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Morphological and molecular Characterization of *Pythium* s.l. species from Khyber Pakthunkhwa province, Pakistan, with some new records and description of *Globisporangium ghaffarianum* sp. nov.

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Abstract

Pythium s.l. is an ecologically important taxon of the phylum Oomycota that lives in terrestrial and aquatic ecosystems as saprobes. Many species are facultative parasites of plants, causing losses worldwide. Thirty-six putative isolates of the genus from the District Bajaur were characterised morphologically and molecularly during the present studies. *Globisporangium ghaffarianum* sp. nov. is introduced as a novel taxon. Besides this, *G. orthogonon, G. paroecandrum, G. selbyi, G. schmitthenneri, Pythium kashmirense, P. aristosporum,* and *Phytopythium helicoides* are new records for Pakistan. *Globisporangium ultimum* var. *ultimum, Pythium dissotocum, P. catenulatum, P. acanthicum, P. oligandrum, Phytopythium sindhum, Ph. vexans,* and *Ph. litorale* are new records for the province.

Keywords – molecular phylogeny – new records – new species – *Pythium*

Introduction

Species of *Pythium* s. l. live as saprobes in terrestrial and aquatic ecosystems, where they play an important ecological role as decomposers. Many others are, however, serious pests of plants, causing heavy losses to agriculture and forestry (Derevnina et al. 2016, Radmer et al. 2017, Golińska & Świecimska 2020). *Pythium insidiosum* causes pythiosis in mammals, including humans (De Cock et al. 1987). *Pythium* species have been recovered from infectious keratitis ulcers in Covid-19 patients (Manohar et al. 2021, Gurnani et al. 2022). Some species, such as *P. oligandrum,* are mycoparasitic and can be used as a biocontrol agent against soil-borne pathogens (Emoghene & Futughe 2016).

The Genus *Pythium* s.l. contains more than 300 species (Golińska & Świecimska 2020). Conventionally, the identification of *Pythium* species is based on morphological characteristics (Van der Plaats-Niterink 1981, Dick 1990), however, due to biological and ecological diversity, the morphological characteristics are highly variable and hence species-level identification is a major challenge (Shahzad et al. 1992, Mostowfizadeh-Ghalamfarsa 2015). The incorporation of DNA molecular techniques in identification and classification has helped to overcome the problem.

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Based on the phylogeny derived from analysis of ITS sequences, the genus was defined as 11 major clades labeled A – K (Lévesque & De Cock 2004). The genus has now been split into five genera, *viz*., *Elongisporangium*, *Globisporangium*, *Phytopythium* (*Ovatisporangium*), *Pilasporangium* and *Pythium* s. stricto (Bala et al. 2010, Uzuhashi et al. 2010).

Pythium is among the less studied genera of fungi in the Khyber Pakthunkhwa (KP) province of Pakistan. In a previous work, eight species from Bajaur have been reported (Abdul & Shahzad 1998, Abdul Haq et al. 2015, Abdul Haq 2021), which appears to be the only systematic study of the genus in the province. This work is also restricted to Bajaur, one of the newly merged districts in KP. It lies in the Hindu Kush Mountain range on the Afghanistan-Pakistan border. A fair number of *Pythium* species have been reported from Sindh province (Lodhi et al. 2004a, 2004b, 2005a, 2005b, 2005c, Bala et al. 2010). Serious losses to various crops caused by *Pythium aphanidermatum* have been reported in the province of Sindh (Lodhi et al. 2013). This study will add to the systematics and diversity aspects of *Pythium* s.l. species in the area.

Materials & Methods

Isolation and Identification

Soil and water samples were collected from time to time at various parts of the district and subjected to baiting and direct inoculation techniques for the isolation of oomycetes. Various materials like grass blades, hemp and sesame seeds, insects' body parts, and citrus, pear and apple fruits were used as baits. The isolated fungi were further purified on solid media amended with pentachloronitrobenzene (PCNB), nystatin, ampicillin, and rifampicin. Various morphological features were recorded, mostly on baits in the water. In the case of oospore forming *Pythium* species, biometric values, i.e., Aplerotic Index, Ooplast Index, and Wall Index (Shahzad et al. 1992), were calculated (n = 20). The growth rate was determined on cornmeal agar (CMA) at room temperature (20–25ºC). All the taxa were identified up to the species level, following identification keys provided by Dick (1990) and Van der Plaats-Niterink (1981). The voucher specimens were deposited in the Pests and Disease Research Laboratory (PDRL), Department of Agriculture and Agribusiness Management, University of Karachi, Karachi.

Table 1 Isolates number, source, locality and sequence accession number of the species. Taxa marked with three asterisks = new species, two asterisks = new record for Pakistan, one asterisk = new record for Bajaur, with no asterisks = already reported. RS= Rhizosphere soil.

S. No	Isolate/ voucher	Taxon name	Source	Locality	Accession
1.	277_AH	*G. ghaffarianum* sp. nov.***	Liverworts	Kotwalo	MG799186
2.	284_AH	*G. orthogonon***	Soil	Khar	MH016276
3.	312_AH	-	*Dahlia* RS	Khar College	MG799187
4.	315_AH	-	Tomato RS	Chinar Gat	MG799188
5.	318_AH	*G. paroecandrum***	*Sausurea* RS	Khar College	MG799189
6.	AB_108	-	Citrus RS	Khar College	MZ207918
7.	296_AH	*G. selbyi***	*Tuja* RS	Khar College	MG799190
8.	297_AH	*G. schmitthenneri***	Tomato RS	Kotki	MG799191
9.	299_AH	-	Chili RS	Kotki	MG799192
10.	309_AH	-	Eggplant RS	Kotki	MG799193
11.	327_AH	-	Tomato RS	Halki	MG799194
12.	260_AH	*G. spinosum*	Maize field	Tangi	MG799195
13.	280_AH	-	Paddy RS	Yusuf Abad	MG799196
14.	302_AH	-	Onion RS	Kotwalo	MG799197
15.	AB_106D	*G. ultimum* var. *ultimum**	Water	Hasham	MZ206375
16.	AB_105	-	Water	Sro Wano	MZ379196

Table 1 Continued.

S. No.	Isolate/ voucher	Taxon name	Source	Locality	Accession
17.	275_AH	*Pythium kashmirense***	Wheat	Yusuf Abad	MG799199
18.	381_AH	*P. aphanidermatum*	Corn field	Kotki	MG799200
19.	322_AH	-	Potato field	Loi Jowar	MG799201
20.	285_AH	*P. aristosporum***	Tomato	Arang	MK420046
21.	317_AH	*P. dissotocum**	Soil	Khar	MG799202
22.	271_AH	-	Citrus RS	Khar	MG799203
23.	272_AH	-	Water	Charmang	MG799204
24.	307B_AH	-	Paddy field	Chakothra	MG799205
25.	286_AH	*P. catenulatum**	Grass RS	Khar Khwar	MH016275
26.	288_AH	*P. acanthicum**	Maize field	Tangi char	MG799206
27.	289_AH	-	Wheat field	Bhatwar	MG799207
28.	300_AH	*P. oligandrum**	Tomato RS	Kotki	MG799208
29.	383_AH	-	Maize field	Khar Khwar	MG799209
30.	307_AH	-	Onion field	Kot walo	MG799210
31.	290_AH	*Phytopythium sindhum**	Paddy field	Batwar	MG799211
32.	269_AH	*Ph. helicoides***	Soil	En. Khwaro	MG799212
33.	298_AH	*Ph. vexans**	Water	Gaber	MG799213
34.	259_AH	*Ph. litorale**	Water	Gaber	MG799214
35.	310_AH	-	Water	Kotki	MG799215
36.	AB_101	-	Water	Khar Khwar	MZ206375

DNA isolation, PCR and phylogenetic analysis

With the slight amendment, DNA was extracted following the protocol described by Möller et al. (1992), using mycelium harvested from a 5–10 days old culture grown on pea or V8 broth in 100 ml Erlenmeyer flasks at room temperature. The broths were prepared by boiling 120 grams of frozen peas or 200 grams of eight different vegetables for half an hour, strained through a double cheese cloth, and then adjusted to 1000 ml. The ITS region of the nuclear rRNA gene was amplified using universal primers: UNup18S42 (Bakkeren et al. 2000) and UN-lo28S22 (Lévesque & De Cock 2004) as forward and reverse primers, respectively. The 20µl PCR reaction mixture contained 50–100 ng of template DNA, 0.2 mM dNTPs, 0.2 µM each of forward and reverse primers, 2.5 mM $MgCl_2$, 1U of Taq polymerase, and 1X reaction buffer. Thermo cycler program was optimized as the initial denaturation step of 94 ºC for 4 minutes, followed by 35 cycles of 94 ºC for 30 s, 60 ºC for 35 s for annealing, 72 ºC for 90 s for extension, and finally one cycle at 72 ºC for 7 minutes. After purification, the amplified product was sequenced using the PCR primers. Sequences were edited and aligned using MEGAX software (Kumar et al. 2018). Nucleotide sequences were selected on the basis of the BLAST search of the GenBank (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi) and were aligned using the ClustalW program. The alignment matrix is available at TreeBASE (https://www.treebase.org/) under ID 29722. A maximum likelihood phylogenetic tree was constructed. The strength of the internal branches from the resulting trees was statistically tested by bootstrap analysis (Felsenstein 1985) with 500 replicates. The trees for *Globisporangium*, *Pythium* s. s and *Phytopythium* were out-grouped through taxa outside the respective genus.

Results

Morphological and phylogenetic analyses

Based on morphological features and sequence analysis, 36 isolates of *Pythium* s. l. (Table 1), were identified at the species level. The evolutionary history was inferred using the maximum likelihood method and the Hasegawa-Kishino-Yano model (Hasegawa et al. 1985). The tree with the highest log likelihood is shown. The percentage of trees in which the associated taxa clustered is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by

applying neighbor-join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete gamma distribution was used to model evolutionary rate differences among sites (3 categories (+*G*, parameter = 1.6169)). The rate variation model allowed some sites to be evolutionarily invariable ([+*I*], 10.78% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The sequences of barcoded taxa were submitted to the GenBank, and accession numbers were obtained (Table 1). The important morphological features of the recorded taxa have been tabulated and illustrated (Table 3). A new specific taxon is described.

Taxonomy

Globisporangium

The Genus was represented by seven species (Figs 2, 3), including a new species, *G. ghaffarianum*. Four species, *viz.*, *G. selbyi*, *G. schmitthenneri*, *G. orthogonon*, and *G. paroecandrum* are reported for the first time from Pakistan (Ahmad et al. 1997, Abdul & Shahzad 1998, Lodhi et al. 2004a, 2004b, 2005a, 2005b, 2005c). *Globisporangium ultimum* var. *ultimum* is a new record for the province while *G. spinosum* has already been reported from its district Bajaur (Abdul & Shahzad 1998).

Globisporangium ghaffarianum Abdul Haq and Shahzad sp. nov. Fig. 2 (B-K)

Index Fungorum – IF 900062

Holotype – Fig. 1B-K (277_AH), isolated from the rhizosphere soil of *Marchantia* sp. on hemp seed halves, Khar, District Bajaur, Khyber Pakhtunkhwa, Pakistan, 2012, Alt: 834m, Lat: 34.723253°N, Lon: 71.525182°E, cladogram; (Fig. 2A), GenBank Accession: MG799186

Etymology –The taxon is named after the late Dr. Abdul Ghaffar, the great mycologist, plant pathologist and professor at the Department of Botany, University of Karachi.

Description – Colonies on cornmeal agar and potato-carrot agar were submerged without any pattern with growth rate of 10 mm/day. Hyphae up to 5.5 µm wide. Zoosporangia and zoospores not observed; globose to lemon-shaped hyphal swellings produced in water and on solid media, 12–25 µm ($\bar{x}$ = 19.5 µm, n = 20) in diameter. Oogonia 13–27 µm ($\bar{x}$ = 22.25 µm, n = 20) in diameter, intercalary, occasionally terminal, globose. Antheridia 1–5 per oogonium, monoclinous, occasionally diclinous. Oospores 12–26 µm ($\bar{x}$ = 21.2 µm, n = 20) in diameter, mostly plerotic, sometimes aplerotic. Oospore wall 1.5–4 µm ($\bar{x}$ = 2.5 µm, n = 20) thick. The biometric values were Aplerotic Index = 75.2%, Ooplast Index = 29.8% and Wall Index = 54.1%.

Sequence Analysis

In nucleotide BLAST search and maximum likelihood analysis (Fig. 2A), 277_AH showed sequence similarity with *G. camurandrum*, *G. rostratum*, and *G. rostratifingens* from the *Pythium* clade E. All these taxa had 27.5-35.5% sequence divergence with 277_AH (Fig. 2B). A GenBank accession KP843586, submitted as *G. parvum* (Syn; *P. parvum*) from the United States of America (Crocker et al. 2015) showed 99.2% sequence similarity with that of 277_AH. This submission appears to be a misidentification, as the sequence of the authentic X-type culture of *G. parvum* (CBS 225.88 Acc; AY598697) had only 72% sequence similarity with 277_AH and KP843586 (Fig. 2G). Morphologically, 277_AH is different from *G. parvum* as the latter has smaller oogonial dimensions, thinner oospore walls, and hypogynous antheridia. The comparison of some important morphological features of the above-mentioned taxa (Table 2) and phylogenetic analysis (Fig. 1G-H) confirms that isolate 277_AH is a novel taxon.

Table 2. Morphological comparison of *G. ghaffarianum* with closely related species. terminal=T, intercalary=I, monoclinous=*mc*, diclinous=dc, hypogynous =hg, chrysanthemum= chrys, not observed=no

Diagnostic features	***G. rostratifingens***	***G. rostratum***	***G. camurandrum***	***G. parvum***	***G. ghaffarianum***
Growth /day	6.0 mm	8.0 mm	9.0 mm	-	10.0 mm
Growth Pattern	chrys	chrys	chrys	chrys	no pattern
Hyphal width	5.0 µm	8.0 µm	7.0 µm	6.0 µm	5.5 µm
Sporangia	no	globose	globose	no	no
Zoospores	no	produced	rare	no	no
Antheridia	mc/dc/hg	mc/dc	mc/dc/hg	hg	mc/dc
Oogonia	T/I	I/T	I/T	I/T	I/T
Oogonial size	14.0–22.0 x̄=16.7 µm	19.0–24.0 x̄= 21.5 µm	11.0–22.0 x̄=17.4 µm	10.0-20.0 x̄=14.3 µm	12.0-25.0 x̄=22.2 µm
Oospore/ oogonia	1.0-2.0	1.0	1.0-2.0	1.0-2.0	1.0
Oospore wall	1.5 µm	2.0 µm	0.4-1.3 µm	0.5 µm	1.5-4.0 x̄=2.5 µm

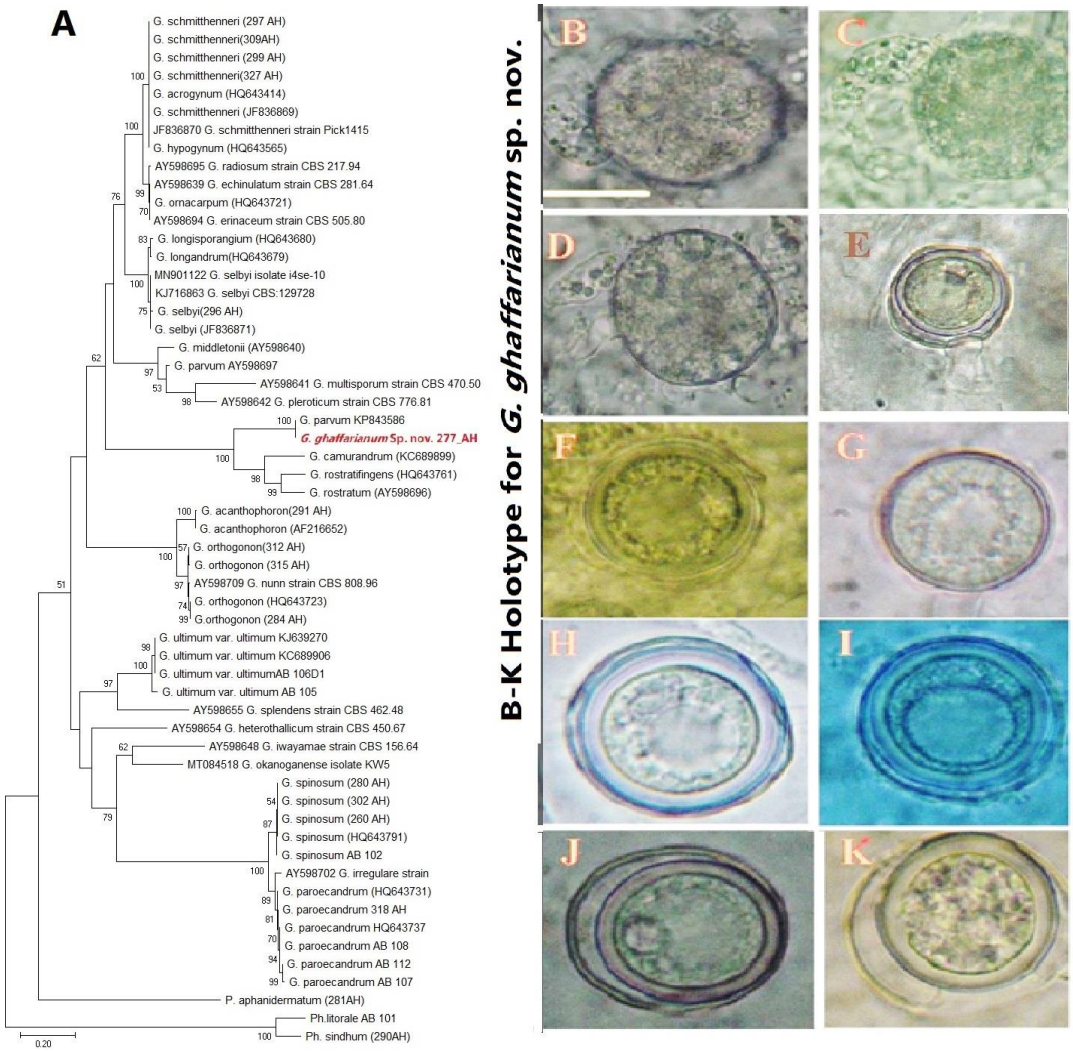

Fig. 1 – A maximum likelihood tree of the Genus *Globisporangium* showing the phylogeny of *G. gaffarianum* sp. nov (277_AH) and the taxa isolated in this study (in bold). B–K (Holotype) *G. gaffarianum* oogonia and oospores. Scale bar = 10 µm.

Globisporangium selbyi (Fig. 2E-H) was isolated from the rhizosphere soil of *Thuja* sp., showing severe root rot and wilt symptoms. The main hypha was 4.5–7 µm wide. It was reported as

a new species and the causal agent of seedling and root-rot disease of corn in Ohio (Elis et al. 2012). Our isolate was identical in morphology and ITS sequence to the type specimen (Fig. 1A and Fig. 2E-H). *Globisporangium longandrum* and *G. longisporangium* are morphologically close to *G. selbyi*, however, the plerotic nature of oospores and hypogynous antheridia distinguish it from the other two. *Globisporangium longisporangium* lacks zoospores as well (Paul et al. 2005). The isolate 296_AH showed >99.5% sequence similarity to that of the original specimen (JF836871) and 97.6% and 96.8% with *G. longandrum* and *G. longisporangium*, respectively. Probably, this is its first report outside the USA.

Globisporangium schmitthenneri (Fig. 2A-D) was isolated from solanaceous plants. It has similarity in the DNA sequence of ITS region with *G. acrogynum* (99.9%) and *G. hypogynum* (99.8%), but differs in morphology from both. *Globisporangium acrogynum* has papillate oogonia, which are smooth in *G. schmitthenneri*. The discharge tube of *G. schmitthenneri* is narrower and longer (>twice the diameter of the sporangium) than that of *G. hypogynum*. The antheridia are strictly hypogynous in *G. acrogynum* and *G. hypogynum* (Van der Plaats-Niterink 1981).

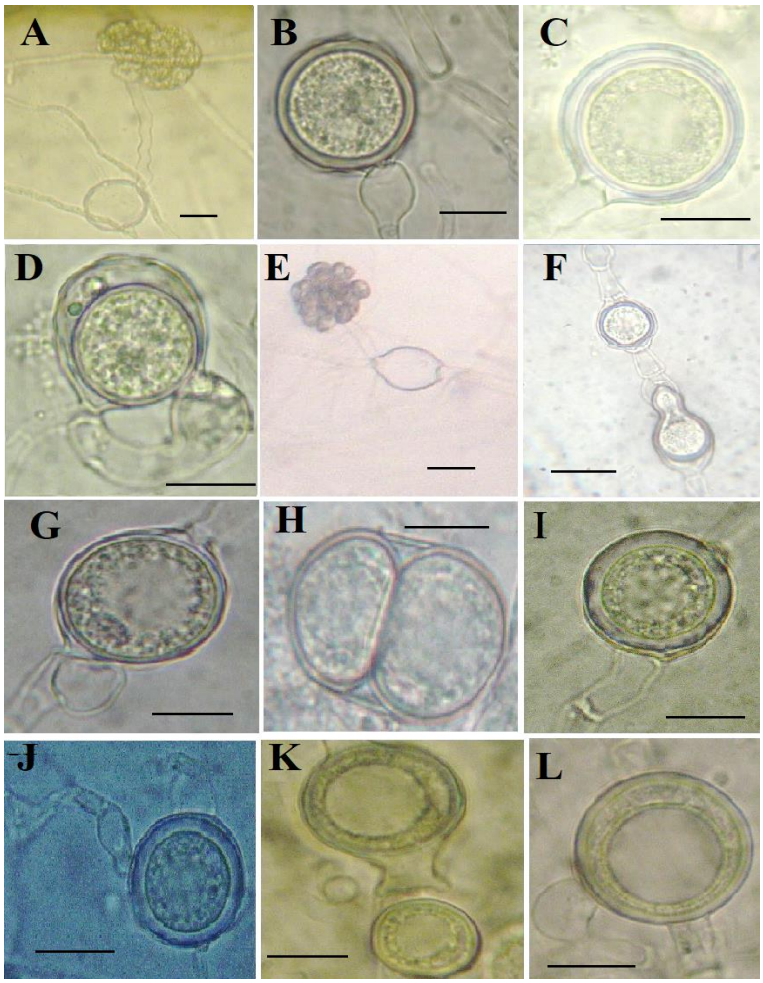

Fig. 2 A–D *Globisporangium schmitthenneri.* A zoosporangial discharge. B–D oogonia with antheridia. E–H *G. selbyi.* E zoosporangial discharge. F–H, D oogonia with attached antheridia. I–J *G. orthogonon* K–L *G. paroecandrum.* Scale bar: = 10µ.

It has been only recently reported from Morocco, where it was isolated from olive trees with severe root and crown rot symptoms (Legrifi et al. 2022). *Globisporangium ultimum* var. *ultimum* is a new record for the province, while *G. spinosum* has already been reported from its district Bajaur (Abdul & Shahzad 1998).

Table 3 Taxonomically important characteristics of the new records of *Pythium* s. l. (globose=g, subglobose=sg, inflated=infl, ovoid=o, sub-ovoid=so, smooth=s, terminal=T, intercalary=I, monoclinous=*mc*, diclinous=dc, hypogynous=hg, not observed=no, no data=nd, not applied=na)

Taxon name	Growth/day (mm)	Sporangial shape	Sporangial size (µm)	Encysted zoospore (µm)	Antheridial origin	Oogonial shape	Oogonial position	Oogonial diameter (µm)	Oospore diameter (µm)	Wall thickness (µm)	Aplerotic Index (%)	Ooplast Index (%)	Wall Index (%)
G. orthogonon	15.0	g/sg	19	9-12	dc/mc	sm	I/T	19.0	17.0	1.5	75	22	48
G. paroecandrum	20.0	g/sg	25	no	mc/dc/hg	sm	I/T	22.0	19.0	1.5	62	17	30
G. schmitthenneri	22.0	g/sg	23	10-12	mc/hg/dc	sm	I/T	22.0	20.0	2.0	76	37	20
G. selbyi	12.0	g/sg	34	8-10	hg/mc/dc	sm	I/T	25.5	23.3	1.7	78	31	37
G. spinosum	35.0	no	no	no	dc/mc	sp	I/T	19.5	17.8	1.2	80	34	28
G. ultimum var. ultimum	30.0	g/sg	25	no	mc/dc/hg	sm	T/I	21.0	17.5	1.3	59	41	39
P. acanthicum	22	no	no	no	mc/dc	sp	T/I	22.2	20.0	1.8	67	22	43
P. aphanidermatum	35.0	infl	nd	10-12	mc/dc	sm	T	23.2	21.0	1.7	64	71	38
P. catenulatum	15	chs	9-22	8-10	dc/mc	sm	T/I	28.5	26.5	1.5	80	41	40
P. dissotocum	12.5	fil	nd	9-10	mc/dc	sm	I/T	23.0	21.0	2.2	62	20	28
P. kashmirense	15.0	fl	nd	10-12	dc	sm	I/T	20.5	18.8	1.0	78	35	14
P. oligandrum	25	infl	nd	9-10	dc/mc	sp	I/T	23.5	20.5	1.5	72	19	38
P. aristosporum	20.5	no	nd	nd	mc/dc	sm	T/I	35.5	33.5	2.2	85	40	17
Ph. helicoides	30	o/so	35x24	10-12	no	no	no	no	no	no	na	na	na
Ph. litorale	20	o/so	28x23	8-10	no	no	no	no	no	no	na	na	na
Ph. sindhum	28	o/so	33x23	9-10	dc/mc	sm	T/I	32.5	30.5	3.5	84	na	52
Ph. vexans	12	g/sg	17x15	8	no	no	no	no	no	no	na	na	na

Pythium s. s

The genus consisted of seven species, *viz.*, *Pythium kashmirense*, *P. aristosporum*, *P. dissotocum*, *P. catenulatum*, *P. acanthicum*, *P. oligandrum*, and *P. aphanidermatum* (Fig. 3A). The first two taxa are new records for Pakistan, whereas the rest, except *P. aphanidermatum*, are reported for the first time from the province.

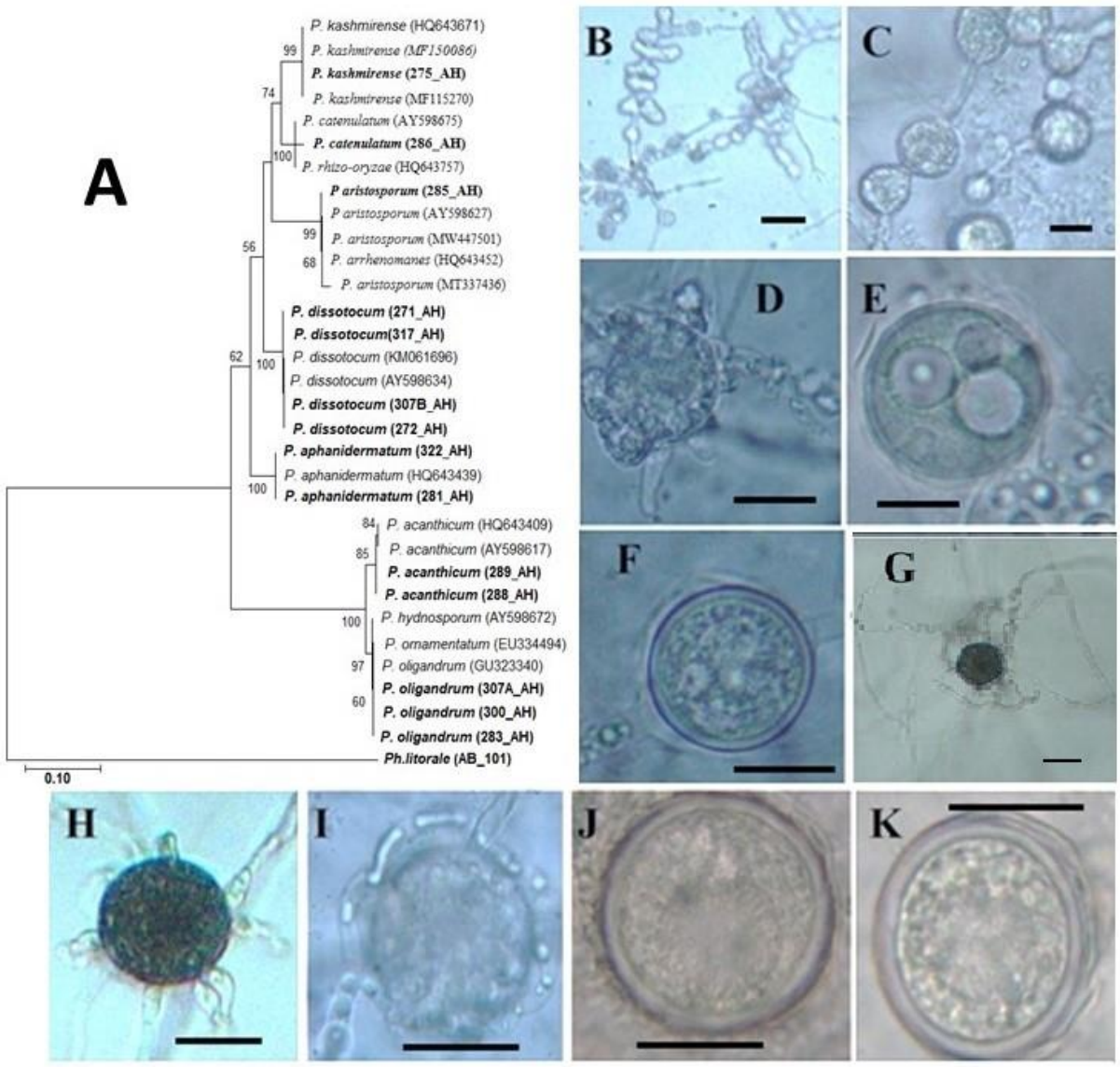

Fig. 3 – Maximum likelihood tree of the genus *Pythium* s. s. showing the phylogeny of the taxa isolated in this study (in bold). B–F *P. kashmirense.* B zoosporangia. C hyphal swelling. D–E oogonia with antheridia. G–K *P. aristosporum.* G–I oogonia with attached antheridia. J–K oospores. Scale bar = 10 µm.

Pythium kashmirense (Fig. 3G-K) was isolated from the wheat rhizosphere soil. It was reported as a new species from India-held Kashmir (Paul & Bala 2008), the Pakistani part of which is adjacent to this province. It is characterized by its inflated sporangia, catenulate hyphal swellings, coiled antheridial branches, and single plerotic oospores per oogonium. Catenulate hyphal swellings are also found in *P. rhizo-oryzae* and *P. catenulatum*, however, with the difference in other morphological features and more than 4% divergence in ITS sequence, *P. kashmirense* considered a distinct taxon. Due to the similarity in morphology and ITS sequence (100% similarity), *P. rhizo-oryzae* appears to be the later homonym of *P. catenulatum.* Benavent-Celma et al. (2021) have demonstrated that inoculations of *P. kashmirense* under controlled

conditions on a number of plants have caused pre-emergence damping-off, as well as seed rot and root rot.

Pythium aristosporum (Fig. 3B-F) was isolated from the tomato fields. Morphologically, it differs from *P. arrhenomanes* by having fewer antheridia per oogonium. Antheridia are monoclinous and diclinous in *P. aristosporum*, and only diclinous in *P. arrhenomanes*. The two taxa have 100% similarity in their ITS sequence. Another related species, *P. graminicola*, has fewer and predominantly monoclinous antheridia and only 90% sequence similarity with the other two taxa. *Pythium aristosporum* has been reported in rice seedlings (Sari Handoko et al. 2022, Liu et al. 2022), zoysia green (Ichitani & Kinoshita 1990), and wheat (Lipps & Bruehl 1978).

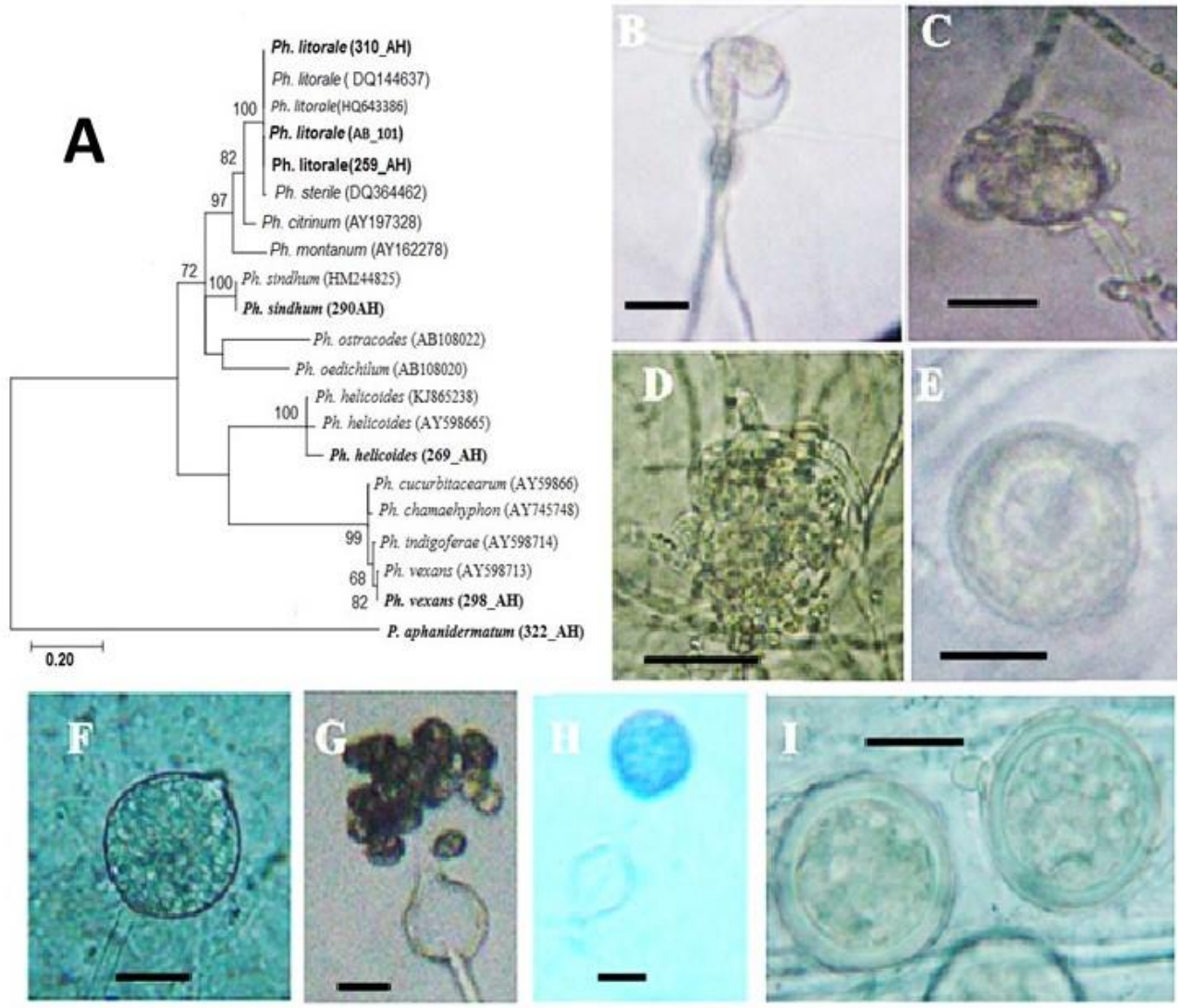

Fig. 4 – A maximum likelihood tree of the genus *Phytopythium* showing the phylogeny of the taxa isolated in this study (in bold). B–E *Ph. helicoides.* B internally proliferating zoosporangium. C–E oogonia. F–I *Ph. sindhum.* F–H zoosporangia. I oospores. Scale bar = 10 µm.

Phytopythium

The genus characteristically has ovoid to globose papillate sporangia (except for *Ph. vexans*), with internal proliferation and zoospore discharge similar to that of *Pythium*. More than 20 species belonging to this genus have been described so far (Tkaczyk 2020). It was represented here by four species, *Ph. helicoides Ph. sindhum*, *Ph. vexans*, and *Ph. litorale* (Fig. 4). *Phytopythium helicoides* (Fig. 4B-E) appears to be a new record not hitherto reported from Pakistan, while the rest are new

records for the province (Ahmad et al. 1997, Abdul & Shahzad 1998, Lodhi 2007, Bala et al. 2010).

The salient features of *Ph. helicoides* are the papillate sporangia and the closely adhering antheridia. It has been reported to cause root and crown rot in strawberries (Ishiguro et al. 2014, Marin et al. 2019) and in roses (Kageyama et al. 2002). *Phytopythium sindhum* (Fig. 4F-I), the type species of this genus, was reported as a new species from the Sindh province of this country (Bala et al. 2010). It is now isolated from the soil of a paddy field in Bajaur. *Phytopythium vexans* and *Ph. litorale* also cause root rot in plants (Beluzán et al. 2022, Pánek & Střížková 2021).

Discussion

The plants play a vital role in the economy of Pakistan. The agriculture sector absorbs 42.3% of the labor force and contributes 18.9% to GDP (Hayat 2019). The plants in the area frequently suffer from diseases, the causal agents of which definitely include soil-born oomycetes belonging to this group. The high species diversity identified during this study in the area, including a new species, demands a more elaborate investigation of these ecologically important fungi. Though the present work was focused on the systematic aspect of these organisms, however, it will provide a base for further studies on aspects like pathogenicity, epidemiology, and control.

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