of the specimens concerned. Consequently, we recognized the presence of cryptic speciation in *E. japonica*. *Quercus robur* (pedunculate oak) proved to be a new host of *E. japonica*.

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2. Materials and methods

2.1. Morphological examination

Specimens were collected in several locations in Japan from 1995 to 2011. Details of host names, collection data, locations, and collectors were recorded. For morphological examinations, mycelia and chasmothecia were stripped off from the leaf surfaces with a clean needle, mounted on a microscope slide, and examined in 3% NaOH using a light microscope with phase contrast and 10×, 20×, and 40× objectives. Thirty chasmothecia, asci and ascospores were measured per sample. Specimens were deposited at the National Museum of Nature and Science (TNS), Japan, Mie University Mycological Herbarium (MUMH), Japan and Herbarium Martin-Luther-Universität, Halle (HAL), Germany.

2.2. Phylogenetic analysis

Whole-cell DNA was extracted from chasmothecia using the chelex method (Walsh et al. 1991) as described in Hirata and Takamatsu (1996). The 5-end of the 28S (including the domains D1 and D2), internal transcribed spacer including the 5.8S (ITS), and intergenic spacer (IGS) rDNA regions were amplified by polymerase chain reaction (PCR) using the respective primer pairs: PM3/TW14 (28S), PM7/ITS4 (ITS fragment 1), ITS5/PM6 (ITS fragment 2), and IGS-12A/NS1R (IGS; Carbone and Kohn 1999).

KOD FX Neo DNA polymerase (Toyobo, Japan) was used in the PCR reaction according to the manufacturer's protocol. The amplicons of 28S, ITS and IGS were sent to SolGent Co. Ltd. (Daejeon, South Korea) for sequencing using primers NL1 and NLP2 (28S), ITS1 and ITS4 (ITS), and IGS-12A and NS1R (IGS).

Representative sequences determined in this study were deposited in DNA databases (DDBJ, EMBL, GenBank) under the accession numbers of AB701300–AB701319. The Sequences were aligned with the sequences of *Erysiphe* species retrieved from DNA databases using MEGA 5 (Tamura et al. 2011). The alignment was deposited in TreeBASE (http://www.treebase.org/) under the accession number of S12462. Maximum parsimony (MP) analysis was done in PAUP* 4.0b10 (Swofford 2002) with the heuristic search option using the 'tree-bisection-reconstruction' (TBR) algorithm. All sites were treated as unordered and unweighted, with gaps treated as missing data. The strength of the internal branches of the resulting tree was tested with bootstrap analysis using 1000 replications (Felsenstein 1985). Tree scores, including tree length, CI, RI, and RC, were also calculated.

3. Results

3.1. Taxonomy

Erysiphe japonica* var. *crispulae Meeboon & S. Takam., **var. nov.** Fig. 1.

MycoBank no.: MB 564666.

Morphologically similar to *E. japonica* var. *japonica* but confined to *Quercus crispula* var. *crispula* and *Q. crispula* var. *horikawae*.

Colonies on leaves hypophyllous, effuse or in large patches, persistent or sometimes evanescent. Hyphae 4–5 μm wide, hyaline. Chasmothecia scattered, blackish brown, (136–)148–191(–224) μm diam. (170 μm diam. on average, $n = 30$), containing 13–21 asci (Table 1). Peridial cells irregular in shape. Gelatinous cells numerous, relatively short, (24–)28–75(–87) × (8–)10–15(–19) μm (53 × 12 μm on average, $n = 30$), in the upper half of the chasmothecium, clavate, apically swollen or narrowed towards the tip, hooked, rarely straight, true appendages absent. Asci (67–)69–86(–91) × (29–)30–40(–44) μm (77 × 36 μm on average, $n = 30$), 7–8-spored, stalked. Ascospores (19–)21–25(–27) × (8–)10–13(–14) μm (23 × 11 μm on average, $n = 30$), ellipsoid-ovoid, hyaline; anamorph not developed.

Materials examined: on *Q. crispula* Blume var. *crispula* (Fagaceae), Japan, Shiga Prefecture, Mt. Odani, 7 November 1999, S. Takamatsu (Holotype: TNS-F-44918 Isotype: HAL2599F, MUMH 890). Additional specimens: Niigata Prefecture, Mt. Yahiko, 25 October 1997, S. Takamatsu (TNS-F-44920, HAL2501F, MUMH 427); Niigata Prefecture, Yuzawamachi, 23 September 1998, S. Takamatsu (TNS-F-44917, HAL2500F, MUMH 513); *Q. crispula* Blume var. *horikawae* H.Ohba, Japan, Toyama Prefecture, Asahi-cho, 26 June 1995, Y. Sato (MUMHs75).

Erysiphe japonica (S. Ito & Hara) C.T. Wei, Nanking J. 11(3): 105, 1942 (var. *japonica*) ≡ *Typhulochaeta japonica* S. Ito & Hara, Bot. Mag. Tokyo 29: 20, 1915.

Colonies on leaves amphigenous, mainly hypophyllous, effuse, forming large patches. Hyphae 3–5 μm wide, hyaline. Chasmothecia scattered, blackish brown, (143–)156–203(–217) μm diam. (181.5 μm diam. on average, $n = 30$), containing 11–22 asci (Table 1). Peridial cells irregular in shape. Gelatinous cells (30.5–)45–56(–62) × (10–)11–14.5(–16) μm (50 × 12.5 μm on average, $n = 30$), *ca* 100–160 per chasmothecium, in the upper half of the chasmothecium, clavate, apically swollen or narrowed towards the tip, straight, true appendages absent. Asci (69–)72–90(–103) × (28.5–)32–41.5(–45) μm (81 × 37 μm on average, $n = 30$), 8-spored, stalked. Ascospores (21.5–)22.5–28(–31) × (9.5–)10–14 μm (26 × 12.5 μm on average, $n = 30$), ellipsoid-ovoid, hyaline; anamorphs not developed.

Materials examined: on *Q. robur* L. (Fagaceae), Japan, Aichi Prefecture, Higashiyama Zoo and Botanical Gardens, 17 November 2011, J. Meeboon & S. Takamatsu (TNS-F-45899, HAL2502F, MUMH 5555); Osaka Prefecture, Botanical Garden of Osaka City University 20 November 2005, S. Takamatsu (TNS-F-44919, HAL2503F, MUMH 4146).

Notes: *Quercus robur* is reported here as a new host for *E. japonica*.

3.2. Phylogenetic analysis

In the 28S rDNA sequence analysis, 807 total characters were used, of which 765 characters were constant, 22 characters were variable and parsimony-uninformative and 20 characters were parsimony-informative. One equally parsimonious tree (Fig. 2) was generated from the MP analysis (TL = 44, CI = 0.955, RI = 0.967, RC = 0.923). In the ITS sequence analysis, 634 total characters were used including 523 constant characters, 78 variable and parsimony-uninformative characters, and 33 parsimony-informative characters. Two equally parsimonious trees (Fig. 3) were generated in this analysis

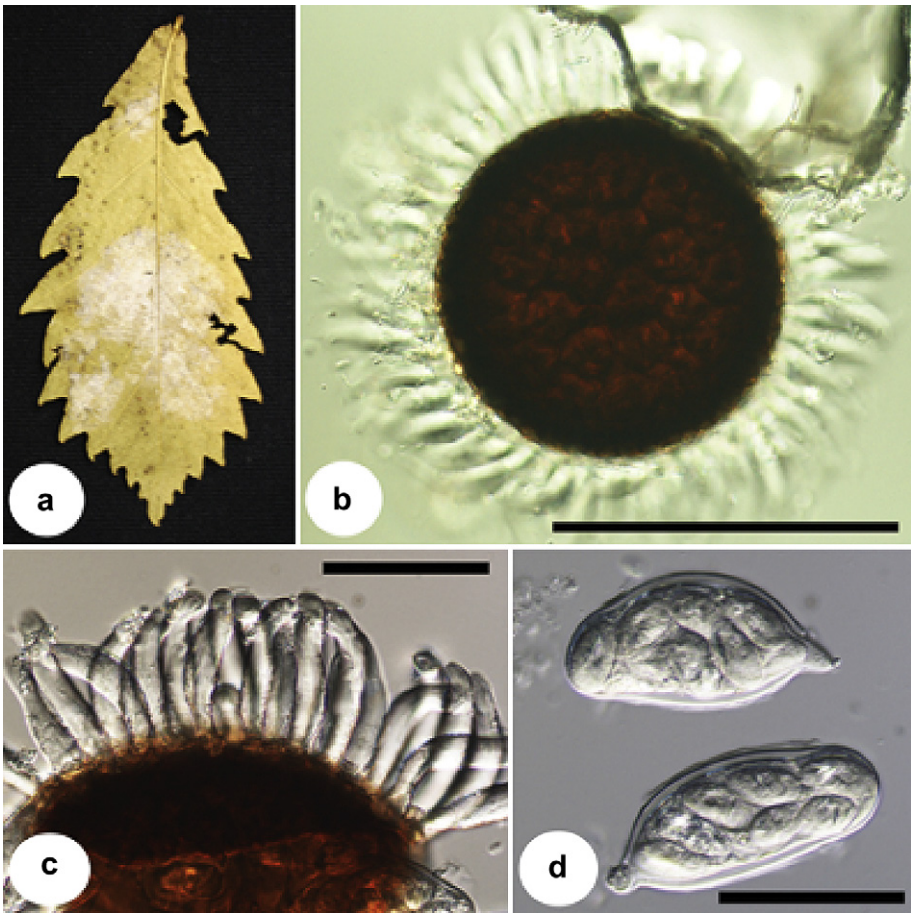

Fig. 1 – *Erysiphe japonica* var. *crispulae* on *Quercus crispula* var. *crispula*. a Hypophyllous symptom on the leaf surface. b Chasmothecium. c Short and clavate appendages. d Asci with 7–8-spored. *Bars* b 150 μm; c, d 50 μm.

(TL = 125, CI = 0.960, RI = 0.896, RC = 0.860). The shortest sequences were generated from the IGS region where 421 total characters were used in the MP analysis. Of them, 352 characters were constant, 58 characters were variable and parsimony-uninformative and 11 characters were parsimony-informative. One equally parsimonious tree (Fig. 4) was generated from the analysis (TL = 72, CI = 1.000, RI = 1.000, RC = 1.000). All phylogenetic trees constructed by the three

Table 1 – Comparison of morphological characteristics of *Erysiphe japonica* on *Quercus* spp.

Character	Host (specimen no./reference)			
	Quercus robur (MUMH 5555)	*Quercus serrata* (MUMH 4582)	*Quercus crispula* var. *crispula* (MUMH 890)	*Quercus serrata* (Ito 1915)
Colonies Chasmothecia	Amphigenous (143–)156–203(–217) μm diam. (182 μm on average), 11–22 asci	Hypophyllous (168–)175–199(–207) μm diam. (186 μm on average), 12–16 asci	Hypophyllous (136–)148–191(–224) μm diam. (170 μm on average), 13–21 asci	Hypophyllous 120–200 μm diam. 5–13 asci
Gelatinous cells	(30–)45–56(–62) × (10–)11–15 (–16) μm (50 × 13 μm on average)	(44–)48–70(–79) × 11–15(–16) μm (60 × 13 μm on average)	(24–)28–75(–87) × (8–) 10–15(–19) μm (53 × 12 μm on average)	45–65 × 10–15 μm
Asci	(69–)72–90(–103) × (29–)32–42 (–45) μm (81 × 37 μm on average), 8-spored	(80–)89–105(–109) × (40–)44–58 (–66) μm (96 × 51 μm on average), 7–8-spored	(67–)69–86(–91) × (29–)30–40(–44) μm (77 × 36 μm on average), 7–8-spored	70–97 × 40–55 μm (6–)8-spored
Ascospores	(22–)23–28(–31) × (9–)10–14 μm (26 × 13 μm on average)	(24–)25–29(–30) × (12–)13–16 (–17) μm (27 × 14 μm on average)	(19–)21–25(–27) × (8–)10–13(–14) μm (23 × 11 μm on average)	18–36 × 12–18 μm

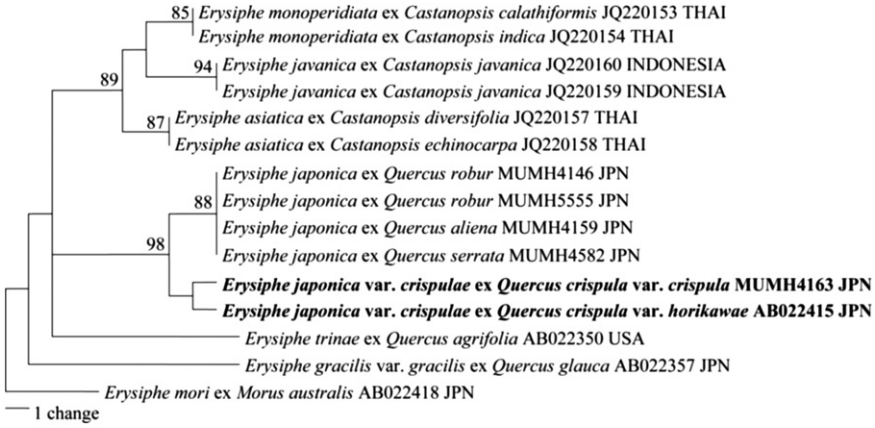

Fig. 2 – Phylogeny of *Erysiphe japonica* var. *crispulae* inferred from the 28S rDNA sequences using the maximum parsimony method. The percentage bootstrap support (1000 replications; ≥50%) are shown on the branches.

DNA regions commonly indicated that *E. japonica* is divided into two phylogenetic groups. Group 1 consists of the powdery mildews occurring on *Quercus aliena*, *Q. robur* and *Q. serrata*, and group 2 the powdery mildews from *Q. crispula* var. *crispula* and *Q. crispula* var. *horikawae*. Group 1 is a monotypic group, consisting of an identical sequence in all three DNA regions. Whereas, the 28S and ITS sequences show that fungus on *Q. crispula* var. *crispula* genetically differs from that on *Q. crispula* var. *horikawae*.

4. Discussion

According to the recent Erysiphales monograph of Braun and Cook (2012), 39 species (including varieties) belonging to four genera and five sections are described as powdery mildews on Fagaceae. Of these, 30 species belonging to four genera and five sections occur on hosts of the genus *Quercus*. There is no other case that such a large number of powdery mildew species occur on a single plant genus (Viennot-Bourgin 1966; Hirata 1968), which suggests especially close relationships between powdery mildews and *Quercus*. Although molecular phylogenetic analyses have not been conducted on all of these species, phylogenetic analyses carried out during the past 15 years (Saenz and Taylor 1999; Mori et al. 2000; Takamatsu et al. 2000, 2003, 2005, 2007, 2008; Limkaisang et al. 2006; Braun et al. 2007; Divarangkoon et al. 2011; Meeboon et al. 2012) indicate that these powdery mildew species on *Quercus* are divided into five large lineages, namely, lineage 1 (*Cystotheca* spp.), lineages 2 (*Parauncinula septata*), lineage 3 (*Phyllactinia* spp.), lineage 4 (species belonging to *Erysiphe* sect. *Microsphaera*), and lineage 5 (species belonging to *Erysiphe* sects. *Californiomyces*, *Erysiphe*, *Typhulochaeta*, and *Uncinula*). Because these five lineages derived each from different ancestors, powdery mildews may have acquired parasitism on *Quercus* at least on five independent occasions during the evolutionary history. Of these lineages, the former four lineages are homogeneous, that is, the members of the respective lineages belong to a single genus or section. On the other hand, lineage 5 includes species belonging to four morphological, non-phylogenetic, sections. Because each of these sections were regarded as different genus in previous taxonomic treatments (Braun 1987; Braun et al. 2002), the above result indicates that species with quite different morphological characteristics form a phylogenetic group by sharing *Quercus* as common host. This suggests that an ancestor of *Quercus*-parasitic powdery mildew adapted its morphology into various directions on *Quercus*. Detailed analyses of this group of powdery mildews may provide a cue to address the causal factors of this close relationship between powdery mildews and *Quercus*.

Erysiphe japonica (≡ *Typhulochaeta japonica*) is the type species of the genus *Typhulochaeta* (currently *Erysiphe* sect. *Typhulochaeta*) having unique morphological characteristics as described in the introduction (Braun 1987; Braun and Cook

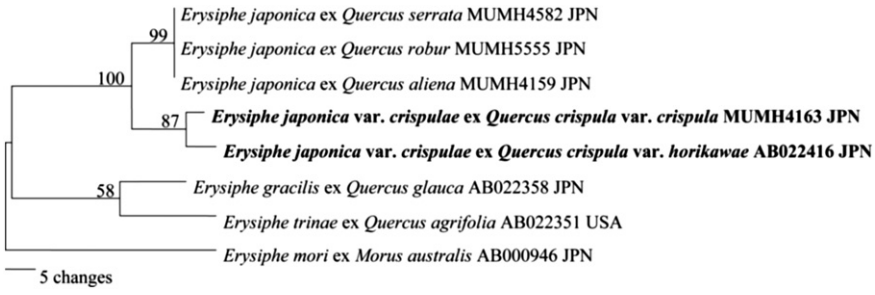

Fig. 3 – Phylogeny of *Erysiphe japonica* var. *crispulae* inferred from the ITS rDNA sequences using the maximum parsimony method. The percentage bootstrap support (1000 replications; ≥50%) are shown on the branches.

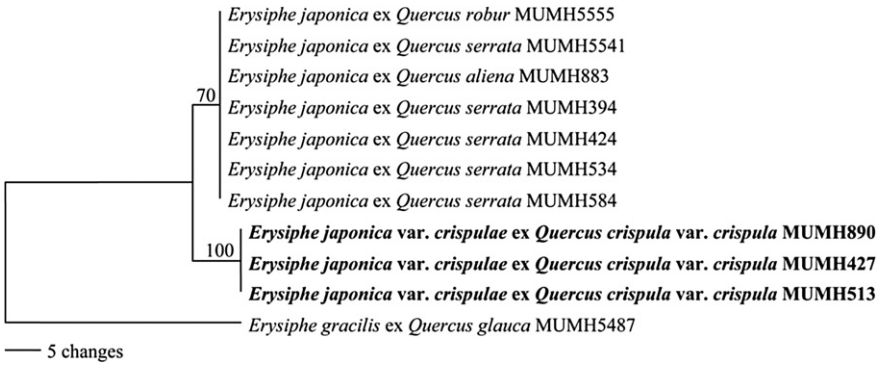

Fig. 4 – Phylogeny of *Erysiphe japonica* var. *crispulae* inferred from the IGS rDNA sequences using the maximum parsimony method. The percentage bootstrap support (1000 replications; ≥50%) are shown on the branches.

2012). Six *Quercus* spp., *Castanopsis* sp., and *Fraxinus japonica* (Oleaceae) are recorded as hosts of this species (Braun and Cook 2012). Of these hosts, *F. japonica* is unproven and its identity is not clear (Braun and Cook 2012). In this study, we report *Q. robur* (pedunculate oak) as a new host of *E. japonica*. *Erysiphe japonica* is distributed only in East Asian countries such as China, Korea, and Japan, whereas the natural distribution area of *Q. robur* is in Europe, North Africa, and East Asia (Manos and Stanford 2001). *Quercus robur* may have been infected by *E. japonica* after being imported to botanical gardens of Japan.

All three phylogenetic trees based on the 28S, ITS and IGS rDNA sequences clearly showed that sequences of *E. japonica* are divided into two different groups: one group consisted of collections on *Q. aliena*, *Q. robur* and *Q. serrata*, and another group encompassed samples on *Q. crispula* var. *crispula* and *Q. crispula* var. *horikawae*. All *Quercus* species involved belong to section *Quercus* (Nixon 1993; Manos et al. 2001). The question why *E. japonica* was divided into two genetic groups cannot be answered only based on host phylogeny. There is niche separation between *Q. crispula* var. *crispula* and *Q. crispula* var. *horikawae*, and *Q. aliena* and *Q. serrata* (Kanno et al. 2004; Okaura et al. 2007). *Quercus crispula* var. *crispula* and *Q. crispula* var. *horikawae* are distributed from sea level to an altitude of more than 1000 m in the northeastern part of Japan and distributed at elevation >800 m in southwestern part of Japan. On the other hand, *Q. aliena* and *Q. serrata* grow at a lower elevation and they are better adapted to a warmer climate than *Q. crispula*. Cryptic speciation within *E. japonica* might be explained with this kind of niche separation. Distinct morphological differences between the two genetic groups of *E. japonica* are not yet evident in teleomorph, and this species lacks anamorph. This suggests that the process of speciation has not yet reached the level of phenotypic deviations. However, because all three genetic regions commonly support the segregation of the two genetic groups and each group can be clearly delimited by its host range, we propose to distinguish them on intraspecific level as varieties.

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