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Original Article

Re-evaluation of the specificity of *Pyricularia oryzae* diagnostic primers and introduction of a cost-effective, species-level screening method for *Pyricularia* isolates using an ISSR marker

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ABSTRACT

Pyricularia oryzae is a devastating pathogen affecting rice and a wide range of cereal crops. Accurate and specific detection of this species is essential for population genetics, epidemiological studies, and the enforcement of quarantine measures. In this study, the specificity of several *P. oryzae*-specific diagnostic primer pairs, including pfh2a/pfh2b, Pot2a-L2/Pot2a-R2, mif23_01F/mif23_01R, MIF-forward/MIF-reverse, MHP1F/MHP1R, and PoM-1F/PoM-1R were re-evaluated using *in silico* and PCR assays. In addition, the efficacy of an ISSR marker was assessed as a cost-effective, species-level screening method for a large set of *Pyricularia* isolates. The results of *in silico* analysis revealed that PoM-1F/PoM-1R primer pair was not species-specific, while four other primers showed probable specificity for *P. oryzae* detection. However, PCR assays revealed that none of the tested *P. oryzae*-specific primers could reliably distinguish this species from other formally accepted species within the genus *Pyricularia*. These results highlight the urgent need to develop novel molecular markers that can unambiguously differentiate *P. oryzae* from related *Pyricularia* species. Such markers would significantly improve the diagnosis and surveillance of blast diseases. Meanwhile, the ISSR-PCR banding patterns produced from 36 strains representing seven formally accepted *Pyricularia* species were reproducible and congruent with phylogenetic species recognition. Therefore, in studies with large numbers of isolates, where cost-effective grouping is required prior to multi-gene sequencing, this marker represents a highly beneficial tool for selecting representative isolates corresponding to distinct species.

KEYWORDS

Cereal Blast, *Magnaporthe oryzae*, Molecular detection, *Pyriculariaceae*, Species-specific primers.

INTRODUCTION

The genus *Pyricularia* (*Pyriculariaceae, Magnaporthales*) was first described by Saccardo in 1880 with *P. grisea* as the type species. Historically, it was classified primarily based on morphological features of its asexual morphs and host association (Ellis 1971, 1976, Ou 1985, Bussaban et al. 2003, 2005). However, these criteria have proven unreliable due to overlapping morphological characteristics and shared host ranges among species (Klaubauf et al. 2014). Molecular phylogenetic studies have revealed that *Pyricularia* is polyphyletic, prompting a taxonomic revision (Bussaban et al. 2005, Zhang et al. 2011, Klaubauf et al. 2014). Using multi-locus phylogenetic analyses of the large subunit of ribosomal DNA (LSU), the internal transcribed spacer (ITS) region, parts of the largest subunit of RNA polymerase

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II (*RPB1*), actin (*ACT*), and calmodulin (*CAL*) genes, Klaubauf et al. (2014) redefined *Pyricularia sensu stricto*. They recognized seven species (*P. angulata*, *P. ctenantheicola*, *P. grisea*, *P. oryzae*, *P. penniseticola*, *P. pennisetigena*, and *P. zingibericola*), with an eighth species (*P. urashimae*) added later (Crous et al. 2016). In recent years, a limited number of phylogenomic studies have been conducted to further delineate species boundaries within the genus *Pyricularia* (Gladieux et al. 2018, Ascari et al. 2024). However, these studies typically lack comprehensive coverage of all currently accepted species, mainly due to the scarcity of available genome sequences-except for *P. oryzae* and *P. grisea*. Nevertheless, the existing genomic data have generally supported the phylogenetic relationships inferred from multi-locus analyses of reference genes. Although over 60 names have been associated with this genus (www.indexfungorum.org, accessed 17 July 2025), only the eight mentioned species are currently accepted; many others have been synonymized, transferred to other genera, or remain taxonomically unresolved (Klaubauf et al. 2014, Marin-Felix et al. 2017, 2019, Valent et al. 2019). Taxonomically unresolved species are those described solely based on morphological characteristics and lacking molecular data. In some cases, their original descriptions are inconsistent with the current circumscription of *Pyricularia s. str* (Klaubauf et al. 2014), while in others, their morphology may be consistent, but the lack of molecular data prevents confirmation of their identity. Accordingly, the taxonomic placement of these taxa should be reassessed using molecular methods, provided that viable cultures are available.

The genus *Pyricularia* is recognized as one of the most important groups of plant-pathogenic fungi. *Pyricularia oryzae*, *P. grisea*, *P. penniseticola*, *P. pennisetigena*, and *P. urashimae* are known to cause blast and leaf spot diseases in members of the *Poaceae* family (Klaubauf et al. 2014, Luo and Zhang 2022, Baudin et al. 2024). *Pyricularia angulata* causes leaf spot and pitting disease on bananas (*Musaceae*) (Male et al. 2011, Ganesan et al. 2017); *P. ctenantheicola* infects ornamental *Ctenanthe* species (*Marantaceae*) (Pappas and Paplomatas 1998); and *P. zingibericola* has been isolated from *Zingiber officinale* (*Zingiberaceae*) (Klaubauf et al. 2014). *Pyricularia oryzae* (syn. *Magnaporthe oryzae*) is the most extensively studied species within the genus, comprising host-specific lineages that cause devastating blast diseases in various cereals, including rice, wheat, maize, barley, and millets. Among these, rice blast is the most widespread and economically significant, accounting for an estimated 5% loss in global rice production annually (Baudin et al. 2024). Wheat blast was first reported in Brazil in 1985 and has since spread across neighboring countries in South America, with more recent outbreaks documented in Bangladesh (Asia) and Zambia (Africa), causing considerable damage (Ioos and Tharreau, 2025). Blast diseases affecting millets and maize pose a significant threat to agriculture and food security in affected regions (Odeph et al. 2020, Patro et al. 2021**)**. Additionally, outbreaks of blast on turfgrasses can result in significant damage to sports fields and recreational areas (Tosa et al. 2016). The remarkable adaptability of *P. oryzae* to new host species and cultivars represents a major threat to cereal crops worldwide-an especially critical concern given the global reliance on cereals as staple foods (Baudin et al. 2024).

Reliable molecular markers are essential for accurate species identification in population genetics, epidemiological studies, and quarantine enforcement. Over the past two decades, several PCR-based primer sets have been developed for the detection of *P. oryzae*, targeting species-specific sequences from repetitive elements, pathogenicity-related genes, or anonymous genomic regions. These primers include pfh2a/pfh2b (Harmon et al. 2003) and Pot2a-L2/Pot2a-R2 (Pieck et al. 2017), which amplify part of the *Pot2* transposable element; mif23_01F/mif23_01R (Huang et al. 2016) and MIF-forward/MIF-reverse (Chadha and Gopalakrishna 2006, Chadha 2019), which target a fragment of the *mif23* gene encoding an infection structure-specific protein; MHP1F/MHP1R, developed based on the hydrophobin class I (*MHP1*) gene (Su'udi et al. 2013) and PoM-1F/PoM-1R, which amplify part of the *MPG1* gene encoding a hydrophobin-like protein (Kumar et al. 2025). While these primers have been used in diagnostic applications, most were not considered taxonomic revisions of the genus and have not been systematically evaluated against the revised species boundaries. This raises concerns regarding their specificity, particularly when closely related species are present.

Given the relatively high cost of sequencing technologies, especially in developing countries, and the challenges posed by studies involving a large number of isolates, there is a need for scalable, low-cost, and discriminatory molecular tools for preliminary screening and isolates grouping. Initial grouping of isolates using a molecular marker, followed by sequencing a few representative isolates from each group, can greatly reduce both time and cost (Alves et al. 2007). Among the available molecular tools, Inter Simple Sequence Repeat (ISSR) markers have proven useful for differentiating species within fungal genera. Unlike traditional species-specific primers that target unique genomic regions exclusive to individual species, ISSR markers amplify variable regions between microsatellite loci distributed across the genome. This amplification generates polymorphic banding patterns that reflect broader genomic differences (Oliveira and Azevedo 2022). The utility of ISSR markers for species-level differentiation has been demonstrated in several fungal genera. For example, Ahmadpour et al. (2025a) successfully used a single

ISSR primer to differentiate closely related *Alternaria* species from different hosts. Similarly, ISSR profiling has been applied to distinguish species within the *Colletotrichum gloeosporioides* complex (Akbarzadeh et al. 2023), *Aspergillus* species (Ghaderi and Abdollahzadeh 2025), and members of the *Botryosphaeriaceae* family (Zhou et al. 2001; Alves et al. 2007).

Therefore, this study aimed to address two key objectives: (i) to re-evaluate the specificity of existing *P. oryzae*-specific primers in the context of the current taxonomy, using a diverse collection of *Pyricularia* strains, and (ii) to assess the effectiveness of an ISSR marker, previously applied to *Alternaria* and *Colletotrichum*, as a reliable, cost-efficient molecular tool for the preliminary differentiation of *Pyricularia* species before multi-gene sequencing.

MATERIALS AND METHODS

Fungal strains

A total of 36 fungal strains representing seven formally accepted *Pyricularia* species were obtained from the PHIM fungal collection unit at the CIRAD Institute, Montpellier, France. Genomic DNA was extracted from all strains and used for PCR amplification with the primers tested in this study. Detailed information on these strains, including their species identity, host origin, and geographic source, is provided in Table 1.

Phylogenetic Analysis

To illustrate the phylogenetic relationships of the 36 strains examined in this study within the genus *Pyricularia* and the wider family *Pyriculariaceae*, a detailed phylogenetic analysis using ITS, *RPB1*, *CAL*, and *ACT* sequences was conducted. A subset of strains had previously been characterized and included in multi-locus phylogenetic analyses based on four gene regions (ITS, *RPB1*, *CAL*, and *ACT*) by Klaubauf et al. (2014), with their species identities well established. Another group of strains, CH0997, CH0999, CH1003, CH1019, OG0002, OG0005, TH0012, TH0016, and IN0001, had been identified as *P. oryzae* based on sexual compatibility assays and genome-based analyses (Gladieux et al. 2018, Lassagne et al. 2022), but had not been previously included in any multi-locus phylogenetic analysis. Sequences for these strains (except for IN0001) were extracted directly from their whole-genome sequencing data. The *RPB1* sequence for strain IN0001 was obtained through sequencing in this study. PCR conditions and primers for this newly generated sequence followed the protocols described by Ahmadpour et al. (2025b). Sequences for all other strains were retrieved from GenBank (Table 1).

Multiple sequence alignments for each locus were performed using the MAFFT v.7 online program (https://mafft.cbrc.jp/alignment/server/) (Katoh et al. 2019). These alignments were then manually adjusted and trimmed in MEGA 6.06 (Tamura et al. 2013). A concatenated dataset combining all four loci (ITS+*RPB1*+*CAL*+*ACT*) was then assembled in Mesquite v.3.81 (Maddison and Maddison 2023). This multi-locus dataset served as the foundation for all subsequent phylogenetic inferences.

Multi-locus phylogenetic inference was performed using three methods: Maximum Likelihood (ML), Maximum Parsimony (MP), and Bayesian Inference (BI). All analyses were conducted via the CIPRES Science Gateway portal (https://www.phylo.org/) (Miller et al. 2010). ML analysis was carried out using RAxML-HPC BlackBox v.8.2.12 (Stamatakis 2014). We performed 1000 bootstrap replicates with the GTRGAMMA+I substitution model, configuring the analysis to search for the best-scoring tree after bootstrapping. MP analyses were conducted as heuristic searches in PAUP 4.a168 (Swofford 2003). These searches included 1,000 stepwise random addition replicates, employed the tree-bisection-reconnection (TBR) algorithm, and 1,000 bootstrap replicates. BI was performed using the Markov Chain Monte Carlo (MCMC) method in MrBayes 3.2.7 (Ronquist and Huelsenbeck 2003). Four chains were run for 1,000,000 generations, sampling trees every 1,000 generations. The first 25% of trees were discarded as burn-in. The temperature value for the heated chain was set to 0.1, and the analysis run was considered complete when the average standard deviation of split frequencies dropped below 0.01. The best-fit nucleotide evolutionary models for each locus were estimated using MrModeltest v. 2.3 (Nylander 2004), based on the Akaike Information Criterion (AIC). Sequences of *Magnaporthiopsis incrustans* M35 and *Magnaporthiopsis poae* ATCC 64411 (*Magnaporthaceae*) were used as the outgroup taxa to root the phylogenetic trees. Finally, the resulting phylogenetic trees were visualized in FigTree v. 1.4.4 (Rambaut 2019) and graphically formatted using Adobe Illustrator® CC 2024 (Adobe Inc., San Jose, California, USA).

In silico specificity evaluation of species-specific primers

A literature review was conducted to identify previously published primer sets developed for the species-specific molecular detection of *P. oryzae*. All retrieved primers were assessed for *in silico* specificity using NCBI Primer-BLAST (Table 2). The specificity of each primer pair was assessed against the core-nt database with default settings, based on their ability to selectively amplify *P. oryzae* DNA without cross-reacting with non-target fungal species. Primer pairs that showed potential amplification of unrelated fungal taxa were excluded from further analysis. In cases where multiple *Pyricularia* species appeared among the predicted amplicons, their taxonomic identity was carefully verified through a literature review. Only after confirmation of a non-target match, the primer was

Table 1. Fungal strains used for phylogenetic analysis. Strains evaluated using specific primers and the ISSR marker are shown in boldface. 'T' indicates the type strain.

Species	Culture collection	Host/substrate	Location	GenBank accession numbers			
				ITS	*RPB1*	*ACT*	*CAL*
Bambusicularia brunnea	CBS 133599^{T}	*Sasa* sp.	Japan	KM484830	KM485043	AB274449	AB274482
	CBS 133600	*Phyllostachys bambusoides*	Japan	AB274436	KM485044	AB274450	AB274483
Barretomyces calatheae	CBS 129274	*Calathea longifolia*	Brazil	KM484831	KM485045	KM485162	KM485231
Bipyricularia graminis	YNE01013^{T}	Unidentified Poaceae	China	MW479090	MW482852	OQ918100	–
	YNE01016	Unidentified Poaceae	China	MW479091	MW482853	OQ918101	–
Macgarvieomyces borealis	CBS 461.65^{T}	*Juncus effusus*	Scotland	KM484854	KM485070	KM485170	KM485239
Macgarvieomyces juncicola	CBS 610.82	*Juncus effusus*	Netherlands	KM484855	KM485071	KM485171	KM485240
Macgarvieomyces luzulae	CBS 143401^{T}	*Luzula sylvatica*	Ukraine	MG934440	MG934469	MG934462	MG934519
	CPC 31555	*Luzula sylvatica*	Ukraine	MG934441	MG934470	MG934463	MG934520
Magnaporthiopsis incrustans	M35	-	-	JF414843	Genome[a]	Genome[a]	Genome[a]
Magnaporthiopsis poae	ATCC 64411	*Triticum* sp.	USA	Genome[a]	Genome[a]	AF395973	AF396032
Neocordana musarum	CBS 142116^{T}	*Musa* sp.	France	KY173425	KY173577	KY173568	–
Neocordana musigena	CBS 142624^{T}	*Musa* sp.	Morocco	KY979749	KY979886	KY979855	–
Neopyricularia commelinicola	CBS 128307	*Commelina communis*	South Korea	FJ850125	KM485086	KM485174	KM485243
	CBS 128308^{T}	*Commelina communis*	South Korea	FJ850122	KM485087	KM485175	–
Nothopyricularia junci	CBS 148308^{T}	*Juncus effusus*	Netherlands	OK664720	OK651152	OK651127	OK651142
Proxipyricularia zingiberis	CBS 132195	*Zingiber mioga*	Japan	KM484869	KM485088	AB274448	KM485244
	CBS 303.39	*Zingiber officinale*	Japan	KM484871	KM485092	KM485177	KM485247
Pseudopyricularia bothriochloae	CBS 136427^{T}	*Bothriochloa bladhii*	Thailand	KF777186	KY905701	KY905700	–
Pseudopyricularia cyperi	CBS 133595^{T}	*Cyperus iria*	Japan	KM484872	AB818013	AB274453	AB274485
	Cr88383	*Cyperus rotundus*	Philippines	KM484874	KM485094	KM485179	KM485249
Pseudopyricularia festucae	CBS 146629^{T}	*Festuca californica*	USA	MW883447	MW890057	–	MW890044
Pseudopyricularia hagahagae	CPC 25635^{T}	Unidentified Cyperaceae	South Africa	KT950851	KT950877	KT950873	–
Pseudopyricularia higginsii	CBS 121934	*Typha orientalis*	New Zealand	KM484875	KM485095	KM485180	KM485250
Pseudopyricularia kyllingae	CBS 133597^{T}	*Kyllinga brevifolia*	Japan	KM484876	KM485096	AB274451	AB274484
	PH0054 = Cb8959	*Cyperus brevifolius*	Philippines	KM484877	KM485097	KM485181	KM485251
Pyricularia angulata	NBRC9625	*Musa sapientum*	Japan	AY265322	–	–	–
	BRIP 53746	*Musa* sp.	Australia	JF719830	–	–	–
Pyricularia ctenantheicola	**GR0001**	***Ctenanthe oppenheimiana***	**Greece**	**KM484878**	**KM485097**	**KM485181**	**KM485251**
	GR0002^{T}	***Ctenanthe oppenheimiana***	**Greece**	**KM484879**	**KM485098**	**KM485182**	**KM485252**
Pyricularia grisea	**US0043**^{T} **= CBS 138707**	***Digitaria*** **sp.**	**USA**	**KM484885**	**KM485105**	**KM485187**	**KM485258**
	BR0029	***Digitaria sanguinalis***	**Brazil**	**KM484880**	**KM485100**	**DQ240874**	**DQ240890**
	CR0024	***Lolium perenne***	**South Korea**	**KM484882**	**KM485102**	**KM485185**	**KM485256**
	JP0034 = NI980	***Digitaria smutsii***	**Japan**	**KM484883**	**KM485103**	**KM485186**	**KM485257**
	PH0055 = Dc88420	***Digitaria ciliaris***	**Philippines**	**KM484884**	**KM485104**	**DQ240877**	**DQ240893**
Pyricularia oryzae	**BF0028**	***Paspalum*** **sp.**	**Burkina Faso**	**KM484886**	**KM485106**	**KM485188**	**KM485259**
	CBS 255.38	-	Romania	KM484889	KM485109	KM485190	KM485261
	CBS 657.66	***Oryza sativa***	Egypt	KM484893	KM485113	KM485194	KM485265
	CD0156	***Eleusine indica***	**Côte d'Ivoire**	**KM484897**	**KM485117**	**KM485198**	**KM485269**
	CH0997	***Oryza sativa***	**China**	**Genome**[b]	**Genome**[b]	**Genome**[b]	**Genome**[b]
	CH0999	***Oryza sativa***	**China**	**Genome**[b]	**Genome**[b]	**Genome**[b]	**Genome**[b]
	CH1003	***Oryza sativa***	**China**	**Genome**[b]	**Genome**[b]	**Genome**[b]	**Genome**[b]
	CH1019	***Oryza sativa***	**China**	**Genome**[b]	**Genome**[b]	**Genome**[b]	**Genome**[b]
	CR0021	***Panicum miliaceum***	**South Korea**	**KM484899**	**KM485119**	**KM485200**	**KM485271**
	GN0001	***Zea mays***	**Gabon**	**KM484903**	**KM485123**	**DQ240882**	**DQ240898**
	GY0011 = Guy11	***Oryza sativa***	**French**	**KM484904**	**KM485124**	**KC167438**	**AF396024**
	IN0001 = KA7	***Eleusine indica***	**India**	**–**	**PX066817**	**–**	**–**
	JP0017 = C10	***Eleusine indica***	**Japan**	**–**	**–**	**AF395970**	**AF396018**
	OG0002 = KA3	***Eleusine coracana***	**Uganda**	**Genome**[b]	**Genome**[b]	**Genome**[b]	**Genome**[b]
	OG0005 = KA9	***Eleusine coracana***	**Uganda**	**Genome**[b]	**Genome**[b]	**Genome**[b]	**Genome**[b]
	PH0051 = CD88215	***Cynodon dactylon***	**Philippines**	**KM484913**	**KM485133**	**KM485208**	**KM485281**
	PH0062 = Pd8824	***Paspalum distichum***	**Philippines**	**KM484915**	**KM485134**	**KM485210**	**KM485283**
	TH0012 = TH12	***Hordeum vulgare***	**Thailand**	**–**	**Genome**[C]	**Genome**[C]	**Genome**[C]
	TH0016 = TH16	***Hordeum vulgare***	**Thailand**	**–**	**Genome**[C]	**Genome**[C]	**Genome**[C]
	US0071	**Setaria viridis**	**USA**	**KM484923**	**KM485142**	**KM485217**	**–**
Pyricularia penniseticola	**BF0017**	***Pennisetum typhoides***	**Burkina Faso**	**KM484925**	**KM485144**	**DQ240878**	**DQ240894**
	CD0086	***Pennisetum typhoides***	**Côte d'Ivoire**	**KM484926**	**KM485145**	**DQ240879**	**DQ240895**
	CD0143	***Digitaria exilis***	**Côte d'Ivoire**	**KM484927**	**KM485146**	**KM485219**	**–**
	CD0180	***Pennisetum*** **sp.**	**Côte d'Ivoire**	**KM484928**	**KM485147**	**DQ240880**	**DQ240896**
	ML0031^{T} **= CBS 138603**	***Pennisetum typhoides***	**Mali**	**KM484929**	**KM485148**	**KM485220**	**–**
Pyricularia pennisetigena	**BR0067**	***Cenchrus echinatus***	**Brazil**	**KM484931**	**KM485150**	**KM485222**	**–**
	BR0093	***Echinochloa colona***	**Brazil**	**KM484932**	**KM485151**	**KM485223**	**KM485292**
	CBS 133596 = NI981(Cc-1J)	*Cenchrus ciliaris*	Japan	KM484934	KM485152	KM485224	AB274475
	ML0036^{T} **= CBS 138604**	***Pennisetum sp.***	**Mali**	**KM484935**	**KM485153**	**KM485225**	**KM485294**
	US0045 = 84P-19	***Pennisetum glaucum***	**USA**	**KM484938**	**KM485155**	**KM485228**	**–**
	PH0047= Ce88454	*Cenchrus echinatus*	Philippines	KM484936	KM485154	KM485226	KM485295
Pyricularia urashimae	CBS 142117^{T}	*Urochloa brizantha*	Brazil	KY173437	KY173578	KY173571	KX524100
	JP0036 = CBS 133598	***Leersia oryzoides***	**Japan**	**KM484940**	**KM485156**	**AB274440**	**AB274473**
Pyricularia zingibericola	**RN0001**^{T} **= CBS 138605**	***Zingiber officinale***	**La Réunion**	**KM484941**	**KM485157**	**KM485157**	**KM485297**
Pyriculariomyces asari	CPC 27442	*Asarum* sp.	Malaysia	KX228290	MG934472	KX228360	–
	CPC 27444^{T} = CBS 141328	*Asarum* sp.	Malaysia	KX228291	KX228368	KX228361	MG934541
Utrechtiana arundinacea	CPC 33994^{T}	*Phragmites* sp.	Netherlands	MG934461	MG934473	MG934468	MG934542
Utrechtiana roumeguerei	CBS 128780^{T}	*Phragmites australis*	Netherlands	JF951153	KM485047	KM485163	KM485232
Xenopyricularia zizaniicola	CBS 133593^{T}	*Zizania latifolia*	Japan	KM484947	KM485161	KM485230	AB274479
	CBS 132356	*Zizania latifolia*	Japan	KM484946	KM485160	AB274444	AB274480

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[a] Genome assemblies available in NCBI (https://www.ncbi.nlm.nih.gov/): *Magnaporthiopsis incrustans* M35 (GCA_003049425.1), *Magnaporthiopsis poae* ATCC 64411 (GCA_000193285.1).

[b] Genome sequences not publicly available yet; generated in previous studies and provided by Didier Tharreau

[c] Genome assemblies available in https://genome.jouy.inra.fr/gemo/

Table 2. Published primers evaluated in this study for the detection of *Pyricularia oryzae*.

Primer Name	Sequence (5'–3')	Target Region	Expected Amplicon size (bp)	Primer-BLAST Result	Reference
Pot2a-L2	GCAATTTCATGCAACCGAAA	*Pot2* transposon	394	Specific to *P. oryzae*	Pieck et al. 2017
Pot2a-R2	CGTACGCCAACCAGATTGAA				
pfh2a	CGTCACACGTTCTTCAACC	*Pot2* transposon	687	Specific to *P. oryzae*	Harmon et al. 2003
pfh2b	CGTTTTCACGCTTCTCCG				
MHP1F	TCGATGCCGACAACTTCTCCGA	*Magnaporthe oryzae* Hydrophobin gene	161	Specific to *P. oryzae*	Su'udi et al. 2013
MHP1R	ACCCTGGTCAAGCTGTTCGATTGT				
mif23_01F	CCCTCGCTACCAAGTATCAATG	infection structure-specific protein (*mif23*) gene	101	Specific to *P. oryzae*	Huang et al. 2016
mif23_01R	TGGAGGCAAGAGCAGACAA				
MIF-forward	GGATCCATTGAGCATGCGTT	infection structure structure-specific (*mif23*) gene	390	No target templates were found	Chadha and Gopalakrishna 2006, Chadha 2019
MIF reverse	GGATCCAATACGATCACTCG				
PoM-1F	CTCCCTCAAGACCGTTGTCC	Hydrophobin-like protein gene	520	Lacks specificity; also amplifies *P. grisea*	Kumar et al. 2025
PoM-1R	GGCTCCCTCACAGAAACTCC				

Table 3. PCR thermal cycling conditions used for species-specific and ISSR amplifications.

Primer Name	Initial denaturation	Denaturation	Annealing	Extension	Final Extension	Number of Cycles
Pot2a-L2/Pot2a-R2	95 °C, 5 min	95 °C, 45 s	61–57 °C, 45 s	72 °C, 1 min	72 °C, 7 min	35
pfh2a/pfh2b	95 °C, 5 min	95 °C, 45 s	61–57 °C, 45 s	72 °C, 1 min	72 °C, 7 min	35
MHP1F/MHP1R	95 °C, 5 min	95 °C, 45 s	60 °C, 45 s	72 °C, 1 min	72 °C, 7 min	35
mif23_01F/mif23_01R	95 °C, 5 min	95 °C, 45 s	58 °C, 45 s	72 °C, 1 min	72 °C, 7 min	35
MIF-forward/MIF reverse	95 °C, 5 min	95 °C, 45 s	60 °C, 45 s	72 °C, 1 min	72 °C, 7 min	35
ISSR $(GA)_5YC$	95 °C, 5 min	95 °C, 45 s	43 °C, 45 s	72 °C, 90 s	72 °C, 7 min	35

considered non-specific. The remaining primer sets, which demonstrated probable specificity for *P. oryzae*, were selected for laboratory validation using genomic DNA from a representative panel of 36 *Pyricularia* strains, encompassing seven different species. Details of the evaluated primers, including their target regions, expected amplicon sizes, and Primer-BLAST results, are provided in Table 2.

DNA extraction, PCR amplification, and primer evaluation

Genomic DNA was extracted from freshly harvested mycelia of 10-day-old fungal cultures grown on potato dextrose agar (PDA). Harvested mycelium was homogenized in a standard lysis buffer containing sodium dodecyl sulfate (SDS), followed by chloroform extraction and isopropanol precipitation, as described by Ahmadpour et al. (2021). The quality of the extracted DNA was assessed using 1% agarose gel.

PCR amplifications were performed using the species-specific primers selected from the *in silico* screening (Table 2). Each reaction was carried out in a 20 µL volume containing 10 µL of a ready master mix (Taq DNA polymerase 2X Master Mix Red, 2 mM $MgCl_2$, Ampliqon Company, Denmark), 0.4 µM of each primer, and approximately 10 ng of template DNA. For each primer pair tested, a negative control reaction lacking template DNA was carried out simultaneously. Amplifications were conducted under the thermal cycling conditions optimized for each primer set, as listed in Table 3. For the primer pairs Pot2a-L2/Pot2a-R2 and pfh2a/pfh2b, the annealing temperature was decreased by 0.5 °C per cycle during the first 10 cycles. PCR products were separated on 1.5% agarose gels stained with FluoroStain™ DNA Fluorescent Staining Dye (SMOBIO Corp., Taiwan) and visualized under UV light. DNA ladder SMOBIO DM3200 1 KB Plus

(SMOBIO, Taiwan) was used as a molecular size marker.

In addition to the species-specific primers, the inter-simple sequence repeat (ISSR) primer $(GA)_5YC$ (Ahmadpour et al. 2025a) was included to assess its potential for discriminating among *Pyricularia* species based on polymorphic banding patterns. The reaction mixture contained 4 µL of a ready master mix, 0.8 µL primer (10 pM), and 1.2 µL of template DNA (approximately 10 ng) in a final volume of 10 µL. Thermal cycling conditions for both species-specific and ISSR assays are provided in Table 3. Each strain was scored for the presence or absence of each amplicon. Genetic similarities were calculated using Dice's similarity coefficient. A dendrogram was constructed using the unweighted pair group method with arithmetic average (UPGMA) by the program Sequential, Agglomerative, Hierarchical, and Nested clustering methods (SAHN) of the software package NTSYS-pc 2.1. To ensure the repeatability of the results, all amplifications, using both the species-specific primers and the ISSR primer, were performed at least twice in independent runs.

RESULTS

Phylogenetic analysis

The concatenated dataset, comprising the ITS, *RPB1*, *CAL*, and *ACT* gene regions, consisted of a total of 2744 nucleotide positions. Of these, 1375 sites were constant, 1212 sites were parsimony-informative, and 157 sites were parsimony-uninformative. Table 4 summarizes the phylogenetic statistics and substitution models selected for their analysis. The overall topologies obtained from ML, MP, and BI analyses were highly congruent. The ML analysis yielded a best-scoring tree with a final log-likelihood of –25594. The MP analysis resulted in a tree with a total length of 5186 steps, a Consistency Index (CI) of 0.477, a Retention Index (RI) of 0.775, and a Homoplasy Index (HI) of 0.523. The BI tree showed similar branching patterns, with high posterior probabilities supporting the major clades. The ML tree was selected for visualization and is presented in Fig. 1. Phylogenetic trees reconstructed using ML, MP, and BI methods placed all studied strains within the genus *Pyricularia* (Fig. 1), and grouped them into seven formally accepted *Pyricularia* species lineages according to their type or representative strains.

In silico and PCR evaluation of *P. oryzae* species-specific primers

The results of Primer-BLAST showed that among the six primer pairs tested (Table 2), the primer pair PoM-1F/PoM-1R was deemed non-specific, as it showed a predicted match to the target region in the *P. grisea* strain NI907. The identity of this strain as *P. grisea* was confirmed by Hirata et al. (2007). Consequently, this non-specific primer pair was excluded from further laboratory evaluations. In contrast, four primer pairs, Pot2a-L2/Pot2a-R2, pfh2a/pfh2b, mif23_01F/mif23_01R, and MHP1F/MHP1R, had no predicted match to other *Pyricularia* spp. nor other fungi, apparently confirmed their specificity to *P. oryzae* (Harmon et al. 2003, Su'udi et al. 2013, Huang et al. 2016, Pieck et al. 2017). These four primer pairs were used in subsequent laboratory evaluation with 36 strains representing seven *Pyricularia* species. Although no target was identified for the MIF-forward/MIF-reverse primer pair in the Primer-BLAST search, it was also included in laboratory PCR testing.

The results of PCR amplifications using four primer pairs, Pot2a-L2/Pot2a-R2, pfh2a/pfh2b, mif23_01F/mif23_01R, and MHP1F/MHP1R, produced fragments of the expected size in all examined strains (Fig. 2), regardless of species, demonstrating a lack of species specificity for these primers. One exception was the primer pair pfh2a/pfh2b, which failed to amplify any product in the *P. ctenantheicola* strain GR0001. However, in the ex-type strain (GR0002), the expected product was successfully amplified. Also, the primer pair MIF-forward/MIF-reverse failed to amplify the expected product (390 bp) in any of the tested strains, including *P. oryzae* strains (Fig. 2), despite testing under multiple PCR conditions (data not shown). Instead, a faint band around 100 bp was consistently observed in all strains, including strains

Table 4. Phylogenetic statistics and substitution models for individual and concatenated gene regions.

Parameter	ITS	*RPB1*	*ACT*	*CAL*	Combined
Number of taxa	71	72	71	61	75
Total characters	536	1030	552	626	2744
Constant sites	340	558	270	207	1375
Variable sites	196	472	309	419	1369
Parsimony informative sites	159	439	243	371	1212
Parsimony uninformative sites	37	33	39	48	157
AIC substitution model*	GTR+I+G	GTR+I+G	HKY+I+G	HKY+I+G	GTR+I+G
Lset nst, Rates	6, invgamma	6, invgamma	2, invgamma	2, invgamma	6, invgamma
−lnL	3852.1	8641.4	5198.6	7254.4	25594

* Substitution models were selected based on the Akaike Information Criterion (AIC) and applied in Bayesian inference analysis.

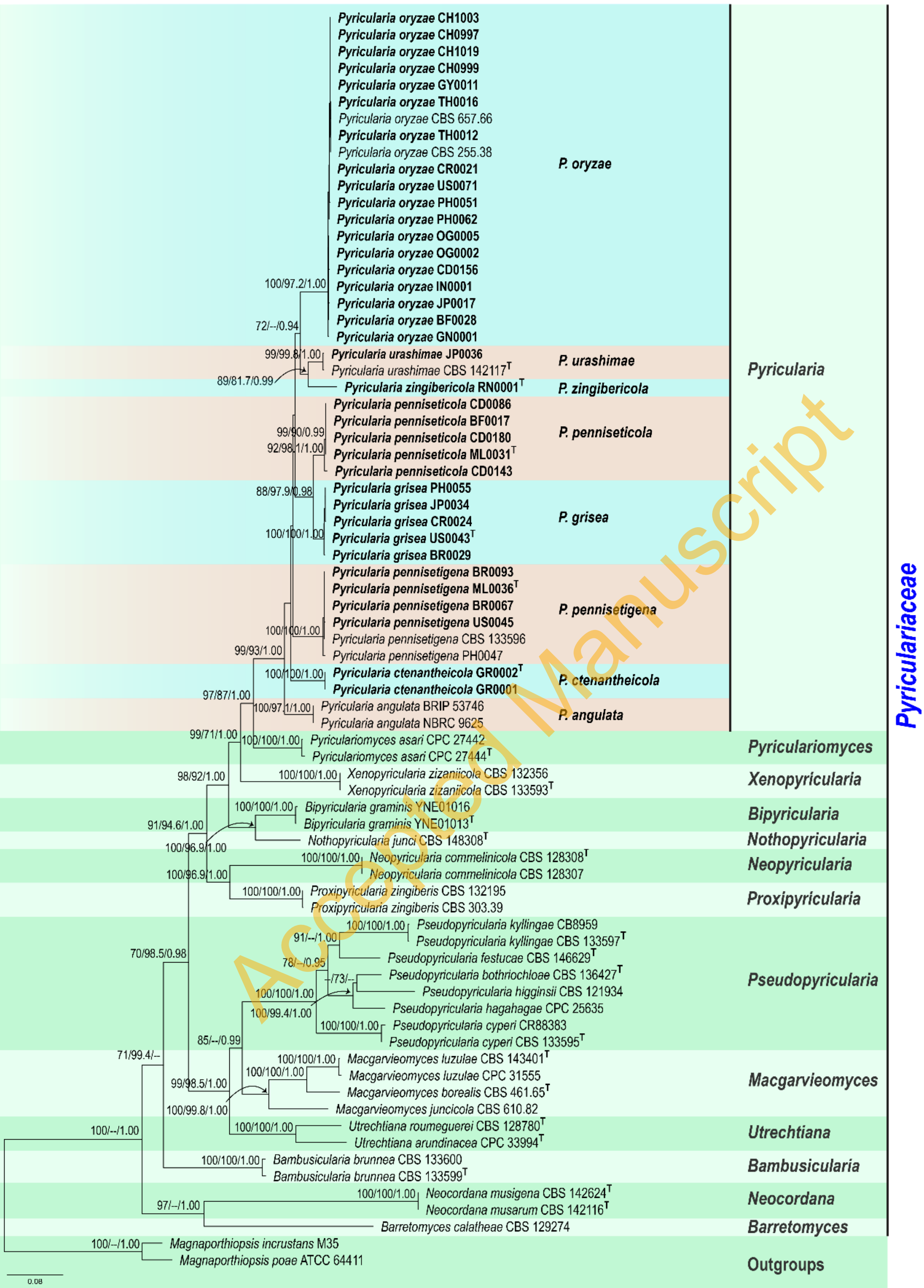

Fig. 1. Phylogenetic tree generated by Maximum Likelihood of the concatenated dataset of ITS+*RPB1*+*CAL*+*ACT* of *Pyriculariaceae* species. The Maximum likelihood (ML) and Maximum parsimony (MP) bootstrap values (≥70%) and Bayesian posterior probabilities (≥0.90) are shown at the nodes (ML/MP/PP). The tree was rooted to *Magnaporthiopsis incrustans* M35 and *Magnaporthiopsis poae* ATCC 64411. The scale bar indicates the number of nucleotide substitutions. The sequenced strain is in blue bold. Strains evaluated using specific primers and the ISSR marker are shown in boldface. 'T' indicates the type strain.

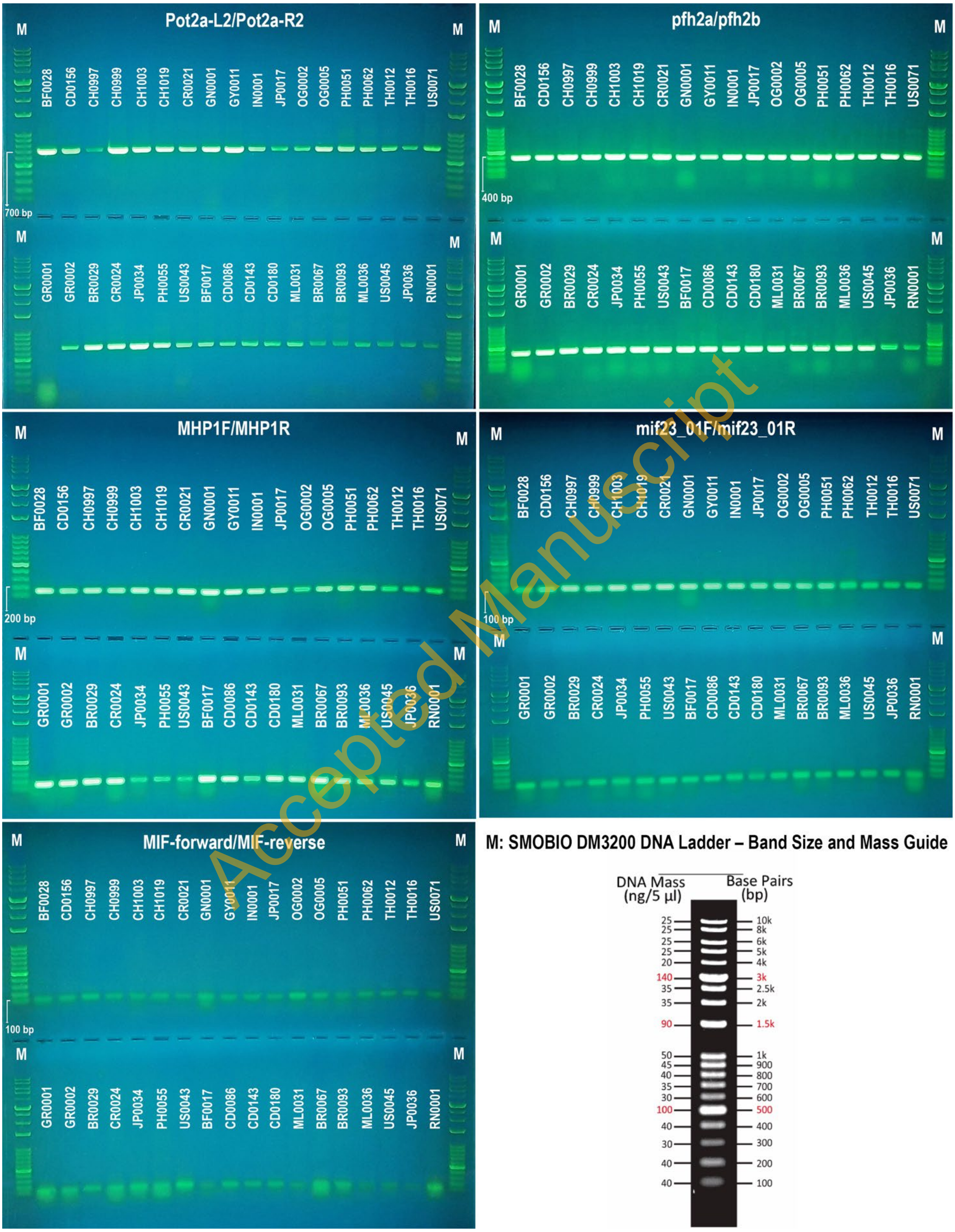

Fig. 2. PCR amplification of 36 *Pyricularia* spp. strains using five species-specific primer pairs. DM3200 1 KB Plus (0.1–10 kb) DNA Ladder was used as the size marker; ladder image reproduced from https://www.smobio.com/

from all seven species. This band may correspond to primer dimer formation or non-specific amplification, but is exactly unrelated to the expected target. Additionally, Primer-BLAST analysis did not return any *P. oryzae*-specific target for this primer pair in the NCBI database, further confirming that the primers lack specificity or do not match any target sequence. Taken together, both *in silico* and *in vitro* results indicate that this primer pair is not a diagnostic marker for the species-specific detection of *P. oryzae*. Negative controls showed no amplifications in any of the reactions (data not shown). Overall, none of the tested primers were suitable for species-specific detection and/or reliable identification of *P. oryzae*.

ISSR fingerprinting

The results of the ISSR-PCR method using (GA)$_5$YC primer generated polymorphic banding patterns in all 36 tested strains representing seven formally accepted species of *Pyricularia*. The banding patterns were reproducible and showed a distinct pattern consistent with the species identity (Fig. 3), as was established through multi-locus phylogenetic analysis (Klaubauf et al. 2014, this study). The UPGMA-based dendrogram also clustered the strains in distinct clusters according to their species identity (Fig. 4). Strains of *P. pennisetigena*, *P. ctenantheicola*, and *P. penniseticola*, as well as *P. zingibericola*, were separated from *P. oryzae* strains at similarity coefficient levels of approximately 0.14 and 0.32, respectively. Strains of *P. grisea* were distinct from *P. oryzae* at a similarity coefficient of about 0.44. The closest species to *P. oryzae* was *P. urashimae*, which differed at a similarity coefficient level of approximately 0.46, in agreement with the phylogenetic analysis results. The high congruence between the banding patterns and species identities suggests that the ISSR-PCR method using (GA)$_5$YC primer could be used as a practical and affordable pre-screening approach for *Pyricularia* species delimitation.

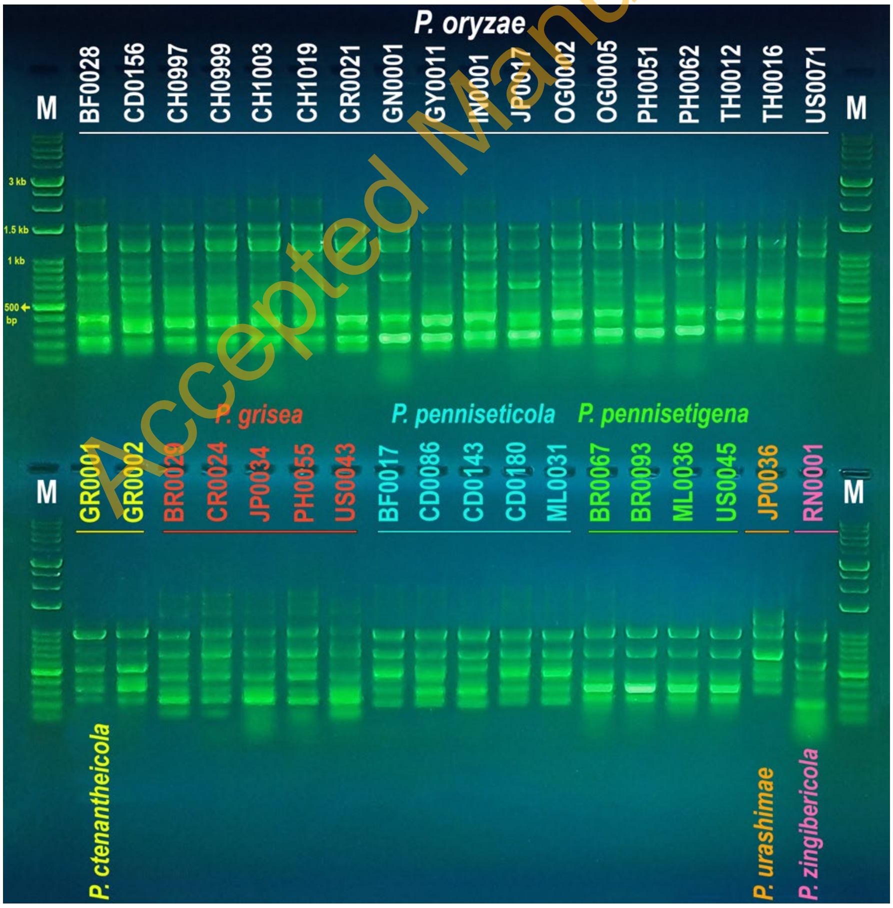

Fig. 3. ISSR banding patterns generated using the primer (GA)$_5$YC for 36 strains of *Pyricularia* spp. M: 1kb marker.

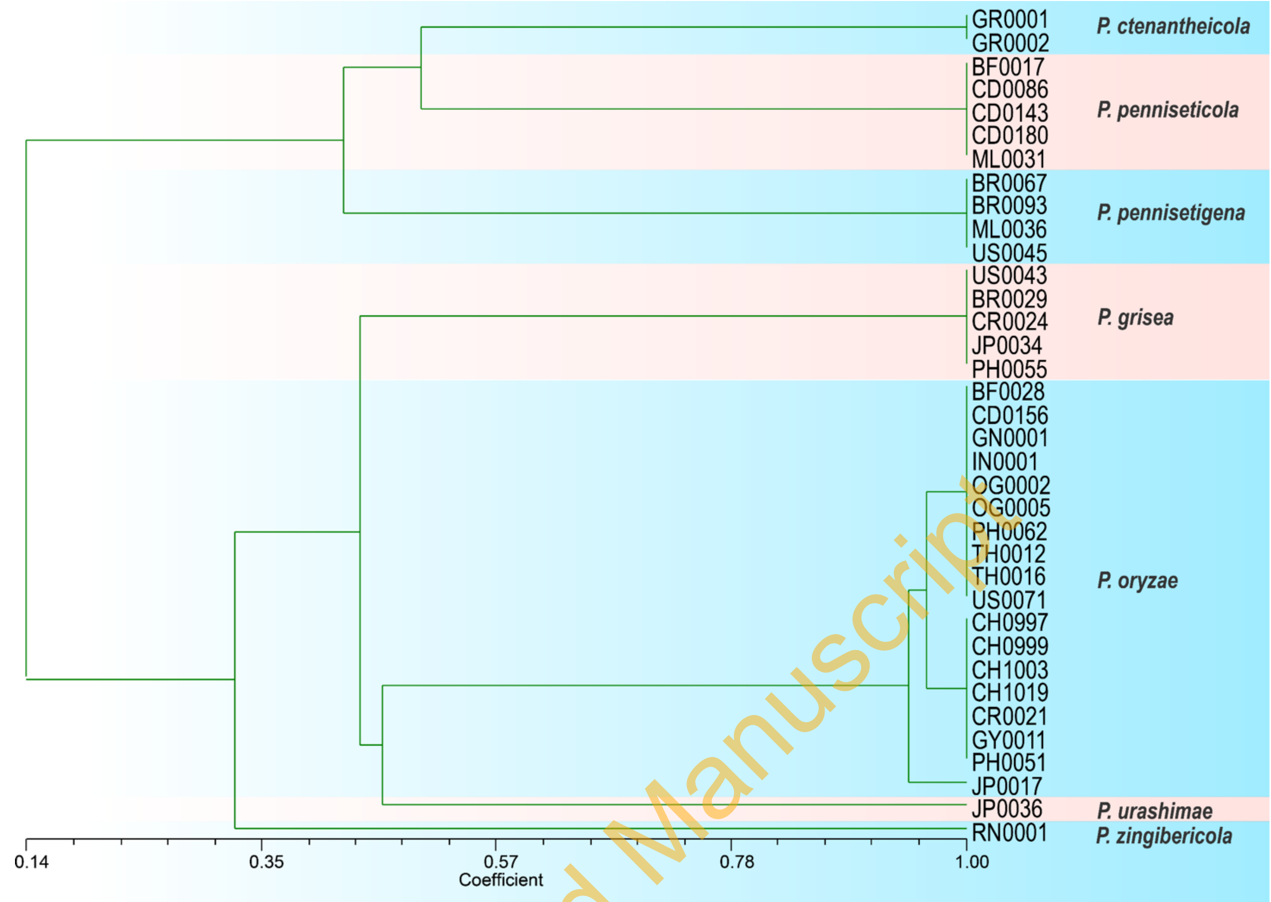

Fig. 4. UPGMA-based dendrogram depicting the genetic relationships among 36 *Pyricularia* strains using Dice's similarity coefficient derived from fingerprints generated by the ISSR primer $(GA)_5YC$.

DISCUSSION

Accurate species-level identification of plant pathogens is essential for effective disease diagnosis, epidemiological studies, and the development of targeted management strategies. This precision becomes especially important in research involving large numbers of isolates, such as population genetics and diversity assessments, where taxonomic resolution directly influences data interpretation. Traditional morphological and cultural methods often fail to distinguish closely related or cryptic species, particularly within genera that contain multiple pathogenic members. Molecular techniques, especially those using species-specific primers, have greatly enhanced the accuracy and reliability of fungal pathogen identification (Jayawardena et al. 2021, Venbrux et al. 2023). However, the development and application of exact species-specific markers require careful validation for both sensitivity and specificity.

In this study, we evaluated species-specific primers for their effectiveness in differentiating *P. oryzae* from other species within the genus *Pyricularia*. Our findings reveal important limitations in the specificity of previously published primer sets: four out of five primer sets produced amplification products in all the tested strains representing seven *Pyricularia* species, indicating broad cross-reactivity and limited diagnostic value at the species level. The MIF-forward/MIF-reverse primer pair that failed to produce the expected product (390 bp), also lacked *in silico* support based on Primer-BLAST analysis, further challenging its validity. The CH7-BAC9 primers, originally designed by Couch et al. (2005) to amplify an anonymous genomic region, were later used by Ascari et al. (2024) as a diagnostic marker for differentiating *P. oryzae* lineages and related species. However, their results showed that this primer also amplified the expected fragment in *P. urashimae*. Similarly, Castroagudín et al. (2016) reported successful amplification of the CH7-BAC9 region in *P. pennisetigena* and *P. grisea* (each represented by a single isolate), further indicating that this marker lacks specificity for species-specific detection of *P. oryzae* when used for simple presence/absence detection by PCR. Consequently, the CH7-BAC9 primer set was excluded from the *in silico* analysis in the present study.

One effective approach for developing species-specific diagnostic tools is comparative genomics, which allows for the identification of unique genomic regions restricted to a target species. This method has been employed to design lineage-specific primers for the wheat-infecting lineage of *P. oryzae* (Villari et al. 2017, Pieck et al. 2017, Thierry et al., 2020a,b), as well as species-specific markers for other pathogens such as *Verticillium* species (Wang et al. 2025) and *Peronospora belbahrii* (Standish et al. 2022). Applying this strategy within *Pyricularia* could facilitate the identification of genomic regions conserved across all *P. oryzae* lineages but absent in other *Pyricularia* species. Such comparative analyses would enable the design of exact species-specific markers, providing a robust and reliable tool for distinguishing *P. oryzae* from its closest relatives. However, while ample genomic resources exist for *P. oryzae* (Baudin et al. 2024), the limited availability of genomic data for other *Pyricularia* species currently limits the use of this approach. Therefore, additional whole-genome sequencing of a diverse range of strains, particularly type strains, across the genus is necessary to identify markers that are definitively species-specific. Furthermore, any diagnostic markers developed through this approach should be thoroughly validated across a broad panel of strains representing multiple *Pyricularia* species from diverse hosts and geographic regions, as well as other genera within the *Pyriculariaceae* family, to ensure their specificity, sensitivity, and practical applicability.

The ISSR marker used in this study demonstrated strong discriminatory power in distinguishing among the analyzed strains of different *Pyricularia* species. The banding patterns observed on agarose gels were highly congruent with species groupings inferred from multi-locus phylogenetic analyses, enabling clear differentiation of all seven species included in our dataset. This high degree of congruence between ISSR profiles and multi-gene phylogenetic results provides compelling evidence for the reliability of this marker in species delimitation within the genus, although confirmation with a larger panel of strains is still needed for some species. The species-specific bands identified in ISSR profiles represent promising candidates for conversion into SCAR (Sequence Characterized Amplified Region) markers, which could facilitate the development of precise and reliable diagnostic tools for individual *Pyricularia* species.

Moreover, this approach offers a cost-effective strategy for selecting isolates for further multi-gene sequencing. When working with a large collection of *Pyricularia* isolates with unknown identities, the ISSR marker can be used for initial grouping based on banding patterns. Then, a few representative isolates from each group can be selected for multi-gene sequencing to determine their exact species identity. This method is particularly useful for large-scale studies such as epidemiological surveys and biodiversity assessments, as it reduces both the time and cost of sequencing while maintaining species-level resolution and optimizing resource use. The successful use of this marker for distinguishing species of *Alternaria* (Ahmadpour et al. 2025) and *Colletotrichum* (Akbarzadeh et al. 2023) has also been reported.

In summary, our study highlights the limitations of existing *P. oryzae* diagnostic primers and the practical value of ISSR markers as rapid, cost-effective screening tools. While whole-genome comparisons remain the gold standard for developing exact species-specific diagnostics, ISSR-based grouping offers an accessible and practical approach for preliminary identification in resource-constrained settings or large-scale studies. Future work should focus on expanding genomic resources for *Pyricularia* and validating new markers with diverse strain panels to support improved pathogen surveillance and management.

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AUTHOR CONTRIBUTION

Conceptualization, Formal analysis, Investigation, Methodology, Software, GenBank submissions, Writing original draft, EH; Funding acquisition, Resources, Supervision, Validation, Review & editing, DT and MJ-N; Review Kh-BF, AMG, HS.

DATA AVAILABILITY

All data are available in online repositories. Requests for more data and materials should be addressed to Esmaeil Hashemlou or Didier Tharreau.

DECLARATION

The authors declare that there is no conflict of interest.

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ETHICS APPROVAL

Not applicable.

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بازنگری اختصاصیت آغازگرهای تشخیصی *Pyricularia oryzae* و معرفی یک روش غربالگری مقرون به صرفه در سطح گونه برای جدایه‌های *Pyricularia* با استفاده از نشانگر ISSR

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چکیده

Pyricularia oryzae بیمارگری مخرب است که برنج و طیف وسیعی از محصولات غلات را تحت تأثیر قرار می‌دهد. شناسایی دقیق و اختصاصی این گونه در مطالعات ژنتیک جمعیت، پژوهش‌های همه‌گیری‌شناسی و همچنین در اجرای اقدامات قرنطینه‌ای ضروری است. در این مطالعه، اختصاصیت چند جفت آغازگر تشخیصی اختصاصی گونه *P. oryzae*، شامل pfh2a/pfh2b، Pot2a-L2/Pot2a-R2، mif23_01F/mif23_01R، MIF-forward/MIF-reverse، MHP1F/MHP1R و PoM-1F/PoM-1R با استفاده از واکاوی‌های رایانه‌ای (*in silico*) و آزمایش‌های PCR مورد بازبینی قرار گرفتند. علاوه بر این، کارایی یک نشانگر ISSR به‌عنوان یک روش غربالگری مقرون به صرفه در سطح گونه برای مجموعه بزرگی از جدایه‌های *Pyricularia* ارزیابی شد. نتایج واکاوی‌های رایانه‌ای نشان داد که جفت آغازگر PoM-1F/PoM-1R اختصاصی گونه *P. oryzae* نیست، در حالی که چهار آغازگر دیگر اختصاصیت احتمالی برای تشخیص این گونه را دارا هستند. با این حال، آزمون‌های PCR نشان دادند که هیچ‌یک از آغازگرهای اختصاصی *P. oryzae* آزمایش شده، قادر به تمایز این گونه از دیگر گونه‌های رسماً پذیرفته‌شده در جنس *Pyricularia* نیستند. این یافته‌ها نیاز مبرم به توسعه‌ی نشانگرهای مولکولی جدید که قادر به تمایز بدون ابهام *P. oryzae* از سایر گونه‌های *Pyricularia* باشد را برجسته می‌کند. چنین نشانگرهایی، تشخیص و پایش بیماری‌های بلاست را به طور قابل‌توجهی بهبود می‌بخشند. همچنین، الگوهای باندی به دست‌آمده از آزمایش ISSR-PCR تولید شده از ۳۶ سویه از هفت گونه رسماً پذیرفته شده *Pyricularia*، تکرارپذیر بوده و با مفهوم تشخیص تبارشناختی گونه مطابقت داشتند. بنابراین، در مطالعاتی که شامل تعداد زیادی از جدایه‌های *Pyricularia* هستند که در آنها، گروه‌بندی اولیه مقرون به صرفه قبل از توالی‌یابی چند ژنی مورد نیاز است، این نشانگر می‌تواند ابزار بسیار سودمندی برای انتخاب جدایه‌های نماینده متناظر با گونه‌های متمایز باشد.

کلمات کلیدی: آغازگرهای اختصاصی گونه، بلاست غلات، شناسایی مولکولی، *Magnaporthe oryzae*، *Pyriculariaceae*.

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