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RESEARCH NOTE

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Designing A Novel Primer Set for *GAPDH* Gene to Enhance Taxonomic and Phylogenetic Studies of *Golovinomyces* Species

Jun Hyuk Park and Young-Joon Choi

Department of Biological Science, Kunsan National University, Gunsan, Korea

ABSTRACT

Golovinomyces (Erysiphaceae, Ascomycota) is an obligate plant pathogenic group causing powdery mildew on diverse angiosperm plants, including economically significant crops. Despite advancements in the taxonomy and phylogeny of *Golovinomyces* species using ribosomal DNA markers (ITS and LSU), several taxonomic issues remain unresolved. Previously, the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene, which exhibits higher nucleotide variation, has been proposed as an additional marker for powdery mildew species. In this study, we designed a new primer set (GoGPD-F and GoGPD-R) to improve the PCR success and efficiency of the *GAPDH* gene across various *Golovinomyces* species and dried herbarium specimens. The primers were successful in amplifying and sequencing the *GAPDH* gene in sixteen *Golovinomyces* species, including six species not previously registered in GenBank and two undescribed species. This development is a significant contribution to future research on the identification, taxonomy, and phylogeny of *Golovinomyces* species, offering a more robust tool for resolving existing taxonomic issues.

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Powdery mildew fungi (Erysiphales; Ascomycota) are obligate plant pathogens responsible for significant economic and ecological impacts on various angiosperm plants worldwide. These fungi are characterized by white conidia and mycelia on the surfaces of leaves, stems, and flowers of their host plants. The genus *Golovinomyces* was initially classified under *Erysiphe* (e.g. *E. cichoracearum*) and later as *Erysiphe* section *Golovinomyces* [1], but subsequent phylogenetic studies elevated it to the genus level [2,3]. This genus includes several notorious species, such as *G. cichoracearum* (syn. *Erysiphe cichoracearum*) parasitic on multiple plant families, *G. bolayi* on lettuce [4], *G. latisporus* on sunflower [5], and *G. tabaci* on cucurbits [4].

Molecular phylogenetic approaches, mainly using ribosomal internal transcribed spacer (ITS) and large subunit (LSU) sequences, have advanced the understanding of taxonomic and phylogenetic relationships among *Golovinomyces* species [4–8]. However, these markers often have limited informative sites, making it challenging to differentiate closely related species [7] and providing insufficient resolution for species complexes within *Golovinomyces*, such as *G. ambrosiae* [5], *G. spadiceus* [5], and *G. bolayi* [4]. Accurate identification of these pathogens is essential for effective disease control and management, necessitating the exploration of alternative genetic markers with higher variability and discriminatory power for this fungal group.

A multi-locus approach has recently been adopted in phylogenetic studies of powdery mildews [5,6,9]. The rDNA intergenic spacer (IGS) region and the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene, in addition to ITS and LSU, have been proposed as supplementary phylogenetic markers [6,9]. Among these, the *GAPDH* gene is particularly effective in distinguishing closely related species due to its higher nucleotide polymorphisms. This gene has been widely used in the taxonomic and phylogenetic studies of various fungal groups [10–12]. Despite its potential, previously designed primers showed low amplification success across *Golovinomyces* species and for dried herbarium specimens, limiting their effectiveness. The present study aimed to design a novel primer set improve their utility in taxonomic and phylogenetic studies.

Powdery mildew samples were collected from diverse host plants in Korea and deposited at the Korea University Herbarium (KUS-F). Genomic DNA was extracted from dried herbarium specimens of *Golovinomyces* and allied genera (Table 1) using the MagListo 5M Plant Genomic DNA Extraction Kit (Bioneer, Daejeon, Korea). To identify the samples, all were amplified with powdery mildew-specific primer sets PM10/ITS4 for ITS and PM3/TW14 for

CONTACT Young-Joon Choi yjchoi@kunsan.ac.kr

Table 1. List of powdery mildew samples collected in Korea.

Species	Host plant	Herbarium No.	Date	Geographic origin	GenBank Acc. No.
Golovinomyces adenophorae	*Adenophora triphylla* var. *japonica*	KUS-F31898	Jul 31, 2020	Seoul	PP952265
G. ambrosiae	*Dahlia pinnata*	KUS-F32139	Nov 06, 2020	Uijeongbu	PP952266
G. arabidis	*Sisymbrium luteum*	KUS-F31062	Jul 02, 2019	Pyeongchang	PP952267
	Sisymbrium luteum	KUS-F30985	May 31, 2019	Chuncheon	PP952268
G. artermisiae	*Artemisia annua*	KUS-F28074	Sep 02, 2014	Yeoncheon	PP952269
	Artemisia indica	KUS-F29848	Jul 04, 2017	Gapyeong	PP952270
	Artemisia rubripes	KUS-F26935	Sep 16, 2012	Hoengseong	PP952271
	Artemisia rubripes	KUS-F31020	Jun 20, 2019	Gangwon	PP952272
G. asperifolii	*Trigonotis peduncularis*	KUS-F32327	Jul 02, 2021	Seoul	PP952273
G. asterum var. *solidaginis*	*Solidago gigantea*	KUS-F32650	Nov 15, 2021	Buan	–
	Solidago gigantea	KUS-F27219	Nov 08, 2012	Busan	–
G. bolayi	*Lactuca serriola*	KUS-F32451	Sep 06, 2021	Suwon	PP952274
G. chrysanthemi	*Dendranthema zawadskii* var. *latilobum*	KUS-F28872	Oct 06, 2015	Hongcheon	–
	Dendranthema zawadskii var. *latilobum*	KUS-F28391	Oct 14, 2014	Hongcheon	–
G. cichoracearum	*Tragopogon dubius*	KUS-F28801	Aug 31, 2015	Seoul	–
	Tragopogon dubius	KUS-F31010	Jun 13, 2019	Guri	–
G. latisporus	*Helianthus annuus*	KUS-F32146	Nov 10, 2020	Seoul	PP952275
	Helianthus tuberosus	KUS-F32079	Oct 20, 2020	Wonju	PP952276
	Helianthus tuberosus	KUS-F31441	Nov 07, 2019	Wonju	PP952277
G. macrocarpus	*Achillea alpina*	KUS-F29264	Jun 28, 2016	Daegu	PP952278
	Achillea millefolium	KUS-F27956	Jul 29, 2014	Wonju	PP952279
	Matricaria chamomilla	KUS-F22504	Nov 26, 2006	Taean	PP952280
G. magnicellulatus	*Phlox subulata*	KUS-F25875	Jul 01, 2011	Yangpyeong	PP952283
	Phlox subulata	KUS-F29166	May 22, 2016	Seoul	PP952284
G. monardae	*Monarda didyma*	KUS-F28319	Oct 07, 2014	Namyangju	PP952285
	Rosmarinus officinalis	KUS-F24012	Mar 17, 2009	Gangneung	PP952286
G. montagnei	*Carduus crispus*	KUS-F29198	Jun 08, 2016	Namyangju	–
	Carduus crispus	KUS-F30644	Jun 20, 2018	Pyeongchang	–
G. orontii	*Capsella bursa-pastoris*	KUS-F29981	Sep 30, 2016	Gongju	PP952287
	Patrinia scabiosifolia	KUS-F29530	Sep 30, 2016	Gongju	PP952288
G. riedlianus	*Galium verum* var. *asiaticum*	KUS-F29268	Jun 28,2016	Daegu	–
	Galium verum var. *asiaticum*	KUS-F29199	Jun 08, 2016	Namyangju	–
G. salviae	*Meehania urticifolia*	KUS-F30693	Jun 16, 2017	Hongcheon	PP952289
G. sordidus	*Plantago asiatica*	KUS-F32690	Nov 29, 2021	Wando	PP952294
	Plantago asiatica	KUS-F31808	Jun 29, 2020	Hongcheon	PP952293
G. tabaci	*Galium spurium*	KUS-F32222	May 24, 2021	Hoengseong	PP952295
	Rubia argyi	KUS-F32108	Oct 27, 2020	Yangpyeong	PP952296
	Rubia argyi	KUS-F31777	Jun 23, 2020	Gimcheon	PP952297
***Golovinomyces* sp. 1**	*Physaliastrum echatum*	KUS-F31881	Jul 21, 2020	Gapyeong	PP952281
	Physalis alkekengi var. *franchetii*	KUS-F28286	Oct 01, 2014	Anyang	PP952282
***Golovinomyces* sp. 2**	*Ixeridium dentatum*	KUS-F29949	Sep 07, 2017	Gimcheon-si	PP952291
	Ixeris stolonifera	KUS-F32561	Oct 25, 2021	Mokpo	PP952292
	Ixeris chinensis subsp. *strigosa*	KUS-F32038	Oct 15, 2020	Pocheon	PP952290
Arthrocladiella mougeotii	*Lycium chinense*	KUS-F32212	Jun 16, 2020	Hoengseong	–
Blumeria graminis	*Bromus japonicus*	KUS-F29679	May 22, 2021	Yangpyeong	–
Cystotheca wrightii	*Quercus glauca*	KUS-F30755	Nov 11, 2016	Seogwipo	–
Erysiphe sp.	*Vaccinium* sp.	KUS-F29783	Jun 02, 2017	Wanju	PP952263
E. quercicola	*Quercus rubra*	KUS-F30755	Aug 22, 2018	Seoul	PP952264
Phyllactinia sp.	Unknown	KUS-F32623	Nov 04, 2021	Wanju	–
Pleochata shiraiana	*Celtis sinensis*	KUS-F32665	Nov 19, 2021	Yeosu	–
Sawadaea nankinensis	*Acer buergerianum*	KUS-F31785	Jun 23, 2020	Seoul	–

LSU rDNA regions [2,13,14]. The PCR products were purified and bidirectionally sequenced by Macrogen (Seoul, Korea) with the same primers used for amplification.

GAPDH gene sequences from twenty *Golovinomyces* species available in GenBank were aligned using SeqMan in the DNASTAR software package (Lasergen, Madison, WI, USA). A suitably variable region of ~323 bp was selected to design new primers, targeting conserved regions flanking the variable sites. Fourteen candidates for the forward primer were designed by modifying the existing primer PMGAPDH1 (GGAATGGCTATGCGTGTACC) [9]. The protein codon site of PMGAPDH1 was confirmed using ExPASy (SIB Swiss Institute of Bioinformatics, Lausanne, Switzerland), and the third nucleotides of each codon, where mutations frequently occur [15], were hybridized to the transition nucleotide. Four reverse primer candidates were designed in regions with low variation among the reference sequences, with lengths between 18 and 22 base pairs. For primer selection, the accuracy and efficiency of the candidate forward and reverse primers were compared through PCR tests and sequencing analysis. As a result, a primer set GoGPD-F (GGAATGGCYATGCGTGTRCC) and GoGPD-R (CAARGARATTCCRGCYTTTGC) was selected for its highest amplification rate and best sequence quality, yielding specific fragments ranging from 219 to 231 bp (excluding primers) of the *GAPDH* gene (Figure 1).

Figure 1. Primer design on *GAPDH* sequences of *Golovinomyces* species. The newly designed primers (GoGPD-F and GoGPD-R) are shown above and below blue bars, while the previously used primers (PMGAPDH1 and PMGAPDH3R) are shown above and below red bars.

Table 2. Sequence analysis of *Golovinomyces* species using the newly developed *GAPDH* primers and the ribosomal ITS and LSU primers.

	GAPDH	ITS	LSU
No. of characters	238	577	735
No. of variables sites	91 (38%)	90 (15%)	38 (5%)
No. of informative sites	64 (27%)	53 (9%)	25 (3%)
Inter-specific variation	0.9–31.3%	0–8.3%	0–3.6%

A gradient PCR was performed to determine the optimal amplification condition, identifying 55.1 °C as the ideal annealing temperature. The touchdown method, which decreases the annealing temperature by 0.2 °C per cycle, further increased amplification efficiency. The final PCR conditions were as follows: initial denaturation at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 55.1 °C (-0.2 °C per cycle) for 40 s, elongation at 72 °C for 40 s, and final elongation at 72 °C for 10 min. The reproducibility of this PCR method was verified using three commercial PCR mixtures: PerfectShot™ Ex Taq (Loading dye mix) (Takara Bio, Shiga, Japan), TOPsimple™ PCR DryMIX-nTaq (Enzynomics, Seoul, Republic of Korea), and AccuPrep® PCR/Gel Purification Kit (Bioneer, Daejeon, Korea). Additionally, all experiments were replicated using two different thermal cyclers in two separate laboratories: the Bio-Rad T100 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA) at Kunsan National University and the Thermo Fisher SimpliAmp™ Thermal Cycler (Thermo Fisher Scientific, Waltham, MA, USA) at Jeonbuk National University.

DNA samples from twenty-one *Golovinomyces* species (Table 1) were PCR-amplified and Sanger-sequenced to compare the efficiency of the newly designed primers (GoGPD-F and GoGPD-R) and previously reported primers (PMGAPDH1 and PMGAPDH3R) [9]. The new primers showed a higher species coverage (76%; sixteen *Golovinomyces* species) compared to the previously reported primers (42%; nine species). They also successfully sequenced six *Golovinomyces* species that had not been previously sequenced: *G. adenophorae*, *G. arabidis*, *G. artermisiae*, *G. bolayi, G. macrocarpus,* and *G. tabaci*. Additionally, the primers amplified two *Erysiphe* species (*E. quercicola* and *E.* sp.) but failed to amplify DNA from other genera such as *Arthrocladiella*, *Blumeria*, *Cystotheca*, *Phyllactinia*, *Pleochata*, and *Sawadaea*. The primers were effective in amplifying DNA from dried herbarium samples collected between 2006 and 2021, demonstrating their ability to amplify DNA from samples up to 18 years old. This broad amplification success underscores the applicability and effectiveness of the new primers for *Golovinomyces* species.

For the sixteen *Golovinomyces* species, the variability and divergence of the *GAPDH* sequences obtained using the newly developed primers were compared with those of ITS and LSU markers. The *GAPDH* exhibited a higher number of variable and informative sites (38% and 27%, respectively) compared to ITS (15% and 9%) and LSU (5% and 3%) regions (Table 2). Using the Kimura 2-parameter model, inter-species genetic divergence of the target gene ranged from 0.9% to 31.3%, which is higher than those of ITS (0–8.3%) and LSU (0–3.6%) markers. This comparative analysis indicates that *GAPDH* sequences provide higher resolution for species discrimination, effectively addressing the limitations of ITS and LSU markers.

A minimum evolution tree was reconstructed using the newly obtained *GAPDH* sequences in MEGA 11 software [16]. The confidence levels of each branch were evaluated using 1000 bootstrap replications. The *GAPDH* tree accurately grouped all analyzed samples

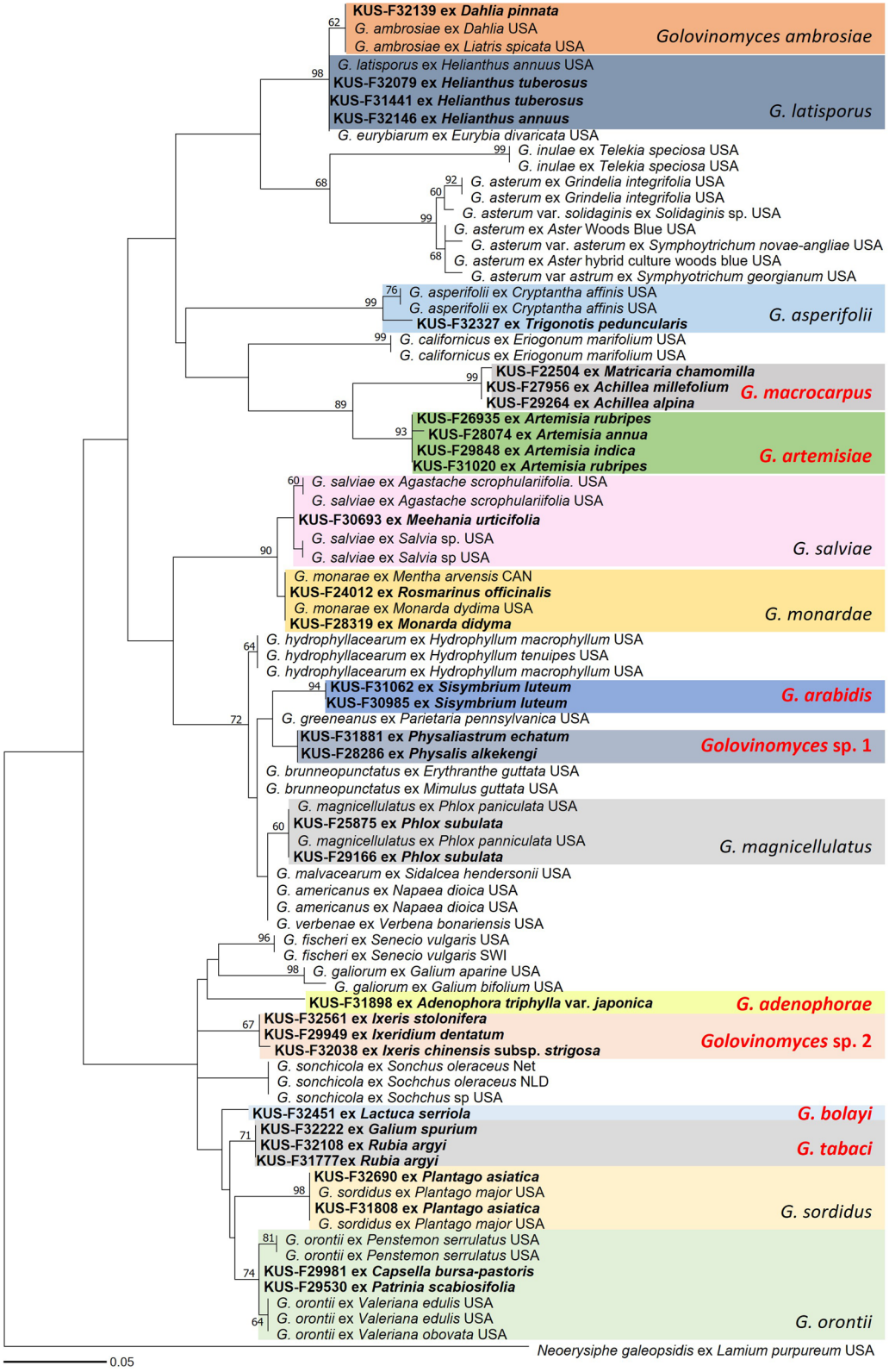

Figure 2. Maximum likelihood tree of *Golovinomyces* species based on *GAPDH* sequences. Bootstrapping support values higher than 60% are given at the branches. Korean specimens sequenced in this study are in bold. *Golovinomyces* species initially sequenced for the *GAPDH* gene in this study are highlighted in red. The scale bar equals the number of nucleotide substitution sites.

into their respective species (Figure 2), demonstrating its high effectiveness in differentiating *Golovinomyces* species. In the phylogenetic tree, the six species (*G. adenophorae*, *G. arabidis*, *G. artermisiae*, *G. bolayi*, *G. macrocarpus*, and *G. tabaci*) for which GAPDH sequences were obtained for the first time in this study formed distinct and unique groups, clearly separated from other known species. Additionally, two new species candidates of *Golovinomyces* were discovered; one parasitic on *Physalis* and *Physaliastrum* spp.

and the other on *Ixeris* and *Ixeridium* spp. Notably, several species, including *G. asperifolii*, *G. salviae*, and *G. orontii*, formed subgroups, associated with specific host plant. Given the high host specificity of powdery mildew fungi, these subgroupings suggest the potential to classify them as separate species. For instance, *G. ambrosiae* s. lat., previously considered a single species based on rDNA sequences, was successfully distinguished by the *GAPDH* sequences it into two species, *G. ambrosiae* s. str. and *G. latisporus*, aligning with the findings of Qiu et al. [5] and Bradshaw et al. [8]. These findings highlight the efficacy of the *GAPDH* marker in resolving taxonomic issues of *Golovinomyces* species.

The present study successfully designed a novel primer set for the *GAPDH* gene, demonstrating a high success rate in amplifying, sequencing, and differentiating a broad range of *Golovinomyces* species. Therefore, the primers could serve as an effective alternative for future research on the taxonomy and phylogeny of *Golovinomyces* species.

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ORCID

Young-Joon Choi http://orcid.org/0000-0002-0909-4723
Jun Hyuk Park http://orcid.org/0009-0004-3070-1227

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