

A reappraisal of the Pyriculariaceae in Iran

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Abstract: The family Magnaporthaceae considered as a single-family placed in the order Magnaporthales. Currently, three distinct clades are phylogenetically distinguished in this order that one is referred as the Pyriculariaceae. The Pyriculariaceae is characterized as a novel family having new genera, new species, new combinations, and neotypification (basonym). To taxonomically evaluate this family, an extensive sampling of several host plants including rice, corn, sorghum, barley and wheat cultivation regions, tea, citrus orchards and forests in the south of Caspian coast from Astara in Guilan Province to Gonbad-e Qabus in Golestan Province, Iran, was conducted for 2 consecutive years. One hundred thirty isolates were obtained from the collected samples showing symptoms of the blast and leaf spot. Morphological characteristics such as shape, color, and the size of conidia and conidiophores and colony color were determined. In order to phylogenetically investigate the gained isolates, the ITS region of ribosomal DNA, DNA replication licensing factor (*MCM7*) and *calmodulin* (*CAL*) genes were utilized as a phylogenetic marker. Three major clades were recognized in the phylogenetic tree constructed based on the ITS and *MCM7* regions. The first clade belongs to the Pyriculariaceae family containing two subclades. Moreover, the Pyriculariaceae clade has two subclades generated based on the analysis of the *CAL* gene.

Key words: *Pyricularia*, new family, morphology, phylogeny.

INTRODUCTION

The family of Magnaporthaceae was proposed by Cannon (1994) to accommodate *Magnaporthe* R. A. Krause & R.K. Webster and its related genera such as *Gaeumannomyces* Arx & D.L. Olivier. This family was originally considered as a single-family placed in the order Magnaporthales and it is closely associated with the Diaporthales and Ophiostomatales based on the phylogenetic studies performed previously (Zhang et al. 2011, Besi et al. 2009, Thongkantha et al. 2009). The family is characterized by nonstromatic black perithecia, usually with long hairy necks, persistent asci, and elongate fusiform or filiform ascospores. Typically, it includes necrotrophic pathogens that are known to infect grasses. The Magnaporthaceae members are hyphomycetes and are diverse but they can be categorized as two morphotypes named as *Pyricularia*-like or *Phialophora*-like. Recently, the phylogenetic study performed by Klaubauf et al. (2014) using a multigene approach (LSU, ITS, RPB1, actin and calmodulin) resulted in the establishment of a new family termed as Pyriculariaceae Klaubauf, Lebrun & Crous.

The Pyriculariaceae was introduced as new family containing new genera, new species, new combinations, and neotypification (basonym). *Pyricularia oryzae* Cavara belonging to the Pyriculariaceae is the causal agent of the rice blast disease, a serious threat for rice (*Oryza sativa* L.) cultivation worldwide. This fungus annually causes yield losses averaging upwards of 30% (Yaegashi 1977, Landschoot & Jackson 1989). A close relative species of *P. oryzae* is *Pyricularia grisea*, which is indistinguishable in terms of conidium, perithecium, and ascospore morphology. *Pyricularia grisea* isolates collected from *Digitaria* were shown to form a distinct clade by the phylogenetic analysis (Couch & Kohn 2002, Hirata et al. 2007). Currently, the Magnaporthaceae *sensu stricto* contains 11 genera including *Buergenerula*, *Bussabanomyces*, *Endopyricularia*, *Gaeumannomyces*, *Harpophora*, *Kohlmeyeriopsis*, *Magnaporthiopsis*, *Nakataea*, *Omnidemtus*, *Pyriculariopsis* and *Slopeiomyces*.

To re-evaluate the systematic relationships among Magnaporthaceae *sensu lato* species and revision of the Pyriculariaceae in Iran, we phylogenetically analyzed relationships among isolates representing a wide range of host plants by using partial DNA sequences of multiple genes such as ITS, *MCM7*, and

calmodulin. Additionally, morphological features of some identified species were described in this study.

MATERIALS AND METHODS

Fungal isolates

During spring, summer and fall of 2012 and 2013, a comprehensive sampling of various host plants including rice, corn, sorghum, barley, wheat, tea, citrus orchards and forests in the south of Caspian Sea (Astara to Gonbad-e Qabus) were conducted (Fig. 1). Furthermore, numerous samples were collected from other provinces such as Khorasan Razavi, Arak, and Qazvin. Subsequently, the collected samples were transferred to the Mycological laboratory of the University of Tehran. For morphological studies, a single spore generated on the surface-sterilized leaf under at 25°C temperatures was transferred to plates filled with water agar and autoclaved rice leaves (WA+ rice leaves). Plates were incubated at 23–25 °C under a regime of 12 h dark/12 h near-ultraviolet light. The *Nakataea* isolates incubated at 23–25 °C

under fluorescent light for sporulation, and examined after 10-15 days. The pictures were taken via the Olympus microscope model BH2.

DNA extraction and sequencing

DNA extraction was done according to the protocol developed by Zhong and Stephenson (2001). PCR amplification of the internal transcribed spacer (ITS) and DNA replication licensing factor (*MCM7*) gene was performed based on the protocols described by Zhang et al. (2011) and for *calmodulin* (*CAL*) gene, the protocols established by Hirata et al. (2007) was employed. Sequence alignments were conducted with Clustal X 1.8 (Thompson et al. 1997). The distance matrix was calculated using Kimura's two-parameter method (Kimura 1980) and analyzed with the Maximum Likelihood (ML) using the software called MEGA 5.10 (Tamura et al. 2011). The statistical accuracy of the tree was tested by bootstrap analysis (1000 repetitions) and the generated sequences have been deposited in the GenBank.



Fig. 1. Schematic representation of the sampling locations in south of Caspian coast

RESULTS

Morphology

One hundred and thirty isolates were collected from leaves having symptoms of the blast and leaf spot. Morphological characterization of the tested isolates, including colony color, shape, color and the size of conidia and conidiophores demonstrated that all the examined isolates were belonged to the six species comprising *Pyricularia oryzae* (80 isolates), *P. grisea* (30 isolates), *Nakataea oryzae* (5 isolates), *Pseudopyricularia higginsii* (8 isolates), *Pseudopyricularia* sp.1 (10 isolates) and *Pseudopyricularia* sp.2 (4 isolates). Species of the *P. oryzae* and *P. grisea* are different in terms of morphological features as well as host plants. Conidiophores sizes are 100-250 µm in *P. oryzae* and 70-175 µm in *P. grisea*; and the conidial size are 16-25×7-10 µm in *P. oryzae*; and 26-31× 6-8 µm in *P. grisea* (Fig. 2. a, b, c, d, e, f).

The genus *Nakataea* has gray to a dark gray colony; some conidiophores are branched, 130-460 µm long; conidia falcate, sigmoid, curved, 45-76 × 10-14 µm, septate and two middle cells brown (Fig.

2. s, v). The genus *Nakataea* has some similarity to *Pyricularia* in overall morphological characters, but it differs in falcate to sigmoid conidia with darker median cells (Luo & Zhang 2013).

Pseudopyricularia higginsii has 3-septate conidiophores with 45-125 µm length; 2-septate conidia with the size of 22–29 × 6–7 µm (Fig. 2. g, i). *Pseudopyricularia* sp.2, conidiophores without septa or rarely 2-septa, the size of 87–140 (–170) × 5–6 µm. Conidia solitary, dry, fusiform or cylindrical, hyaline, 23–29 × 5–8 µm, smooth, 2-septate, hilum often protuberant (Fig. 2. n-r). *Pseudopyricularia* sp.1, conidiophores branch, straight, 1–2(–4)-septate, 65–125(–140) × 4–5 µm. Conidia solitary, dry, obpyriform to obclavate, hyaline, (17–) 22–27(–29) × (5–) 6–8 µm, smooth, 1-septate, hilum often protuberant (Fig. 2. j, m). The *Pseudopyricularia* sp.1 species is similar to *Pyricularia bothriochloae* in having 1-septate conidia. However, the conidia of *Pseudopyricularia* sp.1 are larger than that of *Pyricularia bothriochloae*. Additionally, it differs in conidiophores septa and size.

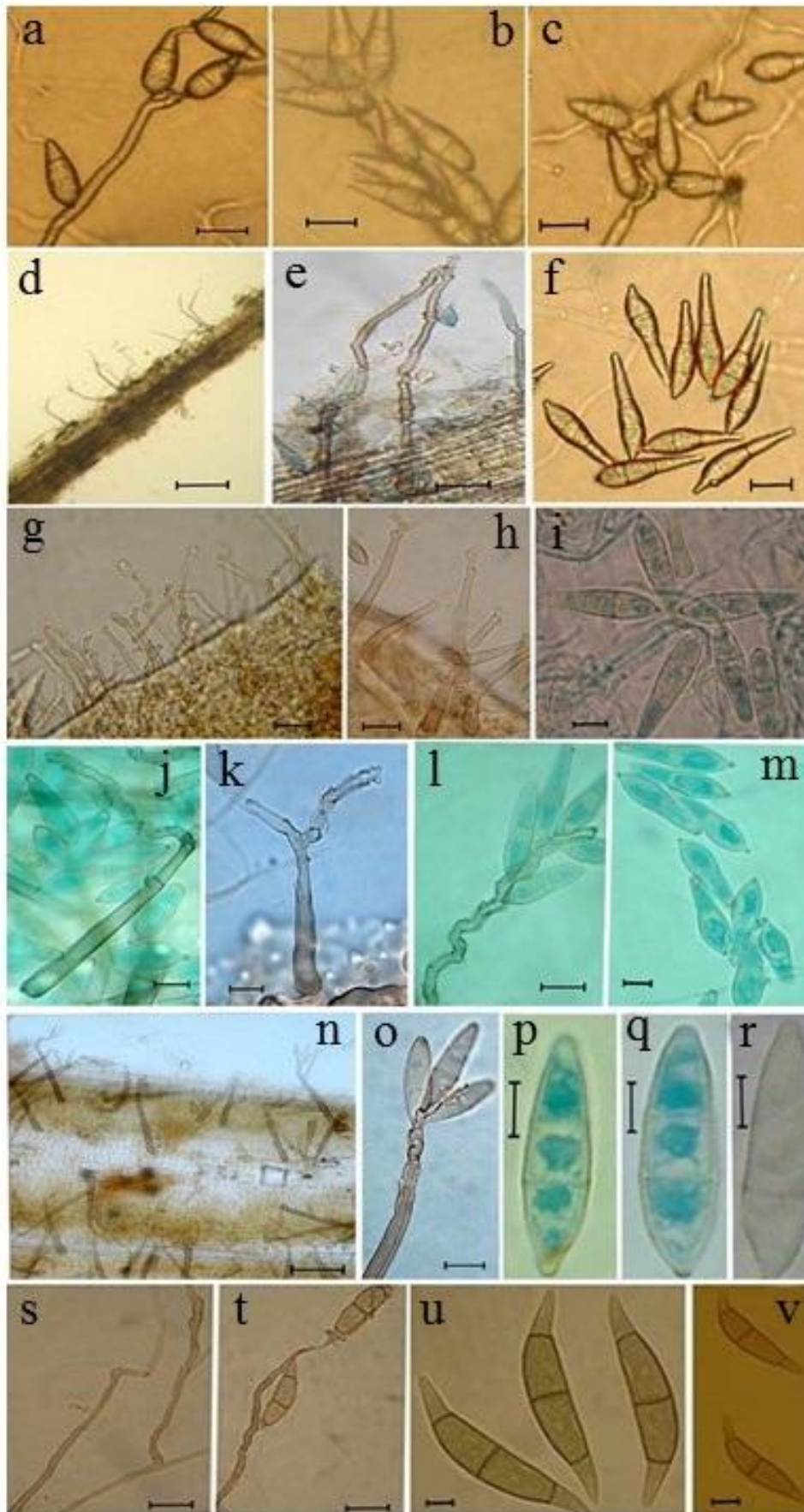


Fig. 2. *Pyricularia oryzae*. **a.** conidiophore; **b-c.** conidia. *Pyricularia grisea* **d-e.** conidiophores; **f.** conidia. *Pseudopyricularia higginsii* **g-h.** conidiophores; **i.** conidia. *Pseudopyricularia* sp. 1 **j-k.** conidiophore; **l-m.** conidia. *Pseudopyricularia* sp. 2. **n-o.** conidiophore; **p-r.** conidia. *Nakataea oryzae* **s-t.** conidiophore; **u-v.** conidia. — Scale bars = 10 μ m.

Phylogeny

A total of 31 strains belonging to 6 species were studied. The isolate numbers, sources, hosts and GenBank accession numbers for the ITS region are included in Table 1.

The results of alignment indicated a total of 512, 410 and 618 nucleotide characters including gaps in ITS, CAL, and MCM7, respectively. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model. Initial tree(s) for the heuristic search were automatically obtained by applying Neighbor-Join and Bio NJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions containing gaps and missing data were eliminated.

There were three main clades in the phylogenetic tree constructed based on the ITS and *MCM7* regions. The Clade A containing ninety-nine species were supported with high bootstrap (99%) whereas that of the Clade B including two species was 100%. In addition, the bootstrap of the clade C possessing five species was 99%. The aforementioned trees were constructed based on the sequencing of the ITS

region. The clade A contained 15 species with high support while both clade B and C had four species supported with 94% and 99% bootstrapping, respectively, in the phylogenetic tree generated based on the analysis of the *MCM7*. In clade A, species *P. oryzae* and *P. grisea* were placed within subclade I. These species are different in phylogeny, morphology, and host plants. Conidiophores and conidial size are diverse in two species. Species of the *Pseudopyricularia higginsii*, *Pseudopyricularia* sp.1, and *Pseudopyricularia* sp.2 were clustered within subclade II. These four species are variable in terms of morphological features.

Magnaporthiopsis poae and *M. rhizophila* were grouped in the clade B in the constructed tree based on the ITS region and the clade C, which was made based on the *MCM7*, respectively. Species positioned into these groups generate *Phialophora*-like anamorph and these species are necrotrophic pathogens on grass root. *Nakataea oryzae* isolates placed in the basal clade of the phylogenetic tree generated based on the ITS region and were placed in the clade B based on the *MCM7*. This species is different from the *Pyricularia* concerning to the anamorphic and phylogenetic characteristics (Fig. 3, 4).

Table 1. Overall characterizations of fungal species used in this study.

Species	Strain code	Source	Host	ITS
<i>Pyricularia oryzae</i>	UTFC-PO1	Mazandaran, Iran	<i>Echinochloa crus-galli</i>	KP144439
<i>P. oryzae</i>	UTFC-PO2	Mazandaran, Iran	<i>Paspalum distichum</i>	KP144440
<i>P. oryzae</i>	UTFC-PO3	Guilan, Iran	<i>Oryza sativa</i>	KP144441
<i>P. oryzae</i>	UTFC-PO4	Mazandaran, Iran	<i>Zea mays</i>	KP144442
<i>P. oryzae</i>	UTFC-PO5	Golestan, Iran	<i>Setaria viridis</i>	KP144443
<i>P. grisea</i>	UTFC-PO6	Mazandaran, Iran	<i>Digitaria</i> sp.	KP144438
<i>P. grisea</i>	UTFC-PO7	Mazandaran, Iran	<i>Digitaria</i> sp.	KP144438
<i>Pseudopyricularia higginsii</i>	UTFC-PO8	Mazandaran, Iran	<i>Cyperus</i> sp.	KP144446
<i>P. higginsii</i>	UTFC-PO9	Mazandaran, Iran	<i>Cyperus</i> sp.	KP144446
<i>Pseudopyricularia</i> sp.1	UTFC-PO10	Golestan, Iran	<i>Cyperus alternifolius</i>	KP144447
<i>Pseudopyricularia</i> sp.1	UTFC-PO11	Golestan, Iran	<i>Cyperus alternifolius</i>	KP144448
<i>Pseudopyricularia</i> sp.1	UTFC-PO12	Golestan, Iran	<i>Cyperus alternifolius</i>	KM207211
<i>Pseudopyricularia</i> sp.2	UTFC-PO13	Golestan, Iran	<i>Juncus</i> sp.	KM207210
<i>Pseudopyricularia</i> sp.2	UTFC-PO14	Golestan, Iran	<i>Juncus</i> sp.	----
<i>Nakataea oryzae</i>	UTFC-MO1	Guilan, Iran	<i>Oryza sativa</i>	KP144444
<i>N. oryzae</i>	UTFC-MO2	Guilan, Iran	<i>Oryza sativa</i>	KP144445

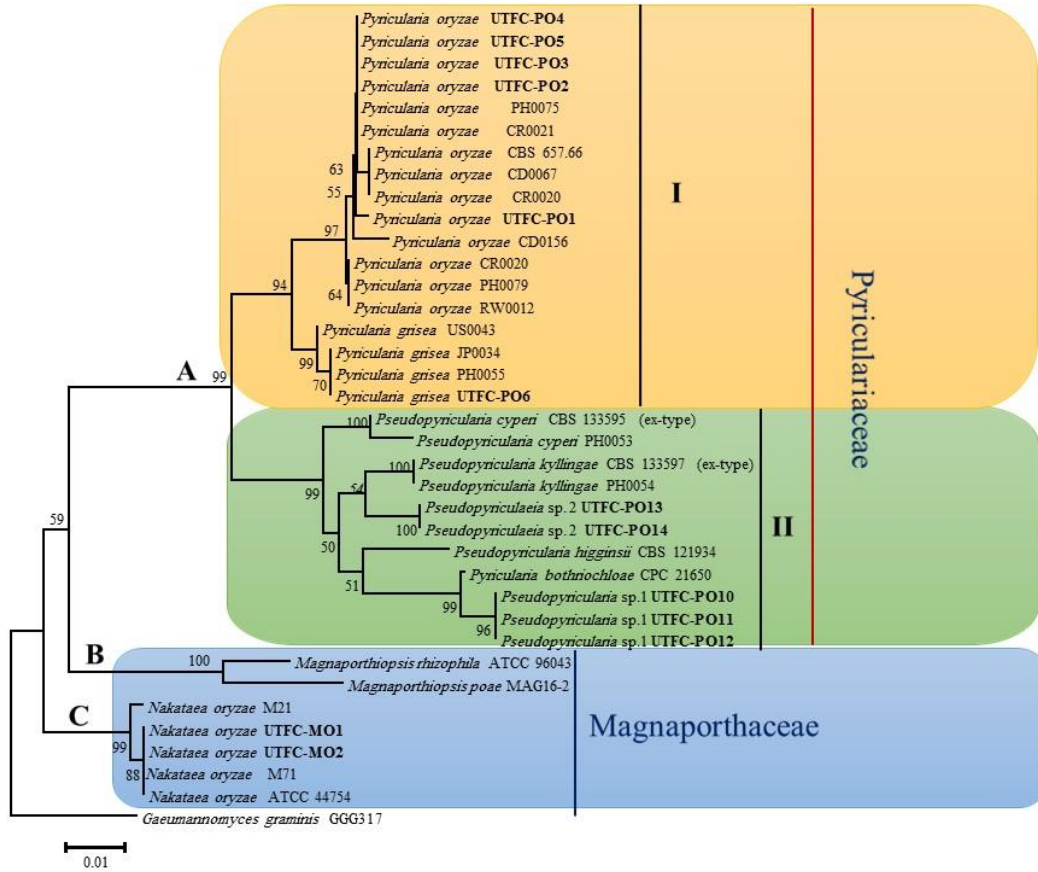


Fig. 3. The maximum likelihood tree inferred from the ITS sequences for 37 species. The numbers above the branches show the bootstrap values in 1000 replicates. The length of branches is proportional to the number of base changes, indicated by the scale bar. *Gaeumannomyces graminis*.

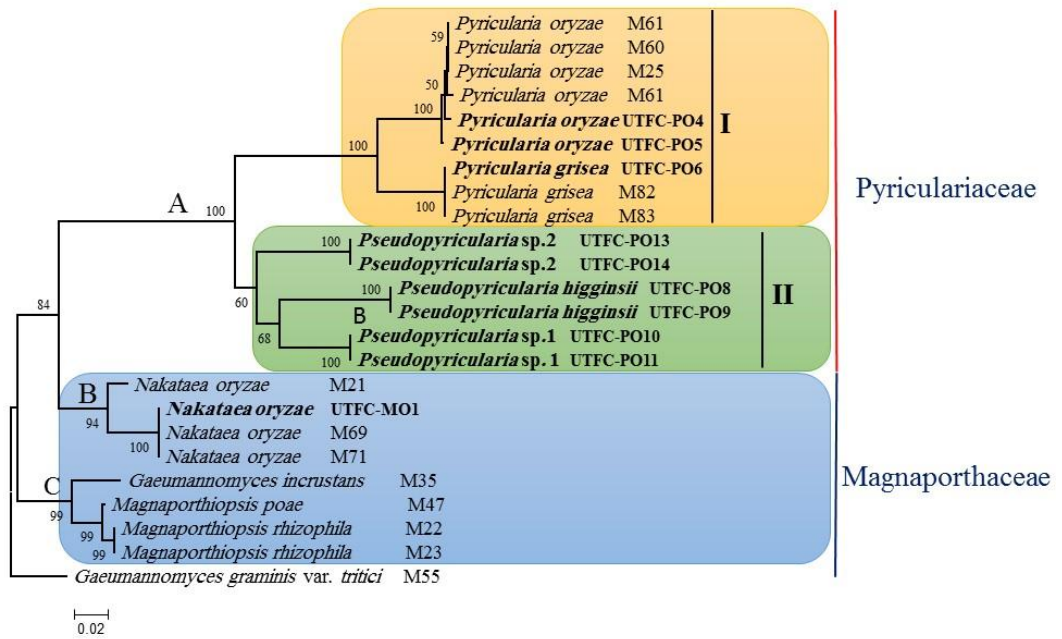


Fig. 4. The maximum likelihood tree inferred from the *MCM7* sequences datasets for 24 species. The numbers above the branches show the bootstrap values in 1000 replicates. *Gaeumannomyces graminis*.

Species belonging to the Pyriculariaceae were grouped in the two distinct clades based on the sequencing of the *CAL* gene. Species of both *P. oryzae* and *P. grisea* were grouped in the clade A, which was supported by the high bootstrap value. These species are morphologically and phylogenetically different and have different host plants. The

species of *Pseudopyricularia higginsii*, *Pseudopyricularia* sp.1 and *Pseudopyricularia* sp.2 were supported with 95% bootstrap in the clade B. All five species were collected from sedge, but these species were different based on morphological characteristics (Fig. 5). It is required to morphologically and phylogenetically investigate this species in further details.

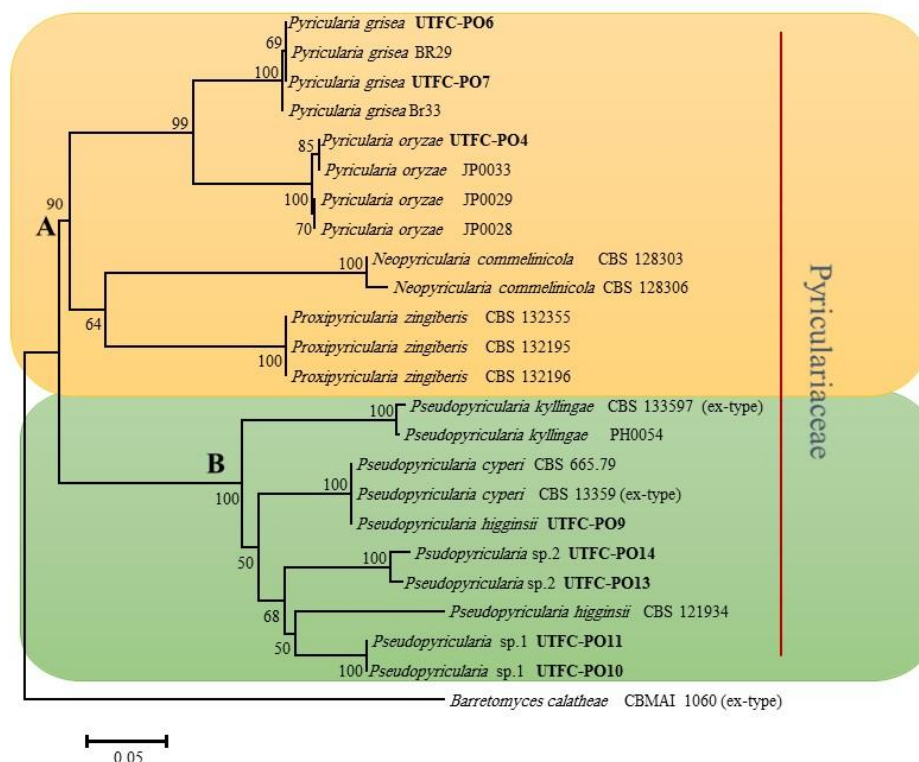


Fig. 5. The maximum likelihood tree inferred from the *calmodulin* sequences datasets for 24 species. The numbers above the branches show the bootstrap values in 1000 replicates. *Barretomyces calathea*.

DISCUSSION

Three distinct families are distinguished in order Magnaporthales, including Magnaporthaceae (based on *Nakataea*), Pyriculariaceae (based on *Pyricularia*), and Ophiocercaceae (based on *Ophioceras*) according to the phylogenetic analysis conducted previously (Zhang et al. 2011, Klaubauf et al. 2014). The Magnaporthaceae and Pyriculariaceae are mainly included fungal plant pathogens such as *Gaeumannomyces*, *Nakataea* and *Pyricularia* causing destructive diseases on cereals and grasses. The Magnaporthaceae is clearly distinguished from the Pyriculariaceae by their asexual morphs, which are phialophora- or harpophora-like, or with falcate versicolored conidia formed on brown and erect conidiophores but the Pyriculariaceae is evidently characterized by pyriform 2-septate conidia and rhexolytic secession (Klaubauf et al. 2014).

Based on the results presented here, we phylogenetically identified three main clades in Magnaporthaceae *sensu lato*. The first one is the

Pyriculariaceae, the early diverging lineage that contains several members such as *Pyricularia oryzae*, *P. grisea*, *Pseudopyricularia higginsii*, *Pseudopyricularia* sp.1, and *Pseudopyricularia* sp.2. Species of *P. oryzae* and *P. grisea* placed in the subclade I. Based on the phylogenetic analysis these species were clustered in two distinct subclades that could be separated based on both host plants and morphological characteristics. Couch and Kohn (2002) described *Magnaporthe oryzae* as a new species, which is distinct from *M. grisea*. In *M. grisea* s. l., two distinct clades were observed, which they are different from each other in terms of host plants. *M. grisea* is able to infect *Digitaria*, a grass grown in tropical and warm temperate regions while *M. oryzae* is cable of infecting *Oryza sativa* and other cultivated grasses. Subclade II included species of the *Pseudopyricularia higginsii*, *Pseudopyricularia* sp.1 and *Pseudopyricularia* sp.2 that they were morphologically different. These abovementioned species were phylogenetically distinct but they were monophyletic and sister groups. The Phylogenetic

analysis demonstrated that the *Pseudopyricularia* species were far away from *Pyricularia* s. str. and they placed in a clade containing the three other known species of *Pseudopyricularia*. This genus has conidia that are solitary, obclavate, pale to medium brown, finely roughened, guttulate, 2-septate; hilum truncate, slightly protruding, unthickened, not darkened and conidiophores are solitary, erect, straight or curved, branched or not, medium brown, finely roughened and septate (Klaubauf et al. 2014).

Previously, several isolates were characterized as the representative of *P. higginsii* belonging to a complex of three related species, *P. cyperi*, *P. kyllingae* and *P. higginsii*, which were classified as *Pseudopyricularia*. Species in this complex are morphologically similar to each other in terms of conidial size and it would be required to resolve the phylogeny of *P. higginsii* (Fig. 3, 4, 5).

Analysis of the ITS and *MCM7* indicated interrelationships of Magnaporthaceae *sensu lato*. Both the second clade constructed based on the sequencing of the ITS region and the third one made based on that of the *MCM7* were mainly contained the Magnaporthaceae *sensu stricto* such as *Magnaporthiopsis poae* and *M. rhizophila*. The species of these groups have *Phialophora*-like anamorph and these species are necrotrophic pathogens on grass root. *Nakataea oryzae* isolates made the basal clade of the phylogeny tree based on the sequencing of the ITS region and the second clade based on that of the *MCM7*. These species are different from the *Pyricularia* genus in both the anamorphic and phylogenetic characteristics. It is different from *Pyricularia* in conidial shape. Based on Melbourne Code (Article 59.1), the name for the rice blast fungus should be *Pyricularia oryzae* while that of the stem rot is *Nakataea oryzae* (Luo et al 2013).

Eventually, this is a preliminary morphological and phylogenetic study on some genus and species of Magnaporthaceae *sensu lato* in Iran. Previous phylogenetic analyses (Zhang et al. 2011, Luo & Zhang 2013, Klaubauf et al. 2014) and also this study shed light on the evolution of Magnaporthaceae *sensu lato*. The taxonomic reappraisal proposed here is a step toward defining monophyletic genera in Pyriculariaceae. More species such as *Pyricularia*, *Pseudopyricularia* and *Nakataea* species should be investigated in the further phylogenetic analysis to gain a better understanding of their evolution and to re-evaluate the current taxonomy.

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ارزیابی جدید از خانواده Pyriculariaceae در ایران

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چکیده: خانواده Magnaporthaceae به عنوان تنها خانواده راسته‌ی Magnaporthales تلقی می‌گردد. براساس بررسی‌های انجام گرفته در سال‌های اخیر سه کلاد اصلی در این راسته قابل تشخیص می‌باشد، که خانواده Pyriculariaceae به عنوان خانواده‌ای جدید از این خانواده مجزا شده است که دارای جنس‌های جدید، گونه‌های جدید، ترکیب‌های جدید و نئوتیپ‌های جدید (بازیونیم) می‌باشد. در بهار، تابستان و پاییز سال‌های ۱۳۹۱ و ۱۳۹۲ از گیاهان دارای نشانه‌های بلاست و لکه‌برگی در مزارع برنج، گندم، جو، ذرت، سورگوم و باغات مرکبات، چای و جنگل‌های نواحی جنوبی دریای خزر- از آستارا در استان گیلان تا گنبد کاووس در استان گلستان- نمونه‌برداری به عمل آمد. در مجموع ۱۳۷ نمونه دارای علائم بلاست و لکه‌برگی جداسازی گردید. صفات مورفولوژیک جدایه‌ها شامل رنگ پرگنه، شکل، رنگ و ابعاد کنیدیوم‌ها و کنیدیوفورها مورد مطالعه قرار گرفت. برای ارزیابی روابط فیلوژنتیکی جدایه‌ها، ناحیه ITS از DNA ریبوزومی، ناحیه ژنی *MCM7* و ناحیه ژنی کالمودولین تکثیر شد. پس از رسم درخت فیلوژنتیکی براساس ناحیه ژنی ITS و *MCM7* سه گروه اصلی و دو زیر گروه در خانواده Pyriculariaceae شناسایی گردید. براساس درخت فیلوژنتیکی رسم شده با ناحیه ژنی کالمودولین دو زیر گروه نیز در خانواده Pyriculariaceae شناسایی گردید.

واژه‌های کلیدی: *Pyricularia*، خانواده جدید، مورفولوژی، فیلوژنی