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MORPHOLOGICAL AND nrDNA-ITS SEQUENCES-BASED IDENTIFICATION AND ECOLOGICAL DISTRIBUTION OF *Volvopluteus earlei* IN PAKISTAN

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Abstract

Volvopluteus is represented by seven species distributed in tropical regions of America, Asia and Europe. They grow as saprobes on wood debris and leaves, in grassy fields and forests. To explore the macrofungal diversity, different localities in Punjab were visited and collections were made. Among these several collections were found similar to *Volvopluteu*s species. They were analyzed morphologically and phylogeneticaly using ITS sequences of nrDNA. Comparative analysis on the basis of morphological and anatomical features revealed that these collections represent *V. earlei*. Maximum likelihood phylogeny also showed the position of current sequences in a same clade with already known *V. earlei* sequences with 74% bootstrap value. *Volvopluteus earlei* is being described from different areas of Pakistan with variety of climatic conditions from warm semi-arid climate of Kasur, Lahore and Sheikhupura districts, humid subtropical climate of Sialkot district, warm desert climate of Muzaffargarh district in Punjab and warm semi-arid to humid subtropical climate of Swat district, Khyber Pakhtunkhwa. The occurrence of *V. earlei* in different climatic zones represents its wide ecological amplitude in the country.

Keywords: *Agaricales*, basidiomata, ITS, molecular phylogeny, semi-arid, taxonomy.

INTRODUCTION

Volvopluteus Vizzini, Contu & Justo is classified in *Pluteaceae* Kotl. & Pouzar (*Agaricales*, *Basidiomycota*) (Vizzini et al., 2024). It is typified by *V. gloiocephalus* (DC.) Vizzini, Contu & Justo and represented by seven species distributed in America, Asia and Europe (Justo et al., 2011a, 2011b; Kaur & Singh, 2014; He et al., 2019; Kaygusuz et al., 2021; Montoya et al., 2021). *Volvopluteus* species occur abundantly in the tropical regions where they come up early in the monsoon season, mostly they are terrestrial, growing as saprobes on wood debris and leaves, in grassy fields and forests (Kaur and Singh, 2014).

Species of *Volvopluteus* are morphologically characterized by the presence of medium-to-large sized basidiomata having volva at the stipe base, free and white to pink or brownish pink lamellae. Anatomically, they are characterized by sub-globose or broadly ellipsoid, thick-walled, inamyloid, basidiospores, an ixocutis pileipellis consists of hyphae which are thin-walled and surrounded by a gelatinous matrix (Justo et al., 2011b; Kaygusuz et al., 2021).

The present study focuses on identification of specimens which were found morphologically similar to *Volvopluteus* species. The detailed morphological and molecular analyses were performed to identify up to species level. The collections were made from three districts (Kasur, Lahore and Sialkot) of Punjab, Pakistan. The climate of Kasur and Lahore district is warm semi-arid while that of Sialkot district is humid subtropical (Köppen climate classification). This work is an effort to explore the fungal taxa and their ecological distribution in various parts of Pakistan.

MATERIALS AND METHODS

During the field study of different regions of Punjab, Pakistan in monsoon season of 2019–2021, basidiomata were collected and morphological characters were noted. Munsell color chart was used for color codes (Munsell, 1975). Specimens were photographed and air dried for preservation. Tissues were rehydrated in 5% KOH and stained with Congo red (1%) and visualized under Xsz 107BN microscope with 100× objective lens. Motic Images Plus 2.0 software (calibrated) was used for measurement of microscopic features. For basidiospores, [s/b/c] represented; s number of basidiospores which were measured from b basidiomata in c collections, l × w represented spore dimensions in terms of length and width, lowest and highest values were shown in parenthesis. Q values represented l/w ratio while Q values for basidiospores were defined following Bas (1969). Average values were represented by av. Drawings were drawn from microscopic images. The collected specimens were deposited in the herbarium (UEH), University of Education, Lahore, Pakistan.

Extract-N-Amp™ kit was used for DNA extraction, following the manufacturer's instructions. PCR amplification of nrDNA-ITS region and its sequencing were carried out from the sequence service using ITS1F as forward and ITS4 as reverse primers. The consensus sequences were made and analyzed in BioEdit sequence alignment editor version 7.2.5 (Hall, 1999). BLAST of the complete ITS region was performed at NCBI to select the sequences with closest match within the genus for phylogenetic analysis. Published sequences of the closest relatives of the species were also included in the final dataset. *Melanoleuca verrucipes* (Fr.) Singer was chosen as the outgroup (Kaygusuz et al., 2021). Multiple alignment was performed using online MUSCLE (Edgar, 2004) and uploaded in MEGA 6.0 software. For maximum likelihood analysis, Jukes-Cantor model (Jukes and Cantor, 1969) was selected as best-fit substitution model approach in MEGA (Tamura et al., 2013).

RESULTS

1- Phylogenetic Analysis

The ITS region amplified during this investigation was 612 and 613 bp long after trimming the sequences at conserved regions. In BLAST results, these sequences were 99% similar to the sequences from India, Italy, Japan, Pakistan, South China, Turkey and USA. The sequences generated from present collections along with other sequences from closely related taxa were downloaded from GenBank (**Table 1**). To reconstruct phylogenetic tree, a final ITS dataset of 73 nucleotide sequences with 868 positions was used to reconstruct phylogeny. Among these, 482 were conserved, 327 were variable, 160 were parsimony informative and 166 were represented as singletons positions. Maximum likelihood analysis clustered the current sequences in a same clade with already known *V. earlei* sequences with 74% bootstrap value (**Figure 1**).

Table 1. Species and specimens used for the molecular phylogeny

GenBank Accession (ITS)	Species	Country	Voucher	Reference
AM286682	*Cryptococcus huempii*	India	DK05	Unpublished
DQ490642	*Melanoleuca verrucipes*	USA	PBM 2289 (WTU) Washington	Matheny et al., 2006; Kaygusuz et al., 2021

NR_119878	*Volvopluteus asiaticus*	Japan	TNS: F-15191	Schoch et al., 2014; Justo et al., 2011a
HM562205	*V. earlei*	Democratic Republic of the Congo	Mamet7	Justo et al., 2011a, 2011b
MH021869	*V. earlei*	Japan	TNS: F-70428	Unpublished
MT353644	*V. earlei*	Pakistan	SWAT001366	Khan et al., 2022
MW033389	*V. earlei*	Turkey	OKA-TR649	Kaygusuz et al., 2021
MW033388	*V. earlei*	Turkey	OKA-TR648	Kaygusuz et al., 2021
MW033390	*V. earlei*	Turkey	OKA-TR650	Kaygusuz et al., 2021
MW033391	*V. earlei*	Turkey	OKA-TR651	Kaygusuz et al., 2021
MW033392	*V. earlei*	Turkey	OKA-TR652	Kaygusuz et al., 2021
MW033393	*V. earlei*	Turkey	OKA-TR653	Kaygusuz et al., 2021
MW033394	*V. earlei*	Turkey	OKA-TR654	Kaygusuz et al., 2021
MW033395	*V. earlei*	Turkey	OKA-TR655	Kaygusuz et al., 2021
MW033396	*V. earlei*	Turkey	OKA-TR656	Kaygusuz et al., 2021
JN086659	*V. earlei*	India	WV-2	Unpublished
JN086658	*V. earlei*	India	WV-1	Unpublished
HM562204	*V. earlei*	Spain	MA22816	Justo et al., 2011a, 2011b
MH021868	*V. earlei*	Japan	TNS: F-70427	Unpublished
MK204987	*V. earlei*	Italy	AGMT n. 69	Giannoni et al., 2018
MK204988	*V. earlei*	Italy	AGMT n. 70	Giannoni et al., 2018
MK204989	*V. earlei*	Italy	AGMT n. 71	Giannoni et al., 2018
MK616346	*V. gloiocephalus*	Spain	V669_1+ITS4	Unpublished
MK616347	*V. gloiocephalus*	Spain	V709_1+ITS4	Unpublished
MW362280	*V. earlei*	Pakistan	LAH35715	Unpublished
MW811445	*V. earlei*	South China	XJ1378	Unpublished
MW811446	*V. earlei*	South China	XJ1379	Unpublished
ON777909	***V. earlei***	**Pakistan**	UEH-F0002	**This study**
ON777910	***V. earlei***	**Pakistan**	UEH-F0003	**This study**
ON777911	***V. earlei***	**Pakistan**	UEH-F0004	**This study**
MW862312	*V. gloiocephalus*	China	HBAU15427	Unpublished
MK616345	*V. gloiocephalus*	Spain	V695_1+ITS1F	Unpublished
MW033404	*V. gloiocephalus*	Turkey	OKA-TR664	Kaygusuz et al., 2021
MW033402	*V. gloiocephalus*	Turkey	OKA-TR662	Kaygusuz et al., 2021
MW033403	*V. gloiocephalus*	Turkey	OKA-TR663	Kaygusuz et al., 2021
MW033397	*V. gloiocephalus*	Turkey	OKA-TR657	Kaygusuz et al., 2021
MW033400	*V. gloiocephalus*	Turkey	OKA-TR660	Kaygusuz et al., 2021
MW033401	*V. gloiocephalus*	Turkey	OKA-TR661	Kaygusuz et al., 2021
MN738645	*V. gloiocephalus*	New Zealand	PDD:103792	Unpublished
HM562202	*V. gloiocephalus*	Spain	AJ239	Justo et al., 2011a, 2011 b
HM562209	*V. gloiocephalus*	Spain	LOU18247	Justo et al., 2011a, 2011b
MW916608	*V. gloiocephalus*	Egypt	EGDA-Volv1	Unpublished
MH930216	*V. gloiocephalus*	Russia	5173	Unpublished
HM562208	*V. gloiocephalus*	Spain	LOU13710	Justo et al., 2011a, 2011b
HM562207	*V. gloiocephalus*	Spain	LOU18619	Justo et al., 2011a, 2011b
MN738634	*V. gloiocephalus*	New Zealand	PDD:95300	Unpublished
MW033398	*V. gloiocephalus*	Turkey	OKA-TR658	Kaygusuz et al., 2021
MW033399	*V. gloiocephalus*	Turkey	OKA-TR659	Kaygusuz et al., 2021
KP826741	*V. gloiocephalus*	France	CM042	Unpublished
MF954700	*V. gloiocephalus*	Canada	UBC F-32227	Unpublished
MK616344	*V. gloiocephalus*	Spain	V678_1+ITS1F	Unpublished

HM562203	*V. gloiocephalus*	California	PBM2272	Justo et al., 2011a, 2011b
MW633065	*V. gloiocephalus*	USA: Arizona, Yavapai Co.	Mushroom Observer 420468	Unpublished
MK729541	*V. michiganensis*	Russia	LE311989	Unpublished
MW242665	*V. michiganensis*	China	HMJAU-CR45	Rao et al., 2021
MG367239	*V. michiganensis*	China	11537	Unpublished
MW242669	*V. michiganensis*	China	HMJAU-CR106	Rao et al., 2021
NR_119876	*V. michiganensis*	USA: Michigan	MICH:11761	Schoch et al., 2014; Justo et al., 2011a
MK729542	*V. michiganensis*	Russia	LE 312006	Unpublished
MW242668	*V. michiganensis*	China	HMJAU-CR46	Rao et al., 2021
MK049912	*V. michiganensis*	Russia	LE311991	Unpublished
MF954699	*V. michiganensis*	Canada	UBC F-32158	Unpublished
MK944280	*Volvopluteus* sp.	China	GDGM74751	Unpublished
MK944281	*Volvopluteus* sp.	China	GDGM73195	Unpublished
MT535739	*Volvopluteus* sp.	Iran	Ghobad-Nejhad 4321	Unpublished
HM246499	*Volvariella acystidiata*	Italy	TO HG1973	Unpublished
HM246496	*Vo. cookei*	Italy	TO AV133	Unpublished
HM246497	*Vo. earlei*	Italy	TO AV134	Unpublished
JN182873	*Vo. gloiocephala*	China	55	Unpublished
HM246495	*Vo. gloiocephala*	Italy	TO AV136	Unpublished
DQ494701	*Vo. gloiocephala*	USA	PBM 2272 (WTU) California	Matheny et al., 2006
HM246498	*Vo. media*	Italy	TO HG2001	Unpublished

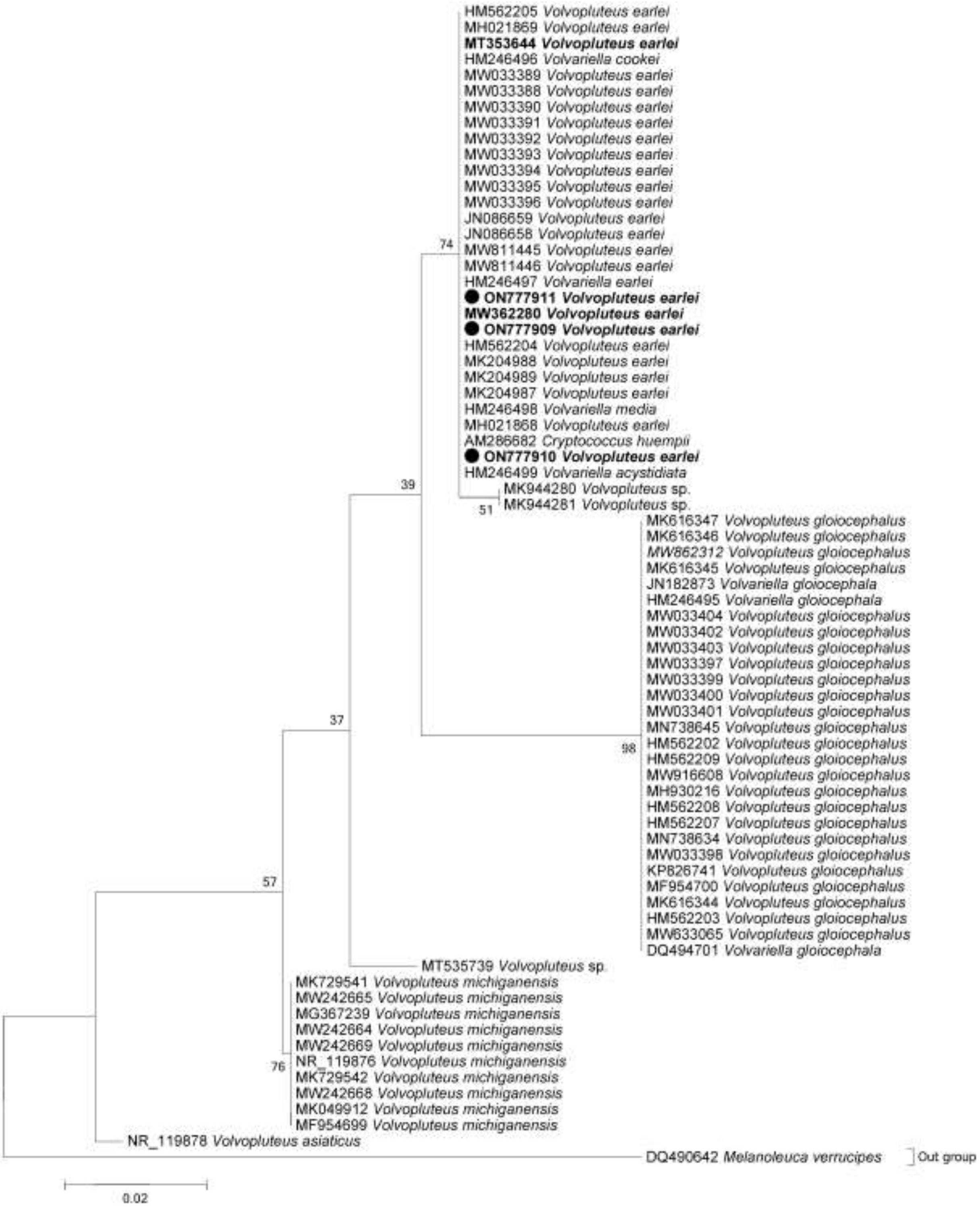

Figure 1. Molecular phylogenetic analysis of ITS sequences of *Volvopluteus earlei*. The sequences from Pakistan are bold face and those generated during this study are marked with bullets.

2- Taxonomy

Volvopluteus earlei (Murrill) Vizzini, Contu & Justo, Fungal Biology, 115(1): 15. (2011). Pileus 2.5–10.4 cm broad, conical to planoconvex, becoming flat at maturity with a low and broad umbo at the center; margins plicate to grooved striate; surface smooth to glabrous or innately radially fibrillose, viscid when young becoming dry with age, white to pinkish (10R 9/1) and greyish (N9), light brown (7.5YR 9/1) at the center. Lamellae free, thin, crowded, ventricose; margin flocculose; white when young, pinkish (7.5R5/2) at maturity, brown when dry (7.5YR 4/2). Lamellulae frequent, of variable lengths. Stipe 5–7.8 × 0.5–0.9 cm, central, cylindrical, sub-bulbous at base with volva; longitudinally striate, white to whitish. Volva sac-like, white, 1–2 cm in length. Annulus absent. Smell indistinct. Not tasted (**Figure 2**).

Basidiospores [80/4/4] (14.8)15.5–19.6(20.2) × (9.9)10.1–14.4(16.3) µm, av = 17.5 × 13.1 µm, Q = (1.24)1.36– 1.49(1.53), avQ = 1.4, broadly ellipsoid to ellipsoid, smooth, thick walled, mono- to poly-guttulate, apiculus prominent. Basidia (34.1)35.1–49.2(49.4) × (12.2)12.4–16.4(17.7) µm, av = 41.7 × 14.9 µm, clavate, 2–4 spored, thin walled, basal clamp absent. Pleurocystidia (21)23.4–52.2(63.6) × (11.5)12.3–18.3(21.8) µm, av = 17.5 × 13.1 µm, infrequent, broadly clavate to clavate to obovoid, thin walled. Cheilocystidia (44.9)51.9–80.3(83.3) × (20.4)21.0–24.2(25.6) µm, av = 64.1 × 23.1 µm, lageniform to broadly lageniform or narrowly utriform, thin walled. Pileipellis ixocutis, hyphae (3.3)4.1–17.3(26.2) µm, av = 14.7 µm in diameter, cylindrical or subcylindrical elements, slightly swollen from terminals, septate, branched, cylindrical, smooth and thin walled. Stipitipellis hyphae (4.1)6.4–16.5(17.0) µm, av =10.5 µm in diameter, elongated cylindrical elements, septate, cylindrical, branched, thin walled. Clamps absent. All tissues hyaline in 5% KOH (**Figure 3**).

3- Material examined

Pakistan, Punjab, Gujranwala division, Sialkot district, Khajoriwala, 256 m asl, from grass lawn, 15 July 2019, Maham Mureed SJ195 (UEH-F0002; GenBank accession: ON777909); along the road, on grass, 29 July 2019, Maham Mureed SJ227 (UEH-F0003; GenBank accession: ON777910); Lahore division, Kasur district, Changa Manga Forest, 192 m asl, on soil, 29 July 2019, Amir Ali SJ57 (UEH-F0004; GenBank accession: ON777911); Lahore district, Punjab University Housing Society, 217 m asl, on grassland soil, 1 August 2021 Hira Ijaz (UEF-F0005).

Figure 2. *Volvopluteus earlei*. A–D. UEH-F0002; E. UEH-F0004; F. UEH-F0003; G–I. UEH-F0005. Scale bars A–F = 5 cm, G–I = 1 cm. Photographs by Maham Mureed and Hira Ijaz.

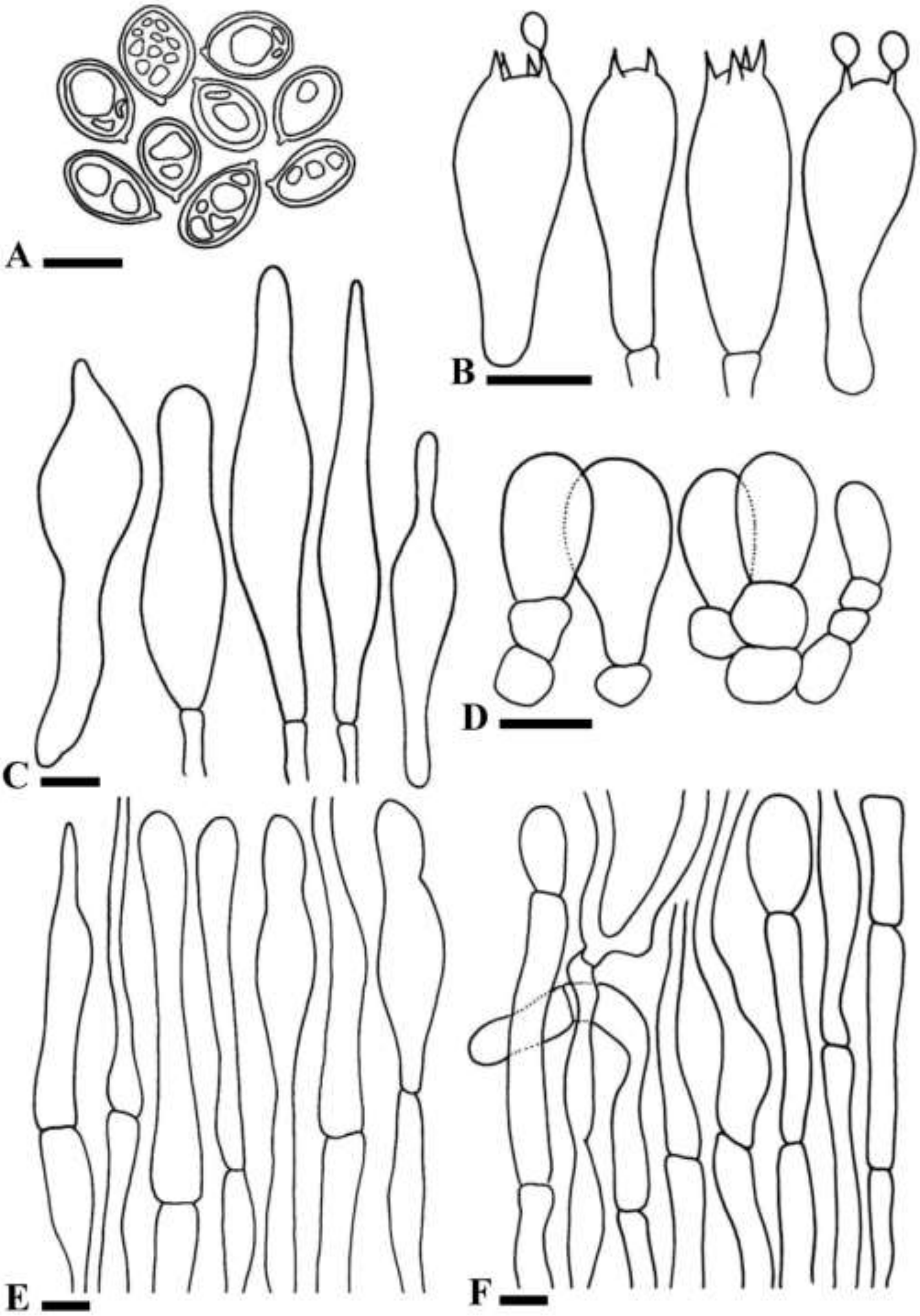

Figure 3. *Volvopluteus earlei* UEH-F0003. A. Basidiospores; B. Basidia; C. Cheilocystidia; D. Pleurocystidia; E. Pileipellis; F. Stipitipellis. Scale bars: A–F = 15 µm. Drawing by Sana Jabeen.

DISCUSSION

Volvopluteus earlei is identified by the medium sized basidiomata with whitish or slightly grey pileus, white to pinkish lamellae, broadly ellipsoid to ellipsoid 15.5–19.6 × 10.1–14.4 µm basidiospores, infrequent pleurocystidia, lageniform to narrowly utriform cheilocystidia, and pileipellis composed of ixocutis (Murrill, 1911; Shaffer, 1957; Justo & Castro, 2010; Justo et al., 2011b; Giannoni et al., 2018; Kaygusuz et al., 2021).

It grows gregarious or solitary on forest soil, grassland, roadsides, and gardens. It mostly occurs on clayey-loamy with medium lime soil that is rich in humus (Kaygusuz et al., 2021; Justo & Castro, 2010). The species is found in boreal and subtropical to tropical regions (Singer, 1986; Justo et al., 2014; Menolli et al., 2015; He et al., 2019; Kaygusuz et al., 2021).

This species was previously described from Cuba and then from USA, Africa, Mexico, Spain, Argentina, Italy and Turkey (Murrill, 1911; Coker, 1947; Heinemann, 1975; Vázquez et al., 1989; Contu, 2006; Justo and Castro, 2010; Niveiro & Albertó, 2012; Giannoni et al., 2018; Kaygusuz et al., 2021). Sequences from India, Japan Pakistan and South China have also been found from GenBank.

Present collections were found growing solitary on loamy soil, grassland and on roadside from different localities in Punjab with different climatic conditions from warm semi-arid of Kasur and Lahore districts and humid subtropical climate of Sialkot district (Köppen climate classification). Records of the same taxon have also been found from two districts of punjab; Muzaffargarh district features warm desert climate and Sheikhupura district with warm semi-arid climate and Swat district in Khyber Pakhtunkhwa having warm semi-arid to humid subtropical climate (Köppen climate classification) (Khan et al., 2022). During this investigation, the morpho-anatomical descriptions and molecular phylogenetic analysis of the *V. earlei* collected from various regions of Punjab, Pakistan represents its wide distribution and ecological amplitude in the country.

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Conflict of Interests

Authors declare no conflict of interest.

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Author’s Contribution

Hira Ijaz wrote the manuscript draft; Maham Mureed collected and described her specimen for her MS research; Dr. Aneela Yasmeen assisted with molecular work and collection; Amir Ali helped with collection; Prof. Dr. Abrar Hussain, as the supervisor of Maham Mureed, reviewed the data; and Dr. Sana Jabeen guided the team members, managed drafts and revisions, handled submission and correspondence, and secured final approval from all authors.

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