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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 seperated 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) are Dicotyledons. 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 characterstics 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-

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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 characters. 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´- GKGCTYTMCGCGT AGT-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´-AGTA ACGGCGAGTGAAGCGG-3´), NL2 (5´-TACTTGTTCGCTATCGGTCT-3´), NL3 (5´- AGACCGATAGCGAACAAGTA- 3´)

Table 1. Sources of fungal materials, hosts, locations and sequence database accession numbers.

Fungal name	Host plant	Location	GenBank Accession no.
Blumeria graminis	*Hordeum* sp.	Guilan, Amarlu	AB103065
Cystotheca wrightii	*Quercus glauca*	Japan	AB022355
Erysiphe betae	*Beta vulgaris*	Iran, ?	AB079684
E. convolvuli	*Convolvulus arvensis*	Karaj	AB102943
E. cruciferarum	*Cardaria draba*	Guilan, Manjil	AB102944
E. galii	*Phuopsis stylosa*	Guilan	AB103365
E. heraclei	*Bifora testiculata*	Guilan, Amarlu	AB103066
E. heraclei	*Conium maculatum*	Guilan, Amarlu	AB103068
E. heraclei	*Chaerophyllum aureum*	Guilan, Amarlu	AB103067
E. heraclei	*Daucus carrota*	Guilan, Mangil	AB103069
E. heraclei	*Pimpinella affinis*	Guilan, Amarlu	AB103366
E. heraclei	*Torilis* cf. *leptophylla*	Guilan, Masuleh	AB103071
E. lycopsidis	*Anchusa ovata*	Guilan, Amarlu	AB103072
E. orontii	*Cucurbita* sp.	Guilan, Talesh	AB103073
E. orontii	*Valerianella* cf. *uncinata*	Guilan, Masuleh	AB077693
E. pisi	*Medicago sativa*	Guilan, Deileman	AB102942
Leveillula lanuginosa	*Daucus carrota*	Guilan, Mangil	AB042641
L. saxouli	*Haloxylon* sp.	Khorasan	AB080469
L. simonianii	*Thevenotia persica*	Esfahan	AB080477
Leveillula sp.	*Chondrilla juncea*	Guilan, Roodbar	AB080478
L. cylindrospora	*Noaea mucronata*	Guilan, Amarlu	AB080468
L. taurica	*Artemisia annua*	Guilan, Roodbar	AB080470
L. taurica	*Impatiens* sp	Karaj	AB080473
L. latucae-serriolae	*Lactuca serriola*	Guilan, Roodbar	AB080476
Microsphaera alhagi	*Alhagi* sp.	Guilan, Roodbar	AB103077
M. multiappendicis	*Berberis vulgaris*	Guilan, Deileman	AB103076
M. trifolii	*Trifolium pratense*	Esfahan	AB103078
Phyllactinia moricula	*Morus australis*	Japan	AB022401
Pleochaeta shiraiana	*Celtis sinensis*	Japan	Ab022403
Podosphaera clandestina	*Cydonia oblango*	Guilan, Roodbar	AB103070
Sawadaea bicornis	*Acer hyrcanum*	Guilan, Amarlu	AB103370
Sphaerotheca fusca	*Cucurbita* sp.	Guilan, Sumeehsara	AB103368
Sphaerotheca fusca	*Xanthium* sp.	Guilan, Talesh	AB103369
Uncinula clandestina	*Ulmus* sp.	Guilan	AB103070

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, *Byssoascus striatosporus* (accesion no.: U17912) and *Hypocrea lutea* (accesion 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

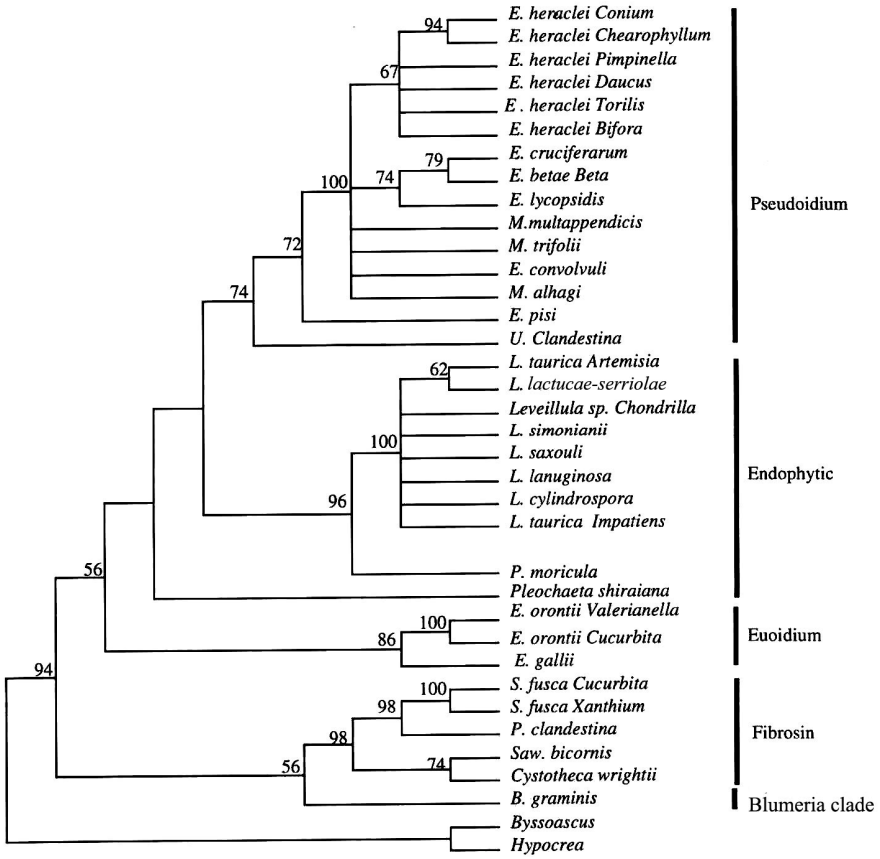

Figure 1. A strict consensus tree inferred from sequences of the 28S rDNA gene. Branch support was determined by 1000 bootstrap replication, shown above the branches. Tree length is 563, the consistency index (CI) is 0.59, the retention index (RI) is 0.46. Bootstrap values below 50% are not shown.

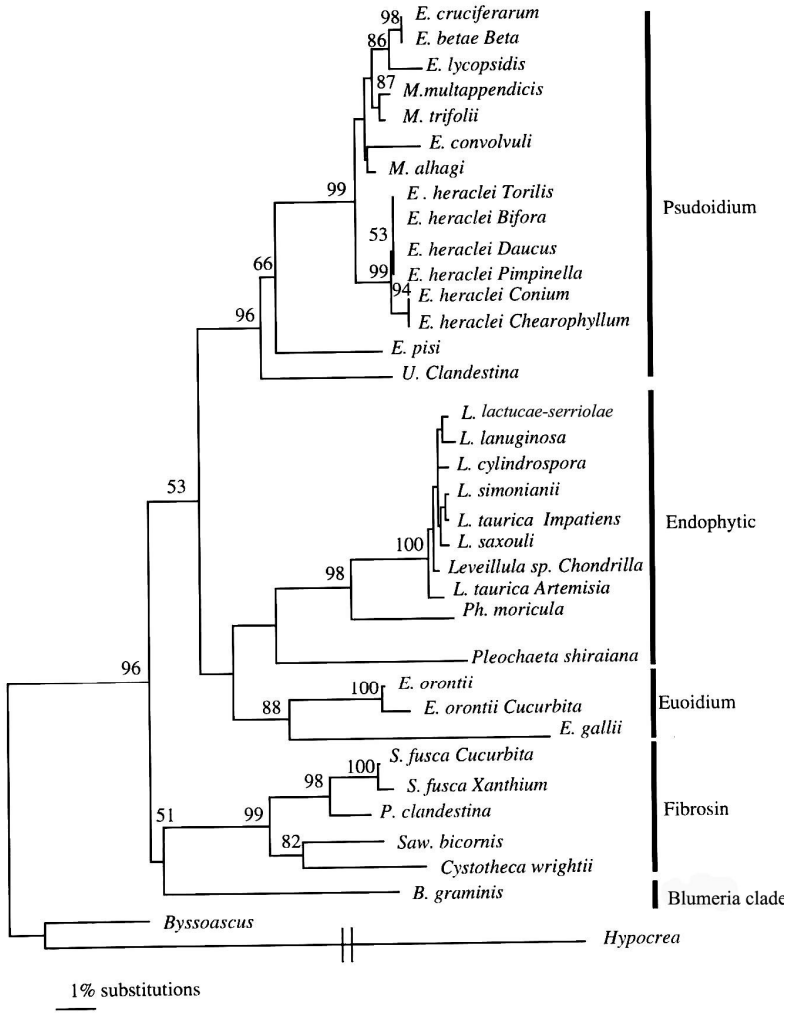

Figure 2. Neighbour-joining tree inferred from sequences of the 28S rDNA gene. Branch support was determined by 1000 bootstrap replication, shown above the branches. Bootstrap values below 50% are not shown.

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 seperated 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
1	*E. cruciferarum*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	*E. lycopsidis*	1.20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	*E. convolvuli*	2.30	2.70	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4	*E. heraclei Daucus*	1.20	1.80	1.70	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5	*E. pisi*	4.40	5.30	4.80	4.40	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6	*M. multappendicis*	1.20	1.80	2.0	0.90	4.50	-	-	-	-	-	-	-	-	-	-	-	-	-
7	*M. trifolii*	1.10	1.70	1.80	0.80	4.40	1.20	-	-	-	-	-	-	-	-	-	-	-	-
8	*M. alhagi*	1.40	2.00	1.50	0.50	4.20	0.90	0.60	-	-	-	-	-	-	-	-	-	-	-
9	*U. clandestina*	5.80	6.10	6.60	6.10	5.50	5.90	6.10	5.90	-	-	-	-	-	-	-	-	-	-
10	*L. taurica Artemisia*	9.50	9.50	9.80	9.60	8.40	9.70	9.10	9.20	8.30	-	-	-	-	-	-	-	-	-
11	*Ph. moricula*	9.60	9.90	10.80	9.90	8.50	10.20	10.40	9.70	8.30	4.40	-	-	-	-	-	-	-	-
12	*Pl. shiraiana*	9.40	9.30	9.90	9.60	7.60	9.40	9.60	9.10	7.70	7.10	5.50	-	-	-	-	-	-	-
13	*G. cicho Cucurbita*	8.60	8.10	9.40	8.90	8.30	9.0	9.20	9.0	8.30	8.60	9.10	7.90	-	-	-	-	-	-
14	*Erysiphe galii*	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	-	-	-	-	-
15	*S. fusca Cucurbita*	9.70	9.50	10.40	9.30	9.0	9.80	9.80	9.80	9.20	10.30	10.70	9.10	10.90	14.10	-	-	-	-
16	*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	-	-	-
17	*Saw. bicornis*	9.50	9.50	10.10	9.90	8.40	9.80	9.80	9.80	8.70	10.70	11.10	9.60	10.20	14.0	5.50	4.70	-	-
18	*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	-
19	*B. graminis*	10.90	10.90	10.90	10.70	9.90	10.60	10.70	10.20	10.20	10.70	11.70	9.80	10.60	12.40	9.60	8.80	9.10	10.80

[a] Some taxa did not included in this table.

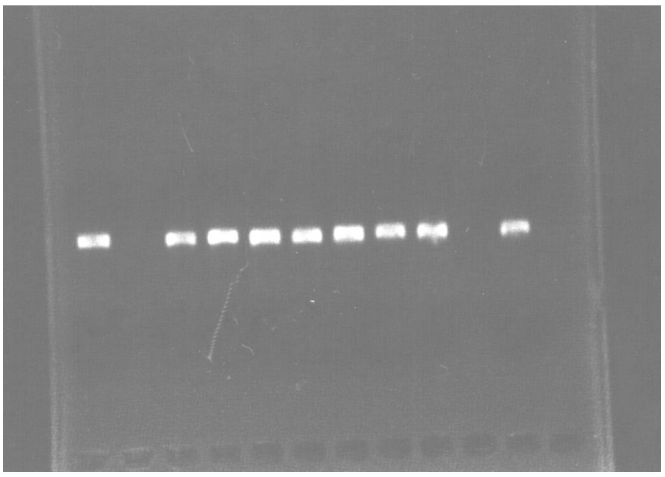

Figure 3. First Polymerase chain reaction (PCR) performed with primers PM3 and TW14 that produced a sharp bands on agarose gel.

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 Psueudoidium 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

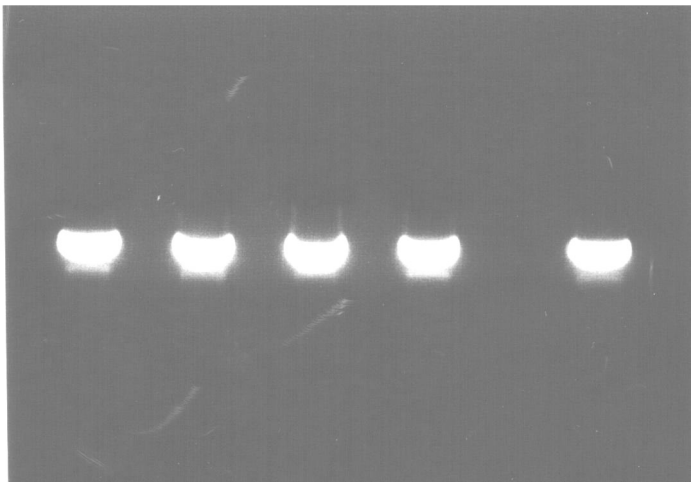

Figure 4. DNA products after second PCR using primers PM3 and NLP2.

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 bristle-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.

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تجزیه و تحلیل فیلوژنتیک جدایه های ایرانی سفیدکهای سطحی بر اساس توالی بخش 28S دی ان ای ریبوزومی

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چکیده

به منظور بررسی مسایل تاکسونومیک قارچهای تیره Erysiphaceae، در این مطالعه ساختار فیلوژنتیک بین ۲۷ تاکسون جمع آوری شده از ایران بر اساس توالی نوکلئوتیدهای بخشی از DNA ریبوزومی مطالعه شد. DNA کل از کلیستوتسیوم و یا میسلیوم با استفاده از روش Chelax استخراج شد. حدود ۶۵۰ جفت باز از انتهای '5 28S rDNA دو بار با واکنش زنجیره ای پلی مراز به کمک آغازگرهایPM3 ، TW14 و NLP2 تکثیر شد. توالی یابی مستقیم محصول PCR در دستگاه توالی یاب Applied Biosystems 373a انجام گردید. براساس نتایج حاصل از تجزیه و تحلیل توالی 28S rDNA، کلیه تاکسونهای مورد مطالعه در پنج گروه قرار گرفتند که این گروه‌ها از نظر مرفولوژی نیز قابل تفکیک هستند. *Erysiphe* sect. *Erysiphe*، *Microsphaera* و *Uncinula* دریک گروه مونوفیلتیک قرارگرفتند (گروهPseudoidium). *Erysiphe* sect. *Galeopsidis* و *Erysiphe* sect. *Golovinomyces* که به دلیل داشتن کنیدیومهای زنجیری از *Erysiphe* sect. *Erysiphe* متمایزمی‌شوند، گروه Euoidium راتشکیل دادند. *Leveillula*، *Phyllactinia* و *Pleochaeta* در گروه اندوفیت قرار گرفتند. *Cystotheca*،*Sphaerotheca*، *Podosphaera* و *Sawadaea* نیز گروه مونوفیلتیک دیگری تشکیل دادند که مهمترین وجه اشتراک این گروه وجود کنیدیومهای زنجیری حاوی اجسام فیبروزین می باشد. *Blumeria graminis* که به دلیل داشتن برخی صفات مرفولوژیک نظیر وجود پایه متورم در روی کنیدیوفر، پنجه ای شکل بودن هوستوریوم و... از سایر تاکسونها به راحتی قابل تشخیص است، گروه پنجم را تشکیل داد و هیچگونه قرابت فیلوژنتیک با گونه های مورد مطالعه *Erysiphe* s. lat. نشان نداد.