Phylogenetic Analysis of Iranian Powdery Mildew Fungi using Nucleotide Sequences of 28S Ribosomal DNA

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ABSTRACT

The nucleotide sequences of 28S nuclear rDNA were determined for 34 powdery mildew taxa mostly collected from Iran in order to infer the phylogenetic relationships of these fungi. Total DNA was isolated from cleistothecia or mycelia using the chelex method. About a 650 nucleotide length of the 5' end of the 28S rDNA was amplified twice by the PCR using a nested primer set, PM3, TW14 and NLP2. Direct sequencing of the PCR product was done in an Applied Biosystems 373A sequencer. The results showed that powdery mildew taxa are divided into five groups, which were distinguished by their morphology. Members of Erysiphe section Erysiphe, Microsphaera and Uncinula clustered together. E. sect. Galeopsidis and E. sect. Golovinomyces were separated from E. sect. Erysiphe and formed the Euoidium without fibrosin bodies group. Leveillula and Phyllactinia showed a close evolutionary relationship and clustered together. The genera Cystotheca, Podosphaera, Sawadaea, and Sphaerotheca formed a monophyletic group (fibrosin body lineage) with 98% bootstrap support. These fungi are well characterized by the presence of fibrosin bodies in their conidia. Blumeria graminis, which is characterized by some unique morphological characters, clustered with fibrosin body lineage with a low bootstrap value. This result shows that B. graminis is not closely related to the Erysiphe species. The nucleotide divergence between the genera analyzed in this study ranged from 0.50 to 14.10%. The lowest nucleotide divergence was found between Microsphaera and E. sect. Erysiphe (0.50–4.50%). Podosphaera and Sphaerotheca showed a low level of divergence, too (2.30–2.60%), which suggests a close relationship between these two genera.

Keywords: Erysiphaceae, Iran, Phylogeny, Powdery mildew, rDNA.

INTRODUCTION

Powdery mildew fungi belong to the family Erysiphaceae (Ascomycota: Erysiphales) which cause serious diseases in a variety of cultivated plants such as cereals, vegetables, fruit trees and ornamental plants. This family consists of 18 genera and about 435 species (Braun, 1987).

According to Amano (1986), over 169 families and 44 orders of flowering plants are infected by powdery mildew fungi, of which about 90% (162 plant families) areDicotyledons. Phylogenetic relationships among the genera of powdery mildews have been proposed by some authors (Neger, 1901; Blumer, 1933; Braun, 1980; 1987). The morphology of cleistothecial appendages has been used for identification and taxonomic treatment of powdery mildew fungi. However, evolutionary analyses based on morphological characteristics have led authors to contradictory phylogenetic and taxonomic treatments. Blumer (1933) and Braun (1987) and several other mycologists regarded simple, mycelioid appendages as an ancestral feature and that other types of complicated appendages are derived. Tradi-
tionally, generic delimitation of powdery mildew fungi is based on some feature such as the number of asci per ascocarp, the structure of appendages and anamorphic characteristics. However, the value of the appendages has often been overestimated by old and modern taxonomists in the taxonomic system of these fungi. The appendages of *Sphaerotheca* and *Podosphaera* are, for instance, different, but the two genera possess several similar morphological characteristics. Genera such as *Erysiphe* section *Erysiphe* and *Microsphaera* are closely related through several transitional species (Braun, 1987 and 1995) but it must be remarked that these genera are separated by the structure of their appendages that are simple and mycelioid in *Erysiphe* and dichotomously branched in *Microsphaera*. Recently, molecular data have been used to infer phylogenetic relationships among powdery mildew fungi and some proposals have been made. Takamatsu et al. (1998) showed that powdery mildews could be divided into four monophyletic lineages using the nucleotide sequences of rDNA ITS sequences. Saenz and Taylor (1999) using rDNA ITS region identified six evolutionary lineages which corresponded well to mitosporic taxa. Mori et al. (2000) showed that *Uncinula septata* occupied the primitive base of the phylogenetic tree and other powdery mildew taxa excluding *U. septata* were split into five major lineages. In this study, we reinvestigated phylogenetic relationships among Iranian powdery mildew fungi using 28S rDNA nucleotide sequences.

**MATERIALS AND METHODS**

**Sample Sources**

A total of 31 Iranian powdery mildew isolates were sequenced in this study. Three sequences, namely of *Phyllactinia moricola*, *Cystotheca wrightii* and *Pleochaeta shiraiana* were obtained from GenBank, which have been previously collected and sequenced in Japan. Fungi used in this study, their hosts, localities and accession number of the nucleotide sequence data bases (DDBJ, EMBL, and GenBank) are listed in Table 1.

**DNA Extraction and PCR Amplification**

Whole cell DNA was isolated from cleistothecia or mycelia using the chelex method (Hirata and Takamatsu, 1996). About 25 cleistothecia or a piece of mycelia were added to 300 µl of 5% Chelex (Bio-Rad) in a 1.5 ml microcentrifuge tube and incubated at 56 °C for several hours. Then the tubes were incubated in a boiling water bath for 8 minutes. The extract was mixed vigorously and incubated in a boiling water bath again. After mixing, the Chelex solution was centrifugated at 15,000 g for 5 minutes. Ten microliters of supernatant were used as template DNA for the first polymerase chain reaction (PCR). The region including about 650 base pairs of the 5’ end of the 28S rDNA were amplified twice by the PCR using nested primer sets. The primer pairs, namely, PM3 (5’- GKGCTYTMCGTGTAAGT-3´; Takamatsu and Kano 2001)/ TW14 (5’- GCTATCCTGAGGGAAACTTC- 3´) and PM3 / NLP2 (5´-GGTCCCAACA GCTATGCTCT- 3´) were used for the first and second PCR amplifications, respectively (Mori et al. 2000). PCR reactions were conducted in 50 µl volumes as previously described (Hirata and Takamatsu 1996).

**DNA Sequencing**

Nucleotide sequences of the PCR products were obtained for both strands using direct sequencing in an Applied Biosystems 373A sequencer. The sequence reactions were conducted using the Prism Dye Terminator cycle sequencing ready reaction kit (Applied Biosystems) following the manufacturers instruction. The primers, NL1 (5’-AGTACGGCGAGTGAAGCGG-3´), NL2 (5’-TACTTGTTCGCTATCGGTCT-3´), NL3 (5’- AGACCGATAGGGAACAAAGTA- 3´)
and NLP2 were used for the sequencing of the 28S rDNA in both directions (Mori et al., 2000).

**Data Analysis**

The sequences obtained were initially aligned using the Clustal V package (Higgins et al., 1992). The data were analyzed using the Parsimony and Neighbour-joining method by PAUP v.4.0b4a (Swofford, 2000). The strength of the internal branches from the resulting trees were tested by bootstrap analysis (Felsenstein, 1985) using 1000 replications. The sequence data of two fungal taxa, *Byssosphaeria striatosporum* (accession no.: U17912) and *Hypocrea lutea* (accession no.: U00739) were obtained from a gene bank and included as out groups.

**RESULTS AND DISCUSSION**

**Multiple Alignment and Sequence Divergence**

The data matrix consisted of 679 characters, of which 140 were phylogenetically informative. The nucleotide divergence of the 28S rDNA between traditional morpho-
logically defined genera ranged from 0.50 to 14.10%. The divergence between species of *Microsphaera* and *Erysiphe* section *Erysiphe* ranged from 0.50-4.50%. 2.30 to 2.60% divergence was found between *Podosphaera* and *Sphaerotheca*. The divergence among *Uncinula* and closely related genera, namely, *E.* sect. *Erysiphe* and *Microsphaera* was moderate (5.50-6.60%). The divergence between *Leveillula* and two other endophytic genera, *Phyllactinia* and *Pleochaeta* was also moderate (4.40-5.50%). Moderate sequence divergence was found between *Cystotheca* and *Sawadaea* and between the former genus and *Podosphaera* / *Sphaerotheca* (4.40-6.60%). The divergence between *E.* sect. *Erysiphe* and two other sections of *Erysiphe* (sect. *Galeopsidis* and sect. *Golovinomyces*) was high (7.80-11.70%) and a 7.90-8.10% sequence divergence was found between *E.* sect. *Galeopsidis* and sect. *Golovinomyces*. *Blumeria graminis*, which is historically classified as *Erysiphe*, was also highly divergent compared with all of the *Erysiphe* species (9.90-12.40%; see Table 2).

**Phylogenetic Analysis**

Resulting phylogenetic trees are shown in Figures 1 and 2. The powdery mildew species in this study were divided into five clades. According to Hillis and Bull (1993), branches receiving greater than 70.0% bootstrap support are correct more than 95% of

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**Figure 1.** A strict consensus tree inferred from sequences of the 28S rDNA gene. Branch support was determined by 1000 bootstrap replication, shown above the branches. Tree length is 563, the consistency index (CI) is 0.59, the retention index (RI) is 0.46. Bootstrap values below 50% are not shown.
the time. These five clades received usually high bootstrap support. One exception was found in the case of the endophytic clade. In this clade Pleochaeta clustered with Phyllactinia and Leveillula with a bootstrap support less than 50% in the neighbour-joining method, whereas the genus was separated from other two genera in most parsimony method.

The five major clades were well characterized with their morphology and can be classified as follows:

**Pseudoidium clade**

This clade includes the E. sect. Erysiphe, Microsphaera and Uncinula. All taxa in this group produce single conidia and, according to Cook et al. (1997), their anamorphs belong to the Oidium subgenus Pseudoidium. Moreover, in this lineage Microsphaera-Erysiphe, and Erysiphe-Uncinula are linked with some morphologically intermediate species. The present
Table 2. Matrix of percentage sequence divergence among 28S rDNA region from some selected powdery mildew species.\(^a\)

| Fungus name       | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 |
|-------------------|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| E. cruciferum     | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  |
| E. lycopsis       | 1.20 | - | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  |
| E. convolvuli     | 2.30 | 2.70 | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  |
| E. heraclei Daucus| 1.20 | 1.80 | 1.70 | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  |
| E. pisi           | 4.40 | 5.30 | 4.80 | 4.40 | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  |
| M. mulaappendicis | 1.20 | 1.80 | 2.0 | 0.80 | 4.50 | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  |
| M. trifolii       | 1.10 | 1.70 | 1.80 | 0.80 | 4.40 | 1.20 | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  |
| M. allagi         | 1.40 | 2.00 | 1.50 | 0.50 | 4.20 | 0.90 | 0.60 | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  |
| U. clandestina    | 5.80 | 6.10 | 6.60 | 6.10 | 5.50 | 5.90 | 6.10 | 5.90 | -  | -  | -  | -  | -  | -  | -  | -  | -  |
| L. taurica        | 9.50 | 9.50 | 9.80 | 9.50 | 8.40 | 9.70 | 9.10 | 9.20 | 8.30 | -  | -  | -  | -  | -  | -  | -  | -  |
| Artemisia         |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| Ph. moricula      | 9.60 | 9.90 | 10.80 | 9.30 | 8.50 | 10.20 | 10.40 | 9.70 | 8.30 | 4.40 | -  | -  | -  | -  | -  | -  | -  |
| Pl. shiraiana     | 9.40 | 9.30 | 9.90 | 9.50 | 7.60 | 9.40 | 9.60 | 9.10 | 7.70 | 7.10 | 5.50 | -  | -  | -  | -  | -  | -  |
| G. cichic Cucurbita| 8.60 | 8.10 | 8.40 | 8.30 | 9.0 | 9.20 | 9.0 | 8.30 | 8.60 | 9.0 | 7.90 | -  | -  | -  | -  | -  | -  |
| Erysiphe gallii   | 10.90 | 11.30 | 11.60 | 11.40 | 9.40 | 11.30 | 11.20 | 10.90 | 11.30 | 11.10 | 11.50 | 9.50 | 7.90 | -  | -  | -  |
| P. clandestina    | 9.80 | 10.0 | 10.70 | 10.20 | 8.10 | 10.10 | 10.10 | 10.10 | 8.30 | 9.90 | 10.20 | 8.90 | 10.40 | 13.0 | 2.30 | -  | -  |
| S. bicorous      | 9.50 | 9.50 | 10.10 | 9.80 | 8.40 | 9.80 | 9.80 | 9.80 | 8.70 | 10.70 | 11.10 | 9.60 | 10.20 | 14.0 | 5.50 | 4.70 | -  |
| Cys. wrightii     | 11.20 | 11.20 | 12.10 | 11.60 | 9.80 | 11.80 | 11.80 | 11.80 | 9.30 | 10.40 | 10.80 | 8.80 | 11.0 | 13.70 | 6.20 | 5.30 | 4.70 |

\(^a\) Some taxa did not included in this table.
phylogenetic analysis also showed that these taxa did not group into separate monophyletic lineage and Microsphaera and E. sect. Erysiphe are closely related to each other.

This analysis coincides well with the results of some other researchers which have been recently published (Takamatsu et al., 1999; Saenz and Taylor, 1999; and Mori et al., 2000).

**Endophytic clade**

This clade includes Leveillula, Phyllactinia and Pleochaeta. Several authors (Braun, 1987; Cook et al., 1997) have considered that Leveillula and Phyllactinia are closely related. Moreover, Braun (1987) placed Pleochaeta as an intermediate genus between Phyllactinia and Leveillula.

This results significantly support a close relationship between Leveillula and Phyllactinia, but the intermediate position of the genus Pleochaeta is under question, because Pleochaeta made a separate clade in the maximum parsimony method and clustered with Phyllactinia and Leveillula with a bootstrap support less than 50% in the neighbour-joining method. Presence of the endophytic mycelia and morphology of the anamorph, which is closely related to mitosporic state of Phyllactinia and Leveillula, showed that Pleochaeta could be a member of the endophytic group, but more likely it comprises more primitive bases than other endophytic genera and could be an ancestral genus in the endophytic group.

**Euoidium clade without fibrosin bodies**

This clade includes E. orontii and E. galii which belong to E. sects. Golovinomyces and Galeopsidis, respectively. Both sections are well characterized with catenate conidia without fibrosin bodies. The genus Erysiphe shares the characteristics of polyascal cleistothecia. However, anamorphic characters support dividing Erysiphe into two or three different genera, which have been proposed by some authors. Sawada (1951, 1959) proposed Ischochaeta for the Erysiphe species with Pseudooidium type anamorph. Golovin (1958) named this genus as Linkomyces.

In our analysis, both sections clustered outside sect. Erysiphe. These results clearly support a polyphyletic origin for Erysiphe s. lat. Moreover, the sequence divergence between the sections Golovinomyces and

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**Figure 3.** First Polymerase chain reaction (PCR) performed with primers PM3 and TW14 that produced a sharp bands on agarose gel.
Galeopsidis was high in the 28S rDNA and similar to those between distantly related genera.

**Euoidium with fibrosin bodies**

This clade comprises the genera Sphaerotheca, Podosphaera, Cystotheca and Sawadaea which was strongly supported by bootstrap analysis. These taxa are well characterized with well-developed fibrosin bodies in their conidia.

Moreover, all genera of this clade possess monoascal cleistothecia except Sawadaea. Podosphaera and Sphaerotheca are closely related morphologically as well as genetically. The sequence divergence between Podosphaera and Sphaerotheca was significantly low (2.3-2.6%) and similar to those between species of respective genera. This result showed close relations between Podosphaera and Sphaerotheca and monophyly of these genera.

**Blumeria clade**

*Blumeria graminis* infects Poaceae (Gramineae) and possesses some unique morphological characters (digitate haustoria, bulbous foot cell of conidiophore and brittle-like secondary hyphae.).

*B. graminis* which has been historically classified as Erysiphe showed to be more distinct evolutionary lineage and clearly clustered outside of the rest of *Erysiphe* s. lat. As suggested by Braun (1987), *B. graminis* seems to be one of the old powdery mildew genera which diverged early in the evolution of powdery mildew fungi.

**REFERENCES**

Phylogenetic Analysis of Iranian Powdery Mildew Fungi


تجزیه و تحلیل فیلوژنیک جدایی های ایرانی سفیدکهای سلخی بر اساس توالی B به

28S دی ای ریبووزومی

س. 1. خداوندست، س. ناکاماتسو و ق. حجارود

چکیده

به منظور بررسی مسائل تاکسونومیک فارچه‌ای تره Erysipheaceae در این مطالعه ساختار فیلوژنیک

Erysiphe s. lat. /dalisolated/alefisolated/dalfinal/nooninitial /noonisolated/aleffinal/sheenmedial/nooninitial .

شده. هر یک از کلیپتستینوم و یا میسیلوم با استفاده از روش Chelax. شده. حاوی 140 جفت

باز از انتمای 5'-28S rDNA 28S rDNA از بار با واکنش نیایش ای پلی همزایی با کمک آغازگرهای PM3، انی جرید. بر اساس نتایج حاصل از تجزیه و تحلیل توالی DNA 28S rDNA کلیه تاکسونوپیا مورد مطالعه در

Erysiphe sect. 

(Pseudoidium) در دیدگاه گروه موتوفیلیک قرار گرفته (گروه Uncinula) و Microsphaera Erysiphe

Erysiphe sect. Golovinomyces و Erysiphe sect. Galeopsidis

Leveillula رانشیکل دادند. Erysiphe sect. Erysiphe

زنجیری از منابعی شوند. گروه Erysiphe sect. Erysiphe

Sawadaea و Podosphaera

در گروه اندوفیت قرار گرفته.

Blumeria graminis

Erysiphe s. lat. 

نگاه گروه موتوفیلیک دیگری تشکیل دادند که مهم‌ترین وجه اشکال زنجیری Erysiphe s. lat. 

Sawadaea و Podosphaera

گروه و چندین گونه زنجیری حاوی اجسام فیروزوئن می‌باشد.

دایر شده برخی صفات موتوفیلیک نظر وجود پایه تومر در روی کنیدوفر، پنج گونه دیده

هوستوریک و... از سایر تاکسونها به راحتی قابل تشخیص است، گروه پنج گونه تشکیل داد و هیچگونه

نیاز نداشته.