FULL PAPER

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Molecular phylogenetic and morphological analyses of *Oidium heveae*, a powdery mildew of rubber tree

Abstract Powdery mildew of rubber tree caused by *Oidium heveae* is an important disease of rubber plantations worldwide. Identification and classification of this fungus is still uncertain because there is no authoritative report of its morphology and no record of its teleomorphic stage. In this study, we compared five specimens of the rubber powdery mildew fungus collected in Malaysia, Thailand, and Brazil based on morphological and molecular characteristics. Morphological results showed that the fungus on rubber tree belongs to *Oidium* subgen. *Pseudoidium*. Nucleotide sequence analysis of the ribosomal DNA internal transcribed spacer (ITS) region and the large subunit rRNA gene (28S rDNA) were conducted to determine the relationships of the rubber powdery mildew fungus and to link this anamorphic fungus with its allied teleomorph. The results showed that the rDNA sequences of the two specimens from Malaysia were identical to a specimen from Thailand, whereas they differed by three bases from the two Brazilian isolates: one nucleotide position in the ITS2 and two positions in the 28S sequences. The ITS sequences of the two Brazilian isolates were identical to sequences of *Erysiphe* sp. on *Quercus phillyraeoides* collected in Japan, although the 28S sequences differed at one base from sequences of this fungus. Phylogenetic trees of both rDNA regions constructed by the distance and parsimony methods showed that the rubber powdery mildew fungus grouped with *Erysiphe* sp. on *Q. phillyraeoides* with 100% bootstrap support. Comparisons of the anamorph of two isolates of *Erysiphe* sp. from *Q. phillyraeoides* with the rubber mildew did not reveal any obvious differences between the two powdery mildew taxa, which suggests that *O. heveae* may be an anamorph of *Erysiphe* sp. on *Q. phillyraeoides*. Cross-inoculation tests are required to substantiate this conclusion.

Key words 28S rDNA · *Erysiphe* · *Hevea brasiliensis* · ITS · *Quercus phillyraeoides*

Introduction

The para rubber tree, *Hevea brasiliensis* (Willd. A.L. Juss.) Muell.-Arg. (Euphorbiaceae), is the most important source of natural rubber for the manufacture of rubber products and latex coagulates. This tree is native to the Amazon region (Brazil, Bolivia, Ecuador, and Peru) and was introduced to tropical regions of Asia. Rubber tree is cultivated by seeds or vegetative material (bud wood) or a combination of both (Wastie 1986). Powdery mildew is an important disease of *Hevea* spp. in rubber plantations worldwide. There are many reports of its outbreak in Malaysia, India, Brazil, and Papua New Guinea (Beeley 1933; Mitra and Mehta 1938; Shaw 1967). The present distribution of this pathogen might have resulted from transport of planting materials (Ramakrishnan and Radhakrishna Pillay 1963). Shaw (1967) also suggested the outbreak from island to island in Papua New Guinea was spread by rubber planting materials. This disease causes defoliation of young shoots and discoloration and curling from the margins on older leaves. In most reports, the powdery mildew fungus infecting rubber tree was reported as *Oidium heveae* B.A. Steinm following the first record of Steinmann (1925). However, detailed morphological data of this fungus have not been published. Some studies only report conidial size, which varies among respective reports (Mitra and Mehta 1938; Thankamma 1968). Moreover, the teleomorphic stage has never been found. Thus, its identity and classification are still uncertain.

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Recently, phylogenetic relationships among powdery mildew fungi were investigated based on analyses of ribosomal DNA internal transcribed spacer (ITS) sequences (Takamatsu et al. 1998, 1999, 2000; Saenz and Taylor 1999; Braun and Takamatsu 2000; Mori et al. 2000; Matsuda and Takamatsu 2003; Takamatsu 2004). This technique is useful to evaluate the morphologically based taxonomic system of powdery mildew fungi and to link anamorphs with their teleomorphs. For example, *O. neolycopersici*, a powdery mildew of tomato, is closely related to *Erysiphe macleayae* and *E. aquilegiae* (Kiss et al. 2001). Also, the two soybean powdery mildews belonging to the same anamorphic group (*Oidium* subgenus *Pseudoidium*) were divided into two distinct species of *Erysiphe*, *E. glycines* F.L. Tai and *E. diffusa* (Cooke & Peck) U. Braun & S. Takam., based on rDNA ITS sequences (Takamatsu et al. 2002). Okamoto et al. (2002) suggested a close relationship of *Oidium* subgenus *Pseudoidium* on prairie gentian (*Eustoma grandiflorum*) with *E. baeumleri* (Magnus) U. Braun & S. Takam. and *E. trifolii* Grev. based on ITS sequence analyses. Moreover, several anamorphic powdery mildews from Australia were identified using molecular data (Cunnington et al. 2003). In this report, we characterized the rubber powdery mildew fungus by combining morphological and molecular data.

Materials and methods

Sample sources

Five rubber powdery mildew specimens collected in Malaysia, Thailand, and Brazil, and two specimens of *Erysiphe* sp. on *Quercus phillyraeoides*, were included in this study. Their herbarium accession number, host plants, locations of collection, and accession numbers of the DNA sequences (DDBJ, EMBL, and GenBank) are given in Table 1.

Morphological study

Herbarium materials were rehydrated before examination by boiling a small piece of infected leaf, with the mycelium downward, in a drop of lactic acid on a slide as described by Shin and La (1993) and Shin (2000). After boiling, the mycelium was scraped off the leaf and mounted in lactic acid for light microscopy. The following information was recorded: size and shape of conidia; presence or absence of fibrosin bodies; nature of conidiogenesis; characteristics of the conidiophore, e.g., size and shape of foot cell, position of the basal septum; shape and position of hyphal appressoria; position of conidial germ tubes, when found; and shape of appressoria formed on conidial germ tubes.

DNA extraction and PCR amplification

Whole-cell DNA was extracted from conidia and mycelia by the chelex method (Walsh et al. 1991; Hirata and Takamatsu 1996). The nuclear rDNA ITS region including the 3'-end of the 18S (small subunit) rRNA gene, the first internal transcribed spacer (ITS1), the complete 5.8S rRNA gene, the second ITS (ITS2), and the 5'-end of the 28S (large subunit) rRNA gene were amplified by the polymerase chain reaction (PCR) using primers ITS5 (White et al. 1990) and P3 (Kusaba and Tsuge 1995). For PCR amplification of the 28S rRNA gene, including the D1 and D2 regions, primer PM3 (Takamatsu and Kano 2001) and TW14 (Mori et al. 2000) were used. PCR reactions were conducted in 50-μl volumes as previously described (Hirata and Takamatsu 1996; Mori et al. 2000). The PCR amplicons were electrophoresed in 1.5% agarose gels in TAE buffer. The desired band was visualized under a long wavelength ultraviolet light and cut from the gel. Purification of the DNA fragment was performed utilizing the JETSORB Kit (Genomed, Oeynhausen, Germany), as described by the manufacturer's protocol.

DNA sequencing and data analysis

For ITS rDNA sequencing, both strands of the amplicons were sequenced using the primers ITS5, ITS4, ITS2 (White et al. 1990), and T4 (Hirata and Takamatsu 1996). The primers PM3 (Takamatsu and Kano 2001), NL1, NL2, NL3, and NLP2 (Mori et al. 2000) were used for 28S rDNA sequencing. Sequence reactions were conducted using the PRISM Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions and run on an Applied Biosystems 373A sequencer (Applied Biosystems).

Table 1. Fungal name, host plant, herbarium accession number, country of origin, and DNA database accession number of internal transcribed spacer (ITS) and 28S rDNA sequence of powdery mildew specimens used in this study

Fungal name	Host plant	Herbarium accession no.[a]	Country of origin	Database accession no.[b]	
				ITS	28S
Erysiphe sp.	*Quercus phillyraeoides*	MUMH124	Japan	AB193590	AB197135
Erysiphe sp.	*Quercus phillyraeoides*	MUMH885	Japan	AB193591	–
Oidium heveae	*Hevea brasiliensis*	MUMH2418	Brazil	AB193606	AB197133
Oidium heveae	*Hevea brasiliensis*	MUMH2419	Brazil	Ab193607	AB197134
Oidium heveae	*Hevea brasiliensis*	MUMH2544	Malaysia	AB193587	–
Oidium heveae	*Hevea brasiliensis*	MUMH2545	Malaysia	AB193588	AB197132
Oidium heveae	*Hevea brasiliensis*	MUMH2602	Thailand	AB193589	AB197136

[a] MUMH, Mie University Mycological Herbarium, Japan
[b] DDBJ, EMBL, and GenBank database accession number of the nucleotide sequence data

Sequences determined in this study (see Table 1) were aligned with sequences of the genus *Erysiphe* obtained from the DDBJ database using the Clustal V package (Higgins et al. 1992). The alignment was visually refined in a word processing program with color-coded nucleotides. Alignment files of the ITS and the 28S rDNA were deposited in TreeBASE (http://www.treebase.org/treebase/) as S1277. Phylogenetic trees were obtained from the data using parsimony and distance methods. For parsimony analysis, we used the maximum-parsimony (MP) method with the heuristic search using PAUP* 4.08 (Swofford 2001). This search was repeated 100 times with different random starting points, using the stepwise addition option to increase the likelihood of finding the most parsimonious tree. All sites were treated as unordered and unweighted. For the ITS partition, gaps were treated as a fifth base. Gaps were treated as missing data for 28S rDNA data set. The branch-swapping algorithm was TBR, the MULPARS option was in effect, and zero-length branches were collapsed. In the distance analysis, the most appropriate evolution model was determined for a given data set using PAUP* and Modeltest 3.06 (Posada and Crandall 1998). A starting tree was obtained with the neighbor-joining (NJ) method (Saitou and Nei 1987). With this tree, likelihood scores were calculated for 56 alternative models of evolution by PAUP*. The output file was then imported to Modeltest to compare the models by using Akaike's information criterion (AIC; Akaike 1974). The general time reversible (GTR; Rodriguez et al. 1990) model was chosen to construct trees with the neighbor-joining method. The strength of the internal branches from the resulting trees was tested by bootstrap analysis using 1000 replications (Felsenstein 1985) in both parsimony and distance analyses.

Results

Molecular phylogenetic study

rDNA ITS sequences were determined for five powdery mildew isolates from rubber trees. The rubber mildew sequences ranged from 556 to 557 bp in length; 220 bp in the ITS1, 154 bp in the 5.8S, and 182–183 bp in the ITS2 region. One base insertion was found in ITS2 of Malaysian and Thai specimens compared with two Brazilian specimens at the 14th site from the 5′-end of the ITS2. Sequences of the two Brazilian isolates were identical to two sequences of *Erysiphe* sp. on *Quercus phillyraeoides*.

These ITS sequences were aligned with 43 sequences covering section *Microsphaera* and some of section *Erysiphe* of the genus *Erysiphe* (anamorph, *Oidium* subgenus *Pseudoidium*) obtained from DNA databases. Sequences of *E. glycines* were used as an outgroup taxon based on Takamatsu et al. (1999). The alignment data matrix consists of 50 taxa and 606 characters, of which 251 (41.4%) characters were variable and 174 (28.7%) characters were informative for parsimony analysis. A parsimony analysis using PAUP* generated 12 equally parsimonious

trees with 627 steps (CI = 0.5789, RI = 0.7647, RC = 0.4427). Tree topologies were nearly identical among the 12 trees, except for minor differences in the branching order of the terminal branches. The tree with the highest log likelihood value is shown in Fig. 1. The tree topology of the NJ tree was nearly identical to the MP tree (tree not shown).

In the MP and NJ trees, all the *Erysiphe* species except for *E. juglandis* and the outgroup taxon *E. glycines* formed a large clade (clade I in Fig. 1) strongly supported by the bootstrap analyses (88% in the MP tree and 94% in the NJ tree). Nearly half the taxa in clade I formed a large subclade (Ia in Fig. 1) with 96% and 98% of bootstrap support in the MP and NJ analyses. The remaining taxa in the clade I branched basally within the clade without bootstrap support. The five isolates of the rubber mildew formed a distinct clade with *Erysiphe* sp. on *Q. phillyraeoides* with 100% bootstrap support in both MP and NJ trees and were placed in subclade Ia. The three rubber mildew isolates from Thailand and Malaysia formed a subclade (65% of bootstrap support in MP analysis).

For the 28S rDNA sequence analysis, the partial sequence of the 28S rRNA gene including the D1/D2 region were determined for four *O. heveae* isolates, i.e., two Brazilian isolates and one isolate from Malaysia and Thailand, and one isolate of *Erysiphe* sp. on *Q. phillyraeoides*. Sequences of the Brazilian isolates differed by two bases from sequences of the Malaysian and Thai isolates, at the 132nd and 517th sites from the 5′-end of the 28S rRNA gene, and differed by one base from the *Erysiphe* sp. sequence at the 132nd site. These sequences were aligned with eight sequences of *Erysiphe* spp. obtained from DNA databases. *Erysiphe glycines* was used as an outgroup. The alignment data matrix consists of 13 taxa and 769 characters, of which 60 (7.8%) characters were variable and 33 (4.3%) characters were informative for the parsimony analysis. A parsimony analysis using PAUP* generated 10 equally parsimonious trees with 156 steps (CI = 0.7115, RI = 0.6538, RC = 0.4652). Tree topologies were nearly identical among the 10 trees, except for minor differences in the branching order of the terminal branches. The tree with the highest log likelihood value is shown in Fig. 2. The results showed that four *O. heveae* isolates grouped with *Erysiphe* sp. on *Q. phillyraeoides* with 91% of bootstrap support. The two *O. heveae* isolates from Malaysia and Thailand formed a subclade with one base difference from this *Erysiphe*.

Morphological study

The morphological features of the rubber powdery mildew fungus are summarized as follows. Mycelia on leaves are amphigenous, mostly epiphyllous, forming irregular patches on the upper and lower sides of leaves. Hyphae are hyaline, septate, thin-walled, sub-straight to flexuous, branching at a right or narrow angle, with a septum 0–7.7 μm from the branching point. Conidia matured one at a time, ellipsoid to cylindrical, without fibrosin bodies, 25.1–43.6 × 13.4–23.3 μm in size, length/width (l/w) ratio 1.4–2.5, with a lobed appressorium (polygoni type) formed on the

Fig. 1. The most parsimonious tree with the highest log likelihood based on the internal transcribed spacer (ITS) sequences data of 50 taxa of *Oidium* subgenus *Pseudoidium*. The tree was obtained by an heuristic search employing the random stepwise addition option of PAUP* (Swofford 2001). Gaps were treated as fifth base. Percentage bootstrap support (1000 replications) is shown *above branches*. Two sequences of *Erysiphe glycines* were used as an outgroup. *Oidium heveae* and *Erysiphe* sp. on *Quercus phillyraeoides* are shown in *boldface*. Tree length = 627, CI = 0.5789, RI = 0.7647, RC = 0.4427
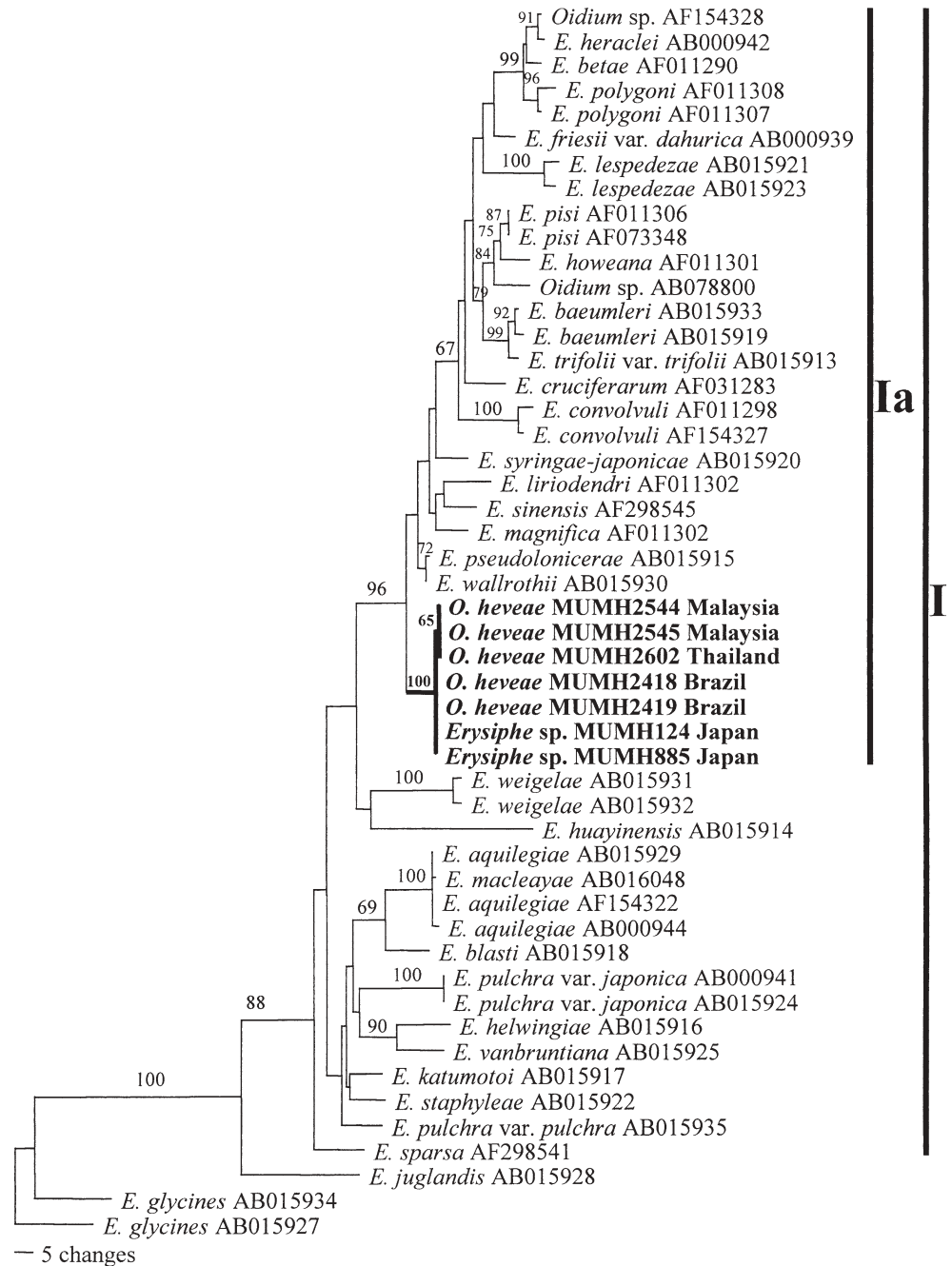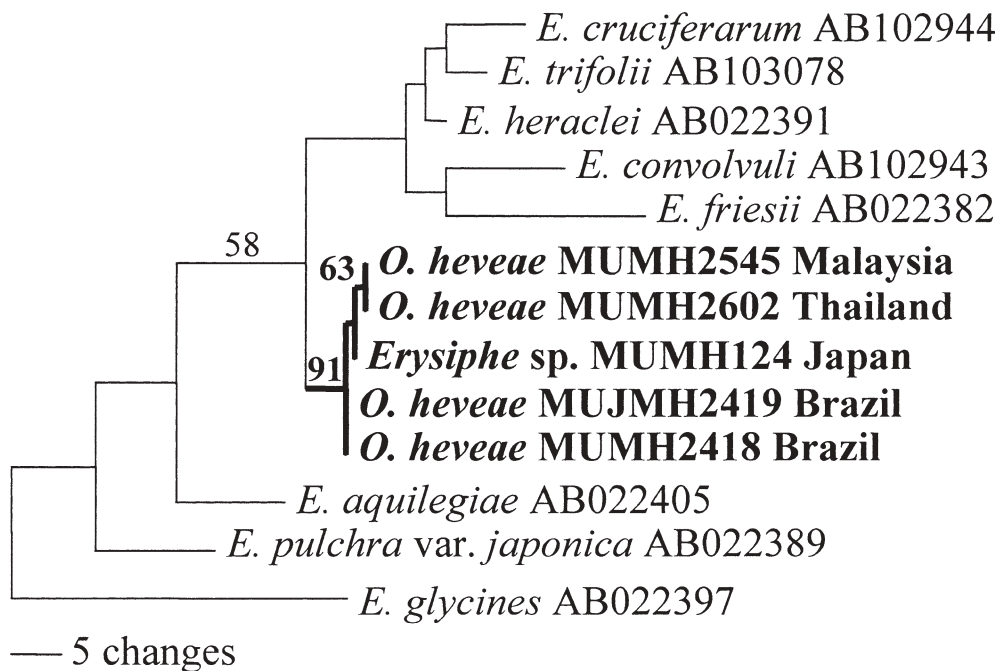
germ tube arising from the end of the conidia. The first conidium formed on a conidiophore (primary conidium) is ellipsoid, rounded at the top part, whereas the subsequently produced ones (secondary conidia) are ellipsoid cylindrical with no round end. Conidiophores are erect. Foot cells are straight, cylindrical, 13.4–61.6 × 7–9.7 μm in size, followed by one to three additional cells (Table 2, Figs. 3–5). Some variation was noted among specimens and between the upper and lower side of a leaf. Usually the size of conidia and length of foot cells were larger on the lower side of leaves (data not shown). These characteristics indicate that the rubber mildew belongs to the anamorphic genus *Oidium* subgenus *Pseudoidium*.

Because the molecular result showed that the ITS and 28S rDNA sequences of *O. heveae* are identical to or only differed by two bases from those of *Erysiphe* sp. on *Q. phillyraeoides*, anamorphic characteristics of the two species were compared. Both species have similar conidial characteristics with ellipsoid to cylindrical conidia and simple to lobed appressoria. Conidiophores arise from the vegetative hyphae with straight and cylindrical foot cells followed by one to three additional cells. Although the conidia of *Erysiphe* sp. on *Q. phillyraeoides* are slightly larger than those of *O. heveae* and the foot cells were shorter than those of *O. heveae*, the l/w ratio is nearly identical between the two species: 1.4–2.5 in *O. heveae* and 1.4–

Table 2. Morphological characteristics of *Oidium heveae* on rubber tree, *Erysiphe* sp. on *Quercus phillyraeoides*, and *E. alphitoides* on *Q. robur*

	Oidium heveae		*Erysiphe* sp.	*E. alphitoides*
	This study	Steinmann (1925)	This study	Braun (1987)
Conidia				
Conidiogenesis	Noncatenate	Catenate	Noncatenate	Noncatenate
Shape	Ellipsoid-cylindrical	Ellipsoid-cylindrical	Ellipsoid-cylindrical	Ellipsoid, ovoid-doliform
Length range (mean)	25.1–43.6 (34.2) μm	28–42 (35) μm	30.8–50 (38.3) μm	25–40 μm
Width range (mean)	13.4–23.3 (18.3) μm	14–23 (17) μm	15.5–21.8 (19) μm	13–23 μm
Length：width ratio range (mean)	1.4–2.5 (1.9)	–	1.4–2.9 (2.1)	1.4–2.3
Germ tube	Polygoni type	–	–	–
Fibrosin bodies	No	–	No	No
Conidiophore				
Foot cell length range (mean)	13.4–61.6 (30.6) μm	–	15.3–46.2 (26.5) μm	15–30 μm
Foot cell width range (mean)	7–9.7 (8) μm	–	7.3–9.7 (8.2) μm	6–9 (–10) μm
Foot cell base	Straight	–	Straight	Straight
No. of additional cells	(1) 2–3	–	(1) 2–3	1–3
Appressoria	Simple lobed	–	Mostly lobed	Lobed

Fig. 2. The most parsimonious tree with the highest log likelihood based on the 28S sequence data of 13 taxa of *Oidium* subgenus *Pseudoidium*. The tree was obtained by an heuristic search employing the random stepwise addition option of PAUP* (Swofford 2001). Gaps were treated as missing data. Percentage bootstrap supports (1000 replications) are shown *above branches*. *Erysiphe glycines* was used as an outgroup. *Oidium heveae* and *Erysiphe* sp. on *Quercus phillyraeoides* are shown in *boldface*. Tree length = 156, CI = 0.7115, RI = 0.6538, RC = 0.4652

Discussion

The powdery mildew fungus of rubber tree is morphologically poorly known (Braun 1987). The rubber mildew was first described by Steinmann (1925) as *Oidium heveae*, in which he reported that the mildew has catenate conidia, often with long chains. In contrast, Peries (1966) reported that the conidia of *O. heveae* are noncatenate. There are some other papers describing the anamorph of the rubber mildew (Thankamma 1968; Mitra and Mehta 1938). However, none mentioned the type of conidial formation. Our present morphological observation clearly showed that the specimens from the rubber tree have noncatenate conidia without fibrosin bodies. In addition, the anamorph possesses simple to lobed hyphal appressoria and a germ tube of the polygoni type, demonstrating that the rubber mildew belongs to *Oidium* subgen. *Pseudoidium* (Table 2, Fig. 5), which supports the report of Peries (1966). In a humid atmosphere, powdery mildews having a *Pseudoidium* anamorph (noncatenate) are often simulated to produce conidia in chains (pseudochains; Boesewinkel 1980). Therefore, the catenate conidia reported by Steinmann (1925) appear to represent pseudochains of *Pseudoidium* produced in high relative humidity.
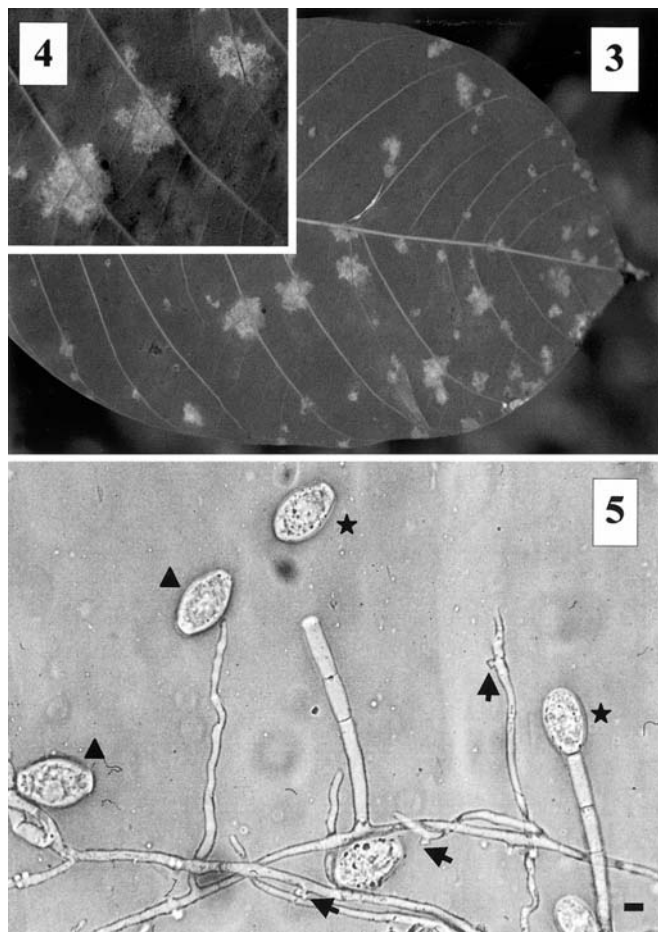
2.9 in *Erysiphe* sp. (Table 2). Therefore, there is no obvious difference in anamorphic characteristics between *O. heveae* and *Erysiphe* sp. on *Q. phillyraeoides*.

Figs. 3–5. Symptoms and anamorph of *Oidium heveae*. **3** Colonies of *O. heveae* on a rubber leaf. **4** Enlargement of **3**. **5** Primary conidia (*stars*), secondary conidia (*triangles*), conidiophores with noncatenate conidia, and hyphae with simple to lobed appressoria (*arrows*) of *O. heveae*. *Bar* 10 μm

Because there is no record of a teleomorph of the rubber mildew, which is necessary to identify the powdery mildew species, we conducted a molecular phylogenetic analysis to clarify the phylogenetic position of *O. heveae* and to link this anamorphic fungus with its allied teleomorph. The result clearly indicated that *O. heveae* is placed in the clade of *Oidium* subgen. *Pseudoidium*, which supports our morphological observation. The phylogenetic analysis of both rDNA regions showed that the *O. heveae* specimens tested form a distinct clade with *Erysiphe* sp. on *Q. phillyraeoides* with 100% bootstrap support. *Erysiphe* (*Microsphaera*) *alphitoides* (Griffon & Maubl.) U. Braun & S. Takam. has been reported as a powdery mildew on *Q. phillyraeoides* in Japan. However, nucleotide sequences of the rDNA ITS region are often different between isolates from different *Quercus* species, and isolates from *Q. phillyraeoides* have a unique ITS sequence (unpublished data). Homma (1937) reported that the appendages of isolates from *Q. phillyraeoides* are shorter than *E. alphitoides* on other *Quercus* species. Therefore, we did not identify the fungus on *Q. phillyraeoides* as *E. alphitoides* in this report. Identification of this fungus will be reported elsewhere.

Comparisons of the anamorphic characteristics of *Erysiphe* sp. on *Q. phillyraeoides* with *O. heveae* were consistent with the present molecular analysis. Consequently, there was no obvious difference in the anamorph of this *Erysiphe* and *O. heveae* (see Table 2). Thus, it is possible that *O. heveae* may be an anamorph of *Erysiphe* sp. on *Q. phillyraeoides*. Cunnington (2002) and Cunnington et al. (2003) reported that the ITS sequence of *O. mangiferae* on mango was identical to that of *E. alphitoides* of *Q. robur*. This report suggests additional close relationships between *E. alphitoides* and anamorphic powdery mildews distributed in tropical and subtropical areas. To further investigate the relationships of *E. alphitoides* and *Pseudoidium* on tropical plants, analyses using many more *Pseudoidium* isolates from a number of tropical plants and cross-inoculation tests are required.

The ITS sequences of the two Brazilian isolates are identical to those of *Erysiphe* sp. on *Q. phillyraeoides*, and only exhibit one base different from the three isolates from Malaysia and Thailand in the ITS2 region, viz., one base deletion in the Brazilian isolates and one adenine insertion in the Southeast Asian isolates at the 14th nucleotide position from the 5′-end of the ITS2. In the 28S sequences, there are two base differences between *O. heveae* isolates from Brazilian and Southeast Asian (Malaysia and Thailand) isolates at nucleotide positions 132 and 517. Both parsimony analyses based on the ITS and 28S rDNA sequences suggest that the Brazilian isolates may be ancestral to the Southeast Asian isolates (see Figs. 1, 2).

The rubber mildew was first recorded in Java in 1918 while the outbreak of this fungus in Brazil first occurred in 1958 (Ramakrishnan and Radhakrishna Pillay 1963). This finding suggests that *O. heveae* spread from Southeast Asia to South America, which conflicts with our present molecular analysis. Breeding programs (Priyadarshan and Goncalves 2003) might have resulted in the spread of the powdery mildew between Brazil and Asia. Because the rubber tree plant originated in the Amazon region of South America and was imported to Southeast Asia (Wastie 1986), it is possible that *O. heveae* was imported from South America to Southeast Asia with rubber trees. A more comprehensive study using isolates from other rubber planting countries is required to understand the genetic diversity and distribution of *O. heveae*.

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