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Morpho-molecular analysis reveals *Appendiculella viticis* sp. nov. (*Meliolaceae*)

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Abstract

A novel species, *Appendiculella viticis*, was collected on freshly fallen leaves of *Vitex canescens* (*Lamiaceae*) in Chiang Rai, Thailand. This species is unique in having vertically striate, conoid cells, without larviform appendages and fusiform to ellipsoidal, guttulate ascospores. Morphological comparison and phylogenetic analysis of combined LSU and ITS sequence data provide evidence that the species is novel. Morphological comparisons of *Appendiculella* species are provided. The molecular data provides evidence to support the distinctness of *Appendiculella*.

Keywords: 1 new taxon, epiphytic fungus, Meliolales, phylogeny, taxonomy

Introduction

Höhnel (1919) introduced *Appendiculella* to accommodate species which have ascomata with larviform appendages (Justavino & Piepenbring 2007). Species Fungorum (2020) listed 92 epithets in *Appendiculella*. However, only two species have molecular data (Zeng *et al.* 2017). *Appendiculella* species are mostly considered host-specific and are widely distributed in tropical regions, such as Taiwan and India (Kirk *et al*. 2008). Some species such as *Appendiculella acaenae* Hansf. recorded from living leaves of *Acaena* and *Appendiculella altingiae* Y.X. Hu & B. Song. from living leaves of *Altingia chinensis* Champ. ex Benth. have been introduced based on host association (Justavino *et al*. 2014).

Appendiculella species are characterized by verrucose ascomatal walls with raised conoid cells, with conical appendages, 2–4-spored, unitunicate asci and 2–3-seriate, fusiform to ellipsoid, 3–4-septate, hyaline to brown ascospores (Hongsanan *et al*. 2015). Their asexual morph comprises ampulliform, alternate or opposite phialides, mixed with capitate hyphopodia (Justavino & Piepenbring 2007).

Appendiculella is included in *Meliolaceae*, Meliolales (Hongsanan *et al*. 2015, Hyde *et al*. 2020, Wijayawardene *et al*. 2018). The first phylogenetic analysis based on *A. lozanellae* Rodr. Just. & M. Piepenbr. (DQ508302), supported its placement in *Meliolaceae* (Hongsanan *et al.* 2015, Justavino *et al*. 2014). Hongsanan *et al*. (2015) showed that *A. lozanellae* is phylogenetically close to *Asteridiella nitidae* Rodr. *Appendiculella* and *Asteridiella* are similar in having ascomata with conical cells, however, some species of *Appendiculella* differ from *Asteridiella* in having larviform

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appendages instead of conoid cells (Hongsanan *et al*. 2015, Hyde *et al*. 2020). According to the observations of Hongsanan *et al*. (2015), *Appendiculella calostroma* (type species of *Appendiculella*) also has conoid cells and absence of larviform appendages. Hansford (1961) differentiated *Asteridiella* from all other genera in *Meliolaceae* using its glabrous perithecia and mycelia (Pereira & Silva 2009). Therefore, these genera are treated as two separate genera (Hongsanan *et al*. 2015, Hyde *et al*. 2020).

The fungi in northern Thailand are remarkably diverse (Hyde *et al*. 2018) and in this study we report a new species of *Meliolaceae*. This family is relatively poorly understood, comprising eight genera (Hyde *et al*. 2020, Wijayawardene *et al*. 2020). Although these genera are morphologically distinct, molecular data, especially of *Meliola* species, have shown them to be polyphyletic (Hongsanan *et al*. 2015). We introduce a novel species in *Appendiculella* which was found on living leaves of *Vitex canescens* Kurz. (*Lamiaceae*). Morphological characters of the new taxon and its phylogenetic placement are provided. The new information will help provide data on the distinction of meliolaceous genera.

Materials and methods

Sample collection, morphological studies and specimen deposition

Freshly fallen leaves of *Vitex canescens* (*Lamiaceae*) with black colonies were collected from Mae Fah Luang University Botanical Garden, Chiang Rai, Thailand. Specimens were examined by using a Motic SMZ 168 series microscope. Hand sections of the fruiting structures were mounted in water and 5% KOH for microscopic studies and photomicrography. Microscopic morphologies were examined using a Nikon ECLIPSE 80i compound microscope and photographed using a Canon 750D digital camera fitted to the microscope. Measurements were made with the Tarosoft (R) Image Frame Work program and images used for figures processed with Adobe Photoshop CS3 Extended version 10.0 software (Adobe Systems, USA). The holotype and isotype are deposited in the Mae Fah Luang University Herbarium (MFLU), Chiang Rai, Thailand. The new taxon was linked with Facesoffungi and Index Fungorum databases as explained in Jayasiri *et al*. (2015) and Index Fungorum (2020).

DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted directly from fresh ascomata using a Forensic Genomic DNA Extraction Kit (OMEGA Bio-tek). The total volume of PCR mixture (50 µL) contained ddH2O (19 µL), 2× PCR Master Mix (QinKe Co., China) (25 µL), DNA template (2 µL) and each primer (2 µL). The complete 28S large subunit rDNA (LSU) and internal transcribed spacer (ITS) genes were amplified using LR0R/LR5 (Vilgalys & Hester 1990) and ITS1/ITS4 (White *et al*. 1990) primers. Polymerase chain reaction (PCR) was set up for initial denaturation of 5 min at 95 °C, followed by 35 cycles of 45 s at 94 °C, 45 s at 52 °C and 90 s at 72 °C, and a final extension period of 10 min at 72 °C (Hongsanan *et al*. 2015). PCR products were viewed on 1% agarose electrophoresis gels, stained with ethidium bromide. Purification and sequencing of PCR products were sent to a commercial sequencing provider, Beijing Liuhe Huada Gene Co. GuangZhou.

Sequence alignment and phylogenetic analyses

Newly generated sequences were assembled and subjected to the standard BLAST search to identify closest matches in GenBank (https://blast.ncbi.nlm.nih.gov). The accession numbers of taxa used in our analyses are shown in TABLE 1. Sequences of LSU and ITS data were aligned using MAFFT v. 6.864b (Katoh *et al*. 2017) and manually improved alignment in BioEdit v. 7.0 (Hall 2004).

Maximum likelihood analysis was performed using RAxML on XSEDE in CIPRES (Miller *et al*. 2010). The optimal ML tree was obtained with 1,000 separate runs under the GTR+GAMMA substitution model resulted from model tests. Bayesian inference (Larget & Simon 1999) was performed using the MrBayes 3.2.2 on XSEDE tool in CIPRES (Ronquist *et al*. 2011). Posterior probabilities (PP) were obtained from Markov chain Monte Carlo Sampling (MCMC) (Rannala & Yang 1996, Ronquist *et al*. 2012) when the average standard deviation of split frequencies fell below 0.01. MCMC chains were run from random trees for 1,000,000 generations and sampled every 100th generations with the burning value of 25%. The remaining trees were used to calculate posterior probabilities values. All trees were visualized in FigTree v1.4.0 (Rambaut 2012) and final layout was done with Microsoft PowerPoint.

TABLE 1. Taxa used in the phylogenetic analyses and their GenBank accession numbers. Newly generated sequences are indicated in red bold, and other ex-type isolates are in black bold.

Species	Culture collections/ Specimen number	Genbank accession number	
		LSU	ITS
Amplistroma caroliniana	BEO9923	FJ532377	N/A
Amplistroma erinaceum	AH 43902	NG058568	N/A
Appendiculella lozanellae	MP3432	DQ508302	N/A
Appendiculella sororcula var portoricensis	VIC32065	KC618640	N/A
Appendiculella viticis	**MFLU 19-1008**	**MT108888**	**MT108889**
Asteridiella atricha	VIC32064	KC618650	N/A
Asteridiella combreticola	**MFLU 17-1041**	**MN74748**	**MN74748**
Asteridiella nitidae	ppMP 796	EF094839	N/A
Asteridiella obesa	**VIC 31239**	**NG057014**	**NR120256**
Asteridiella pittieri	DP114	KC618639	N/A
Chaetosphaeria innumera	SMH 2748	AY017375	N/A
Endomeliola dingleyae	PDD 98304	GU138866	N/A
Irenopsis cornuta	VIC32058	KC618642	N/A
Irenopsis moelleriana	VIC32059	KC618646	N/A
Irenopsis vincensii	VIC 31751	JX133163	N/A
Irenopsis walsurae	MFLU 13-0621	KT021648	NR154075
Melanochaeta hemipsila	SMH2125	AY346292	N/A
Meliola sp.	MFLUCC 15-0047	KR868698	KR868703
Meliola brachyodonta	VIC32066	KC618644	N/A
Meliola caesalpiniicola	VIC32061	KC618641	N/A
Meliola centellae	**VIC 31244**	**NG042650**	**NR137799**
Meliola citri maximae	MFLU 14-0288	KX458474	N/A
Meliola clerodendricola	MFLU 13-0620	KT021647	N/A
Meliola crescentiae	VIC32056	KC618649	N/A
Meliola danielliae	VIC32057	KC618648	N/A
Meliola monnieriae	VIC32062	KC618647	N/A
Meliola niessleana	UBC F23799	KC833049	N/A
Meliola panici	VIC32063	KC618651	N/A
Meliola peruiferae	**VIC 31249**	**NG 060294**	**N/A**
Meliola pseudosasae	MFLU 16-2136	KX845434	N/A
Meliola tamarindi	MFLU 14-C0282	KP744489	N/A
Meliola trichostroma	VIC32068	KC618643	N/A
Meliola variaseta	DRJ 54 (PMA)	EF094840	N/A
Meliola vernaliae	VIC 31240	JX096808	N/A
Ruzenia spermoides	SMH4655	KF765619	N/A
Sordaria fimicola	CBS 723.96	MH874231	MH862606
Synaptospora plumbea	SMH3962	KF765621	N/A

Results

Phylogenetic analyses

LSU and ITS sequence data of representative families comprised 37 strains, including the outgroup taxon *Sodaria fimicola* (CBS 723.96) (*Sodariaceae*, Sordariales). The alignment contained 1687 characters (LSU: 1–942 and ITS: 943–1687) including gaps. The best scoring RAxML tree was selected to represent the relationships among taxa with a final likelihood value of-13524.483628. The matrix had distinct alignment patterns with 45.81% of undetermined characters or gaps. Estimated base frequencies were: A = 0.245945, C = 0.225648, G = 0.316063, T = 0.212344; substitution rates AC = 0.909091, AG = 2.777058, AT = 1.545145, CG = 0.565964, CT = 7.351068, GT = 1.000000; gamma distribution shape parameter α = 0.391518 (FIGURE 1).

Our new taxon clusters with *Appendiculella lozanellae* (MP3432) and *A. sororcula var portoricensis* (VIC32065) with medium statistical support (64% ML, 0.88 BYPP).

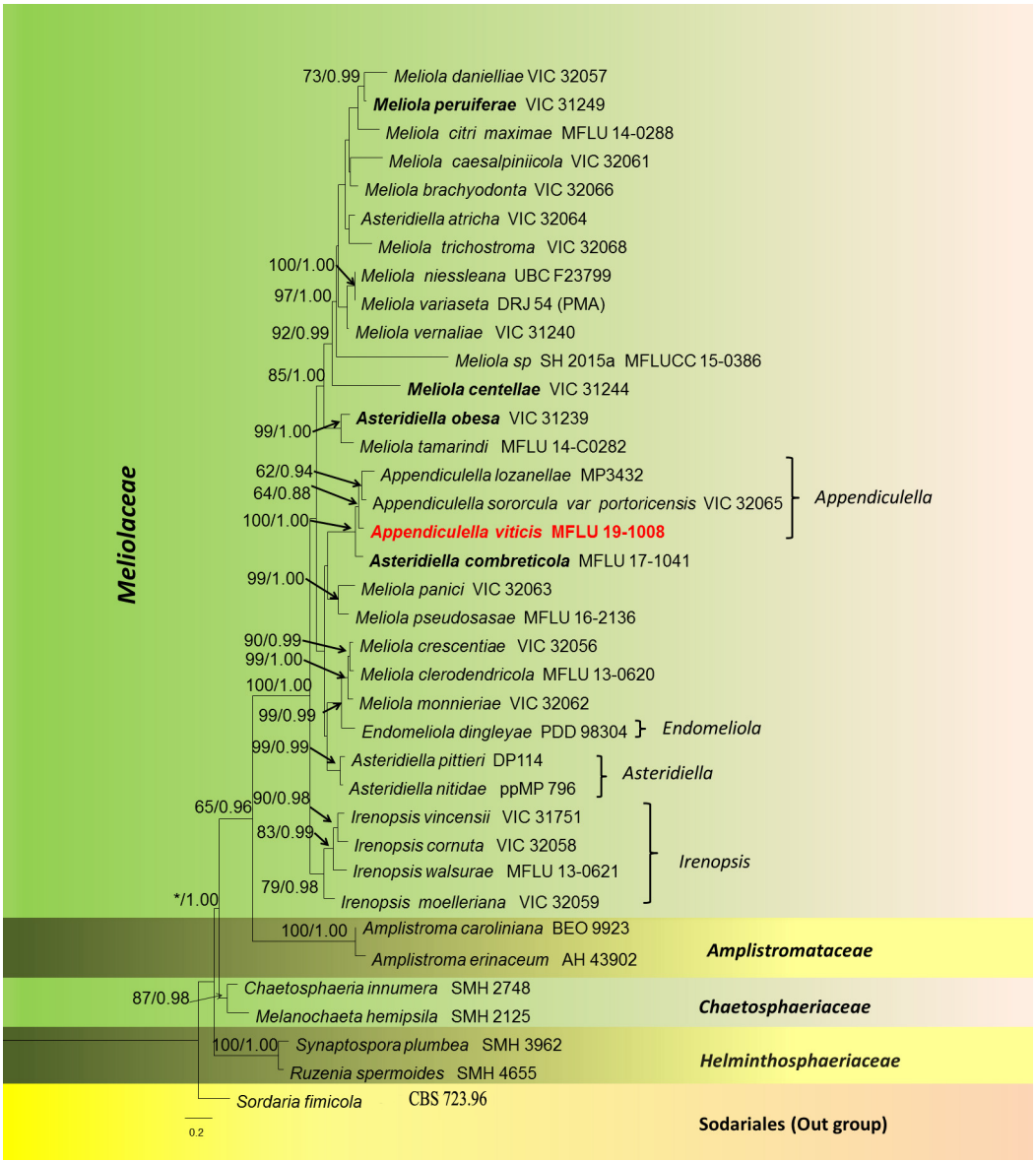

FIGURE 1. Phylogram generated from maximum likelihood (RAxML) based on LSU and ITS matrix. ML bootstrap support (≥60%) and Bayesian posterior probability (≥ 0.80) are indicated above the branches as ML/BYPP. The values of ML (<50%) and BYPP (<0.80) are represented by "*". The tree is rooted to *Sodaria fimicola* (CBS 723.96). Type strains are in bold and the newly generated sequence is in red.

Taxonomy

Appendiculella viticis Marasinghe, Hongsanan, Boonmee & K.D. Hyde ***sp. nov.*** (FIGURE 2)
Index Fungorum number: IF557246; *Facesoffungi number*: FOF07514.

Etymology:—The specific epithet refers to the host genus *Vitex.*

Holotype:—MFLU 19–1008.

Epiphytic on the surface of freshly fallen leaves of *Vitex canescens* (*Lamiaceae*). *Hyphae* 7–15 µm wide ($\overline{x}$ = 9 µm, n = 10), superficial, straight to undulate, branched, septate, darker at the septa, with hyphopodia, hyphal setae lacking. *Hyphopodia* 25–30 × 8–15 µm ($\overline{x}$ = 27 × 11 µm, n = 20), alternate, straight to antrorse; stalk cell 10–15 × 6–10 µm ($\overline{x}$ = 11.4 × 8.6 µm, n = 10) cylindrical, slightly bent; head cell 15–20 × 10–20 µm ($\overline{x}$ = 18.4 × 15.5 µm, n = 10), cylindrical to slightly lobate, sometimes brown spot at the center. **Sexual morph**: *Ascomata* 90–130 × 90–110 µm ($\overline{x}$ = 109.5 × 100.3 µm, n = 5), superficial, mostly gregarious, globose to subglobose, thick-walled, lacking setae; with raised conoid cells, 27–45 × 20–30 µm ($\overline{x}$ = 33 × 24 µm, n = 20), conical, straight to curved, vertically striate. *Peridium* 18–25 µm ($\overline{x}$ = 21 µm, n = 5), comprising dark brown cells of *textura angularis* when viewed in squash mounts, with two strata, outer stratum of brown to dark brown cells of *textura angularis*, inner stratum of hyaline to pale brown flattened cells. *Hamathecium* with evanescent paraphyses. *Asci* 46–65 × 30–40 µm ($\overline{x}$ = 56 × 36 µm, n = 20), 2-spored, unitunicate, oblong to obovoid, lacking an opening mechanism, short pedicellate or apedicellate, evanescent at maturity. *Ascospores* 40–60 × 15–30 µm ($\overline{x}$ = 51.6 × 21.7 µm, n = 30), 2–3-seriate, hyaline to brown, fusiform to ellipsoid, 4-septate, slightly constricted and darker at the septa, guttulate, smooth-walled. **Asexual morph**: *Phialides* 15–25 × 6–12 µm ($\overline{x}$ = 19 × 8.3 µm, n = 10), ampulliform, 2-celled, opposite or alternate, mixed with hyphopodia, conidia not observed.

Material examined:—THAILAND, Chiang Rai, Mae Fah Luang University, Botanical Garden, on freshly fallen leaves of *Vitex canescens* Kurz. (*Lamiaceae*), 16 November 2018, Diana Sandamali, D98 (MFLU 19–1008, holotype).

Notes: *Appendiculella viticis* shares some morphological characters with *A. alchorneae*, *A. araliae*, *A. arisanensis*, *A. calophylli*, *A. castanopsidifoliae*, *A. elaeocarpi*, *A. elaeocarpicola*, *A. engelhardtiae*, *A. lithocarpicola*, *A. malasiae*, *A. konishii*, *A. shettyi*, and *A. sorocula var portoricensis* in having globose to subglobose, thick-walled ascomata, with raised conoid cells, without larviform appendages and 4-septae, dark brown ascospores. However, *A. viticis* differs from these species in having vertically striated conoid cells and fusiform to ellipsoidal, guttulate ascospores. Other species have transversely striated conoid cells and cylindrical to ellipsoidal ascospores without guttules. Phylogenetically, *A. viticis* (MFLU 19–1008) forms a basal branch to *A. lozanellae* (MP3432) and *A. sororcula var portoricensis* (VIC 32065) with 64% MLBT and 0.88 BYPP support. However, *A. lozanellae* and *A. sororcula* have larviform appendages instead of conoid cells (Hansford 1961, Justavino & Piepenbring 2007). *Appendiculella viticis* is also phylogenetically related with *Asteridiella combreticola* X.Y. Zeng, K.D. Hyde & T.C. Wen. showing the polyphyletic nature within the *Meliolaceae*.

Discussion

The new species, *Appendiculella viticis*, is introduced with morpho-molecular evidence. It is the first *Appendiculella* species found on leaves of *Vitex canescens*. Phialides (mucronate hyphopodia/asexual morph) produce small spores which might be involved in asexual multiplication or sexual reproduction (Luttrell 1989, Mueller *et al.* 1991). Species of the *Meliolaceae* are traditionally assumed to be host-specific, but this assertion is not yet supported by adequate molecular evidence (Justavino & Piepenbring 2007). Based on field observations it is assumed that host-specificity within *Appendiculella* is not related to a single host genus, but also to other genera in the same family (Rodrýguez 2001). A comprehensive comparison of morphology within this genus is difficult. This is because informative illustrations and detailed descriptions of some characters in previously described species is lacking. However, detailed morphological comparison with closely related *Appendiculella* species is provided in Table 2. The complexity of species delineation based on host association is also a problem that needs to be resolved. Extensive molecular data may validate the significance of host-specificity for species delineation (Justavino & Piepenbring 2007).

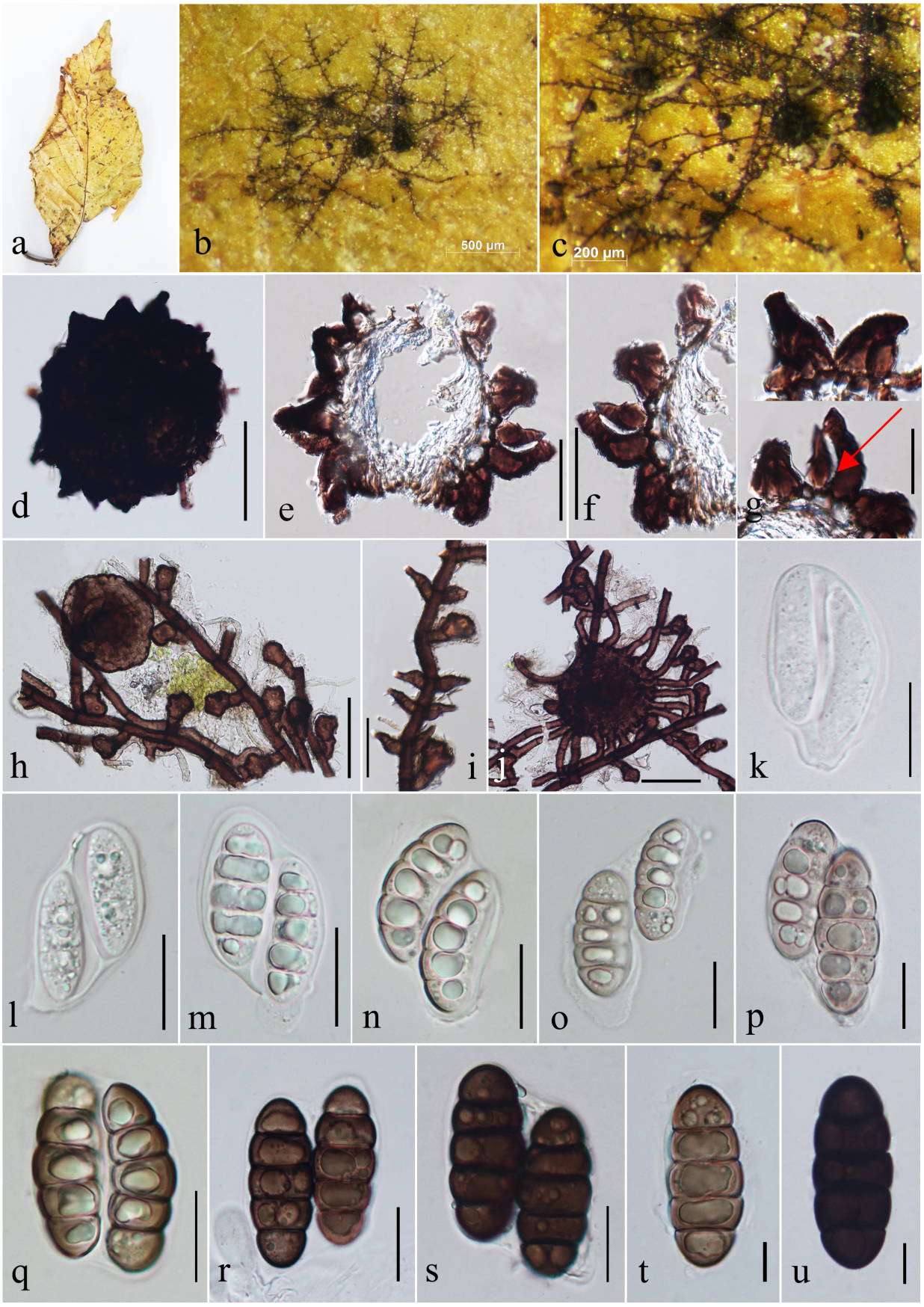

FIGURE 2. ***Appendiculella viticis*** (MFLU 19–1008, holotype). **a** freshly fallen leaf specimen. **b, c** Ascomata on surface of leaves. **d** Appearance of ascoma. **e** Cross section of ascoma. **f** Peridium comprising conical cells. **g** Vertically striate, conoid cells on ascomata (red arrow). **h** Hyphae with hyphopodia. **i** Hyphae with phialides. **j** Immature stage of ascoma. **k–s** Immature to mature asci. **t, u** Immature and mature ascospores. Scale bars: d, e = 100 µm, h, j = 50 µm, f, g, i, k–s =20 µm, t, u = 10 µm.

TABLE 2. Comparison of morphologically similar *Appendiculella* species with conoid cells.

Size and morphology

Species	Hyphopodia		Ascomatal conoid cells (µm)	Ascospores (µm)	Hosts	Country	References
	Head cell (µm)	Stalk cell (µm)					
A. alchorneae	11–15 × 10–14	5–2	65 × 25, subcylindric to obtuse, bent apex subhyaline and brownish	30–35 × 14–17, ellipsoid	*Alchornea* sp.	Guyana	Hansford (1961)
A. araliae	12–22 × 12–17	12–4	90–18 × 23, cylindric, pale translucent brown, transversely striated	38–48 × 18–22, ellipsoid	*Aralia* sp.	England	Hansford (1961)
A. arisanensis	10–14 × 7–9	14–5	23–53 × 14–21, curved, rounded at apex	44–54 × 14–19, ellipsoid to oblong	*Cyclobalanopsis* sp.	Taiwan	Hansford (1961)
A. calophylli	18–31 × 9–12.5	6–31	85 × 20, curved to crooked	43–46.5 × 15–18.5	*Calophyllum apetalum*	India	Toro (1925)
A. castanopsidifoliae	9–14 × 8–10	7–3	21–42 × 23–48, coniform, slightly bent	39–48 × 12–23, oblong	*Synaedrys amygdalifolius*	Taiwan	Hansford (1961)
A. elaeocarpi	12–14 × 7–10	7–5	up to 24 long, straight to curved, horizontally striated, attenuated, broadly rounded at apex	34–38 × 12–14, ellipsoid to oblong	*Elaeocarpus turberculatus*	India	Hosagoudar & Robin (2011)
A. elaeocarpicola	14–22 × 7–10	7–5	up to 24 long, straight to curved, horizontally striated, attenuated, broadly rounded at apex	34–38 × 12–14, ellipsoid to oblong	*Elaeocarpus tuberculatus*	India	Hosagoudar & Robin (2011)
A. engelhardtiae	10–13 × 7–8	6–3	18–30 long, coniform, slightly bent, obtuse	42–54 × 17–23, cylindrical to subellipsoid	*Engelhardtia chrysolepis*	Taiwan	Hansford (1961)
A. konishii	14–17 × 8–10	7–3	44–55 × 14–23, coniform, thick at the base	44–55 × 14–23, ellipsoid to oblong	*Synaedrys konishii*	Taiwan	Hansford (1961)
A. lithocarpicola	9–14 × 9	14–5	21–53 × 19–21, corniform straight to bent thick at base attenuate to rounded truncate apex	48–60 × 14–23, ellipsoid to oblong	*Synaedrys amygdalifolia*	Taiwan	Hansford (1961)
A. malasiae	15–17 × 13–15	7.5–22	20–45 × 20–35, conoid to mammillate, attenuate upwards, obtuse to nearly acute at apex, straight to slightly curved	43–45 × 12–13, cylindrical	*Malaisia scandens*	China	Song & Li (2004)
A. shettyi	9–13 × 8–11	3–7	14–28 × 9–19, subcylindrical to conoid, simple, striated, broadly obtuse	30–40 × 16–17, oblong to obovoid,	*Gordonia obtusa*	India	Biju *et al.* 2005
A. sororcula	13–20 × 9–14	9–5	50 × 20, erect spreading, pale brownish, transversely striated, granulose	40–46 × 18-20, oblong to ellipsoid	*Eupatrorium portoricense*	Porto Rico	Hansford (1961)
A. viticis	15–20 × 10–20	6–10	27–45 × 20–30, coniform slightly bent, vertically striated	40–60 × 15–30, fusiform to ellipsoid, guttulate	*Vitex canescens*	Thailand	This study

ITS base pair comparisons are not possible due to the lack of ITS sequence data for *A. lozanellae* and *A. sororcula var portoricensis.* In our analysis, *A. viticis* clustered with medium statistical support with the reference material of *A. lozanellae* and *A. sororcula*. The *Appendiculella* clade however, is not well-resolved from *Meliola*. *Asteridiella*, *Endomeliola*; *Meliola* species and they are also polyphyletic. These genera are, however, clearly morphologically distinct. Thus, we maintain them as distinct until molecular data of the type species are available (Hongsanan *et al.* 2015).

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