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Neotypification and phylogeny of *Kalmusia*

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Abstract

Kalmusia ebuli, the type species of *Kalmusia*, lacks type material and therefore its phylogenetic position remains unresolved. As a consequence the familial position of *Kalmusia* is based on morphology and molecular phylogeny of species other than the type. A fresh collection of *K. ebuli*, recently obtained from decorticated wood of *Populus tremula* in the foothills of the French Pyrenees is, therefore, designated as neotype to stabilize the application of the species and/or genus name. The holotype of *K. ebuli* f. *sarothamni* represents a synonym of *K. ebuli.* The genus *Kalmusia* is shown to be polyphyletic within the family Montagnulaceae, with *K. ebuli* being distant from *K. brevispora* and *K. scabrispora*, which appear to represent a different genus.

Key words: Montagnulaceae, polyphylogeny, taxonomy, type

Introduction

Kalmusia was formally established by Niessl (1872), with *K. ebuli* as the type species, and the diagnostic characters were considered to be "immersed, globose ascomata with a central, stout papilla, surrounded by hyphae in the substrate, pedicellate asci with septate pseudoparaphyses, and brown, 3-septate inequilateral ascospores" (Barr 1992). After comparing their morphological characters, *Diapleella* Munk (1953: 74) and *Dendropleella* Munk (1953: 125) were considered synonyms of *Kalmusia*, and *Thyridaria* Sacc. (1875: 21) was regarded as closely related (Barr 1992).

At present, *Kalmusia* includes more than 40 species names. *Kalmusia* has been placed in a diverse range of families and thus has an unsettled history based on morphological considerations. von Arx & Müller (1975) assigned *Kalmusia* to the *Pleosporaceae*, Barr (1992) assigned the genus to Phaeosphaeriaceae, while Barr (2001) and Eriksson (2006a) placed it in Montagnulaceae. Kirk *et al*. (2008), Lumbsch & Huhndorf (2010) and Hyde *et al*. (2013) placed the genus in Montagnulaceae. *Leptosphaeria scabrispora* Teng (1934: 378) was transferred to *Kalmusia* (as *K. scabrispora* (Teng) Tanaka (2005: 110), and *L. amphiloga* Petrak (1931: 202) as *K. amphiloga* (Petr.) Eriksson (2006b: 67), because these species lacked scleroplectenchymatous cells in the peridium (Tanaka *et al*. 2005, Eriksson 2006a).

The clavate asymmetric ascospores, as well as the clavate asci with long pedicels of *K. ebuli* do not fit the concept of the Phaeosphaeriaceae as defined by Zhang *et al*. (2012). Most recent phylogenetic studies also indicated that *K. brevispora* (Nagas. & Y. Otani) Y. Zhang ter, *et al*. (2009a: 94) and *K. scabrispora* nest within the Montagnulaceae (Zhang *et al*. 2009a, 2012, Hyde *et al*. 2013). However, these taxa are not the generic type, for which molecular data is lacking, rendering the familial placement of *Kalmusia* uncertain. Because the type material of *K. ebuli* was lost soon after it was introduced (Barr 1992), a neotype needs to be designated to resolve the familial placement of this genus (Zhang *et al*. 2012).

This study therefore (1) designates a suitable specimen of *K. ebuli* with a living culture as neotype; (2) morphologically characterizes the designated neotype; (3) infers phylogenetic relationships of *K. ebuli* with its closely related taxa, and (4) resolves the familial placement of *Kalmusia*.

Materials and methods

A fresh specimen of *Kalmusia ebuli* was collected in May 2007 in southern France (Aude, Belcaire) by J.B. Declercq. The holotypes of *K. ebuli* f. *sarothamni* Mouton and *K. sarothamni* Feltgen were also obtained from the National Botanical Gardens, Belgium (BR) and the National Museum of Natural History (Luxembourg, LUX), respectively. Samples were processed and examined following the method described in Zhang *et al*. (2009b). Observations and photographs were prepared from material mounted in water, chlorazol black, lactic acid or Indian ink and measurements follow the procotol outlined in Zhang *et al*. (2011, 2012). Because of the absence of type material of *K. ebuli*, we relied on the original description by Niessl (1872), together with the description from Barr (1992).

DNA extraction, amplification and sequencing

The ex-neotype culture of *K. ebuli* (CBS 123120) was grown on potato dextrose agar (PDA) and malt extract agar (MEA) and total genomic DNA was extracted from mycelia following the protocols outlined by Zhang *et al*. (2009b). *nu*rDNA gene sequence data has been widely used in systematic studies of Dothideomycetes (Shearer *et al*. 2009, Zhang *et al*. 2009a, 2011, Chomnunti *et al*. 2011, Hirayama & Tanaka 2011, Liu *et al*. 2012, Hyde *et al*. 2013). Here we use 18S, 28S and internal transcribed spacer (ITS) *nu*rDNA sequences to study the phylogenetic relationships among *K. ebuli* and other closely related pleosporalean taxa. DNA amplification and sequencing follow the protocol used by Zhang *et al*. (2009b).

Sequence alignment and phylogenetic analyses

Sequences generated from different primers (NS1/NS4 for 18S *nu*rDNA, LROR/LR5 for 28S *nu*rDNA and ITS4/ITS5 for ITS *nu*rDNA) were analyzed with other sequences obtained from GenBank. A Blast search was performed to find possible sister groups of the newly sequenced taxa. A combined 18S and 28S *nu*rDNA dataset was analysed in this study. Multiple alignment was performed in MEGA 5 (Tamura *et al*. 2011) and analyses were performed in PAUP v. 4.0B10 (Swofford 2002). Designated outgroup taxa were *Venturia populina* and *V. inaequalis*. The resulting alignment was manually corrected by visual inspection to optimize the position of small gaps. Maximum parsimony (MP) and maximum likelihood (ML) analyses were performed in PAUP v. 4.0b10 by using heuristic searches as implemented in PAUP (Swofford 2002). For MP analysis, clade stability was assessed in a bootstrap (BS) analysis with 1,000 replicates, random sequence additions with maxtrees set to 1,000 and other default parameters as implemented in PAUP. For the ML analysis, best-fit model of nucleotide evolution (GTR+I+G) was selected by Akaike information criterion (AIC) (Posada & Buckley 2004) in MrModeltest 2.3. Bootstrap analysis with 1,000 replicates was used to test the statistical support of the branches. With model parameters estimated from the data, a heuristic search with ten random taxon addition sequences and TBR branch swapping was performed. The same substitution model was used in the Bayesian analyses performed with MrBayes v. 3.1.2. The Metropolis-coupled Markov Chain Monte Carlo (MCMCMC) approach was used to calculate posterior probabilities. A preliminary Bayesian inference (BI) analysis using MrBayes software revealed that the Markov Chain Monte Carlo (MCMC) steady state was reached after less than 6,000,000 generations (the average standard deviation of split frequencies was constantly below 0.01). A conservative burn-in of 60,000 dendrograms was chosen and a full analysis of 10,000,000 generations was carried out with sampling every 100 generations. Trees were viewed in TREEVIEW. The nucleotide sequences reported in this paper were deposited in GenBank (Table 1).

TABLE 1. Taxa used in the phylogenetic analysis and their corresponding GenBank accession numbers (newly generated sequences are indicated in bold).

Species	Culture/voucher[1]	SSU	LSU	ITS
Kalmusia sarothamni	CBS 113833	**KF796670**	**KF796671**	**KF796675**
Kalmusia sarothamni	CBS 116474	**KF796672**	**KF796673**	**KF796676**
Bimuria novae-zelandiae	CBS107.79	AY016338	AY016356	
Corynespora olivacea	CBS 114450	-	GU301809	
Didymocrea sadasivanii	CBS 438.65	DQ384066	DQ384103	
Kalmusia scabrispora	MAFF 239517	AB524452	AB524593	
Kalmusia scabrispora	NBRC 106237	AB524453	AB524594	
Kalmusia brevispora	KT 2313	AB524460	AB524601	
Kalmusia brevispora	KT 1466	AB524459	AB524600	
Kalmusia ebuli	CBS 123120	**JN851818**	**JN644073**	**KF796674**
Karstenula rhodostoma	CBS 690.94	GU296154	GU301821	
Letendraea helminthicola	CBS 884.85	AY016345	AY016362	
Letendraea padouk	CBS 485.70	GU296162	AY849951	
Massarina cisti	CBS 266.62	FJ795490	FJ795447	
Massarina eburnea	CBS 473.64	GU296170	GU301840	
Montagnula anthostomoides	CBS 615.86	GU205246	GU205223	
Montagnula opulent	CBS 168.34	AF164370	DQ678086	
Paraconiothyrium minitans	CBS 122788	EU754074	EU754173	
Paraphaeosphaeria michotii	CBS 591.73	GU456305	GU456326	
Paraphaeosphaeria michotii	CBS 652.86	GU456304	GU456325	
Venturia populina	CBS 256.38	GU296206	GU323212	
Venturia inaequalis	CBS 176.42	-	GU348998	
Plenodomus fuscomaculans	CBS 116.16	EU754098	EU754197	
Phaeodothis winteri	CBS 182.58	GU296183	GU301857	
Microdiplodia hawaiiensis	CZ481	GU296183	GU301857	
Macrovalsaria megalospora	CBS 178149	FJ215706	FJ215700	
Macrovalsaria megalospora	CBS 178150	FJ215707	FJ215701	
Keissleriella cladophila	CBS 104.55	GU296155	GU301822	
Katumotoa bambusicola	MAFF 239641	AB524454	AB524595	
Falciformispora lignatilis	BCC 21118	GU371835	GU371827	
Falciformispora lignatilis	BCC 21117	GU371834	GU371826	
Byssothecium circinans	CBS 675.92	GU205235	GU205217	
Kalmusia brevispora	MAFF 239276	AB524459	AB524600	
Kalmusia brevispora	NBRC 106240	AB524460	AB524601	

Results

The combined 28S (LSU) and 18S (SSU) *nu*rDNA dataset consists of 34 strains. The dataset consists of 2,371 characters after alignment. Of the included bases, 271 sites are parsimony-informative. A heuristic search with random addition of taxa (1,000 replicates) and treating gaps as missing characters generates two equally parsimonious trees. All trees are similar in topology and not significantly different (figures not shown). A single Bayesian tree is shown in Fig. 1, with values of the Bayesian posterior probabilities (PP) (equal to or above 0.90 based on 1,000 replicates) from MCMC analysis shown under the branches. Bootstrap support (BS) values obtained from the maximum parsimony (MP, equal to or above 60% based on 1,000 replicates) are shown on the upper branches (Fig. 1).

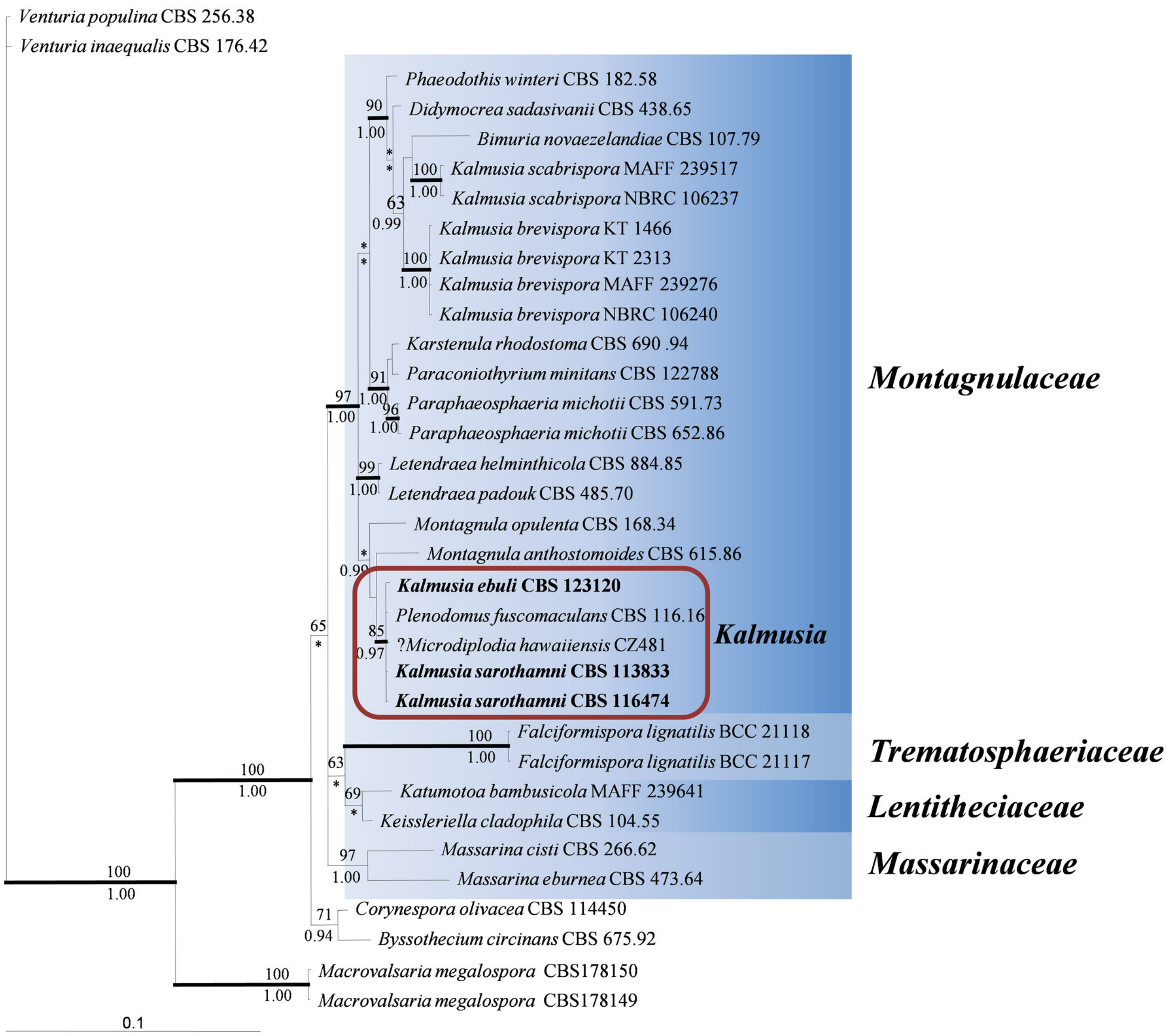

FIGURE 1. Bayesian tree generated from sequence analysis of the combined 28S and 18S *nu*rDNA dataset. Designated outgroup taxa are *Venturia inaequalis* and *V. populina*. Maximum parsimony bootstrap support values above 60% are shown at nodes and based on 1,000 replicates. Bayesian support above 90% is shown under the branches. The thickened branches mean maximum likelihood bootstrap support values above 80%.

FIGURE 2. ITS sequences comparision between *Kalmusia ebuli* and *K. sarothamni*. Note: · indicates identical base pairs, and – indicates absent base.

Taxonomy

Kalmusia ebuli Niessl, Verh. nat. Ver. Brünn 10: 204 (1872). (Figs. 3, 5)
= *Kalmusia ebuli* f. *sarothamni* Mouton, Bull. Soc. Roy. Bot. Belg. 26: 180 (1887).

Ascomata 325–400 µm high × 380–520 µm diam., black, immersed under a thin pseudostroma blackening wood surface, scattered or in small groups, globose to subglobose, opening through a cylindrical, flush to slightly papillate ostiole. *Peridium* 28–38 µm thick, of even thickness, a *textura prismatica* of thick-walled dark brown cells, more pigmented outwardly, with brown hyphal appendages penetrating wood. *Pseudoparaphyses* septate, 1.5–3.5 µm wide at base, containing conspicuous oily guttules, narrowly trabeculate above asci, embedded in gelatinous matrix. *Asci* 115–155 × 10–13.5 µm (pedicel is included when measuring the length of ascus), 8-spored, bitunicate with a small ocular chamber, fissitunicate, narrowly clavate, with a tapering pedicel, 35–48 µm long. *Ascospores* 17.5–19.5 × 5–6 µm, clavate, often slightly curved, 3-septate, slightly constricted at median septum, brown, end cells paler, septa darker, wall finely verruculose, overlapping biseriate in upper part of ascus, no mucilaginous sheath observed in India ink.

Culture characteristics:—Colonies on PDA 40 mm diam. after 2 weeks at 25–27°C, not fast growing, circular, white in first week, the center becoming grey-white after 1–2 weeks, reverse white to pale white after 1 week, becoming brown after 2 weeks, flattened, felty, with sparse aerial mycelium, surface with erose edge, filamentous (Fig. 4).

Materials examined:—BELGIUM. Dolembreux, on branchlets and pieces of stumps of *Sarothamnus scoparius* from woodland, *V. Mouton* (BR 101525–63!, holotype of *Kalmusia ebuli* f. *sarothamni*). FRANCE. Aude, Belcaire, Bois de la Bénague, chemin de Trassoulas, ca. 900 m, 2 May 2007, on decorticated wood of *Populus tremula*, leg *B. Declercq* (BJFC 200201!, **neotype designated here**); ex-neotype living culture deposited at CBS-KNAW Fungal Biodiversity Centre, Netherlands (CBS 123120).

Notes:—Mouton (1887) introduced *K. ebuli* f. *sarothamni* based on it having shorter ascospores than *K. ebuli* (14–15 µm *vs*. 19–20 µm), but also suggested that the two forms may be specifically identical. The holotype of *K. ebuli* f. *sarothamni* (named as *K. ebuli*) was described by Zhang *et al*. (2012), in which the ascospore length is recorded as 15–18 µm. The length of the ascospores (17.5–19.5 µm) of our new collection shows significant overlap between *K. ebuli* f. *sarothamni* and *K. ebuli*. In addition, the immersed ascomata, long pedicellate clavate asci, septate filamentous pseudoparaphyses and slightly clavate ascospores of our new collection fit the protologue descriptions of *K. ebuli* and *K. ebuli* f. *sarothamni* well (Niessl 1872, Zhang *et al*. 2012). Thus, the new collection is designated here as the neotype of *K. ebuli*.

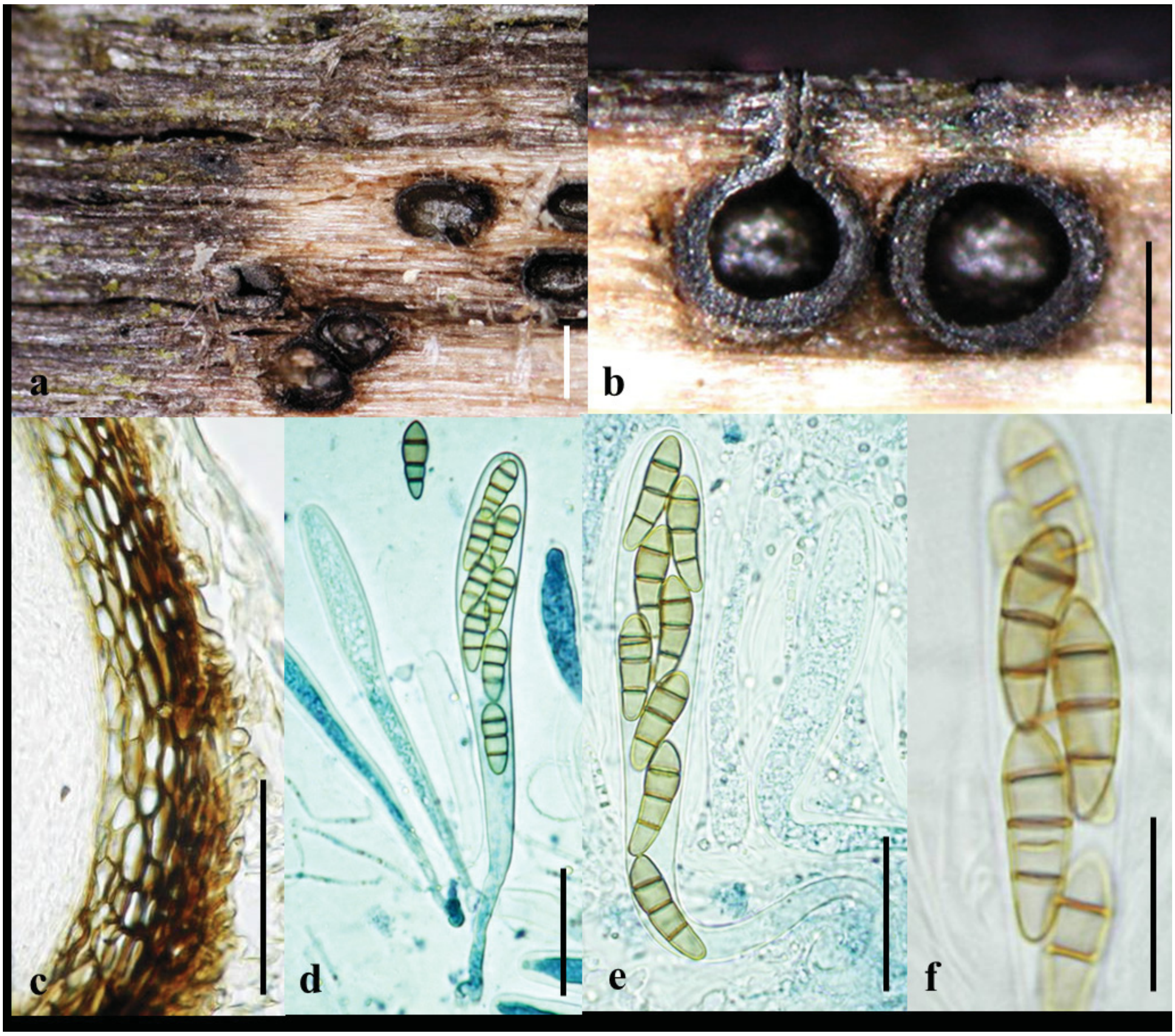

FIGURE 3. *Kalmusia ebuli* (neotype). a. Horizontal section of immersed ascomata scattered on the host surface. b. Section of two ascomata. c. Section of a part of the peridium. Note the thick-walled, dark brown, peridial cells. d, e. Mature and immature clavate asci with eight spores and long pedicels, plus pseudoparaphyses. f. Part of ascus. Scale bars: a, b = 0.5 mm, c–e = 20 µm, f = 10 µm.

Discussion

Kalmusia ebuli, the generic type of *Kalmusia*, together with *K. sarothamni* nests in the well supported clade of Montagnulaceae in this study. The family Montagnulaceae was introduced by Barr (2001) to accommodate some pleosporalean genera with ascomata immersed under a clypeus, a pseudoparenchymatous peridium with small cells, cylindric or oblong asci with pedicels and brown septate ascospores. Three genera were included, *i.e.* phragmosporous *Kalmusia*, dictyosporous *Montagnula* and didymosporous *Didymosphaerella* (Barr 2001). Multigene phylogenetic analysis indicated that species from *Bimuria*, *Didymocrea*, *Kalmusia*, *Karstenula*, *Letendraea*, *Paraphaeosphaeria*, *Phaeosphaeria* as well as *Montagnula* resided in the monophyletic clade of the Montagnulaceae (Schoch *et al*. 2009, Zhang *et al*. 2009a, 2012, Hyde *et al*. 2013). The type species of *Montagnula*, *M. infernalis* (Niessl) Berl. (1896: 68), was illustrated in Zhang *et al*. (2012) and has small to medium sized, immersed to erumpent ascomata which develop in groups, a thick, black peridium, narrowly cellular pseudoparaphyses, bitunicate asci with a long pedicel and reddish brown to dark yellowish brown, muriform ascospores. When Berlese (1896) introduced *Montagnula*, he accepted two dictyosporous species, *i.e*. *M. infernalis* and *M. gigantea* (Mont.) Berl. (1889: 69), both of which were exclusively associated with members of Agavaceae. Subsequently, species with different ascospore morphology, such as phragmosporous species (Leuchtmann 1984) and didymosporous species (Aptroot 1995a) were added to the genus, which rendered it heterogenic. Current familial placement of *Montagnula* is based on phylogenetic analysis of the *nu*rDNA sequences of *M. opulenta* (De Not.) Aptroot (1995b: 340) (Zhang *et al*. 2009a, 2012). The type species of *Montagnula* has not been sequenced and it is imperative that this species is also recollected, epitypified and subjected to phylogenetic study to verify the position of Montagnulaceae.

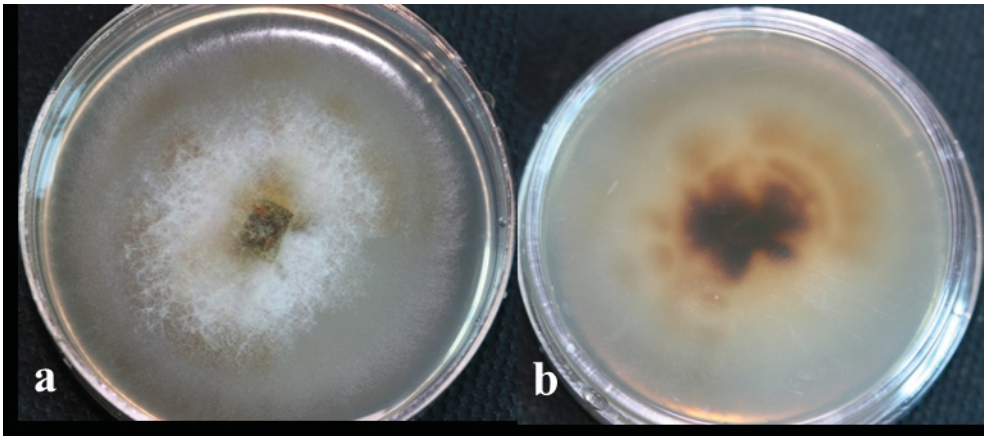

FIGURE 4. *Kalmusia ebuli* colonies on PDA 14 days after inoculation of ex-epitype (CBS 123120). Upper surface (a) and reverse (b).

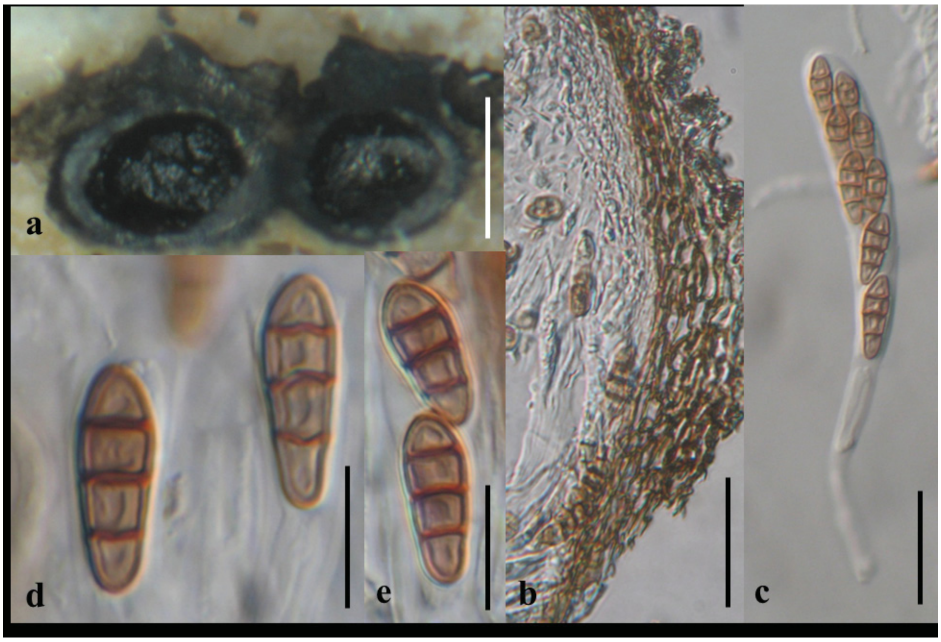

FIGURE 5. *Kalmusia ebuli* **(**holotype of *K. ebuli* f. *sarothamni*). a. Vertical section of two ascomata. b. Section of part of peridium. Note the compressed peridium cells. c. Eight-spored clavate ascus, with long pedicel. d, e. Released ascospores. Scale bars: a = 0.5 mm, b = 50 µm, c = 20 µm, d, e = 10 µm.

Kalmusia ebuli forms a robust clade with an authentic strain of *Plenodomus fuscomaculans* (Sacc.) Coons (1916: 714) (CBS 116.16) and *Microdiplodia hawaiiensis* Crous (2006: 947) (CZ481) as well as *K. sarothamni*, which might indicate that both *P. fuscomaculans* and *M. hawaiiensis* are closely related to *Kalmusia*. In addition, *Microsphaeropsis hirta* (Sacc.) Höhn. and *Hendersonia hirta* (Fr.) Currey (1859: 324) (= *Microdiplodia*) have been reported as the asexual states of *Kalmusia ebuli* (Barr 1992). Although the molecular phylogenetic results obtained from LSU and SSU *nu*rDNA sequence comparisons indicated that *K. ebuli* is closely related to *K. sarothamni*, they are easily distinguished morphologically in that *K. sarothamni* forms multi-loculate ascostromata, and *K. ebuli* forms scattered ascomata. In addition, the ascospore septa of *K. sarothamni* are conspicuously constricted, while those of *K. ebuli* are less so (Figs 3, 6).

It is noteworthy that *K. sarothamni* and *K. ebuli* cannot be separated by the 28S rDNA sequence comparisons (Fig. 1). In addition, the ITS sequence divergence is 2% (treated the gap region from 380 to 412 as a single base pair) between *K. sarothamni* and *K. ebuli* (Fig. 2), which is lower than 3% threshold as mentioned by Nilsson *et al*. (2008), as well as the average intraspecific variation of *Lindgomyces* (Lindgomycetaceae, Pleosporales) (2.5%, Raja *et al*. 2011). The vouchers of *K. sarothamni* used in the phylogenetic analysis in this study, however, could not be obtained for verification. Based on the morphological distinctions of the two respective type specimens, we retain *K. sarothamni* and *K. ebuli* as distinct species.

FIGURE 6. *Kalmusia sarothamni* (LUX-044996, type). a–b. Ascomata on host substrate. c. Section of ascomata. d. Section of ostiole. e. Close up of the peridium. f. Long filiform, paraphyses. g–h. Immature asci. i–j. Asci with long pedicel bearing eight irregularly arranged, partially overlapping ascospores. k–l. Immature ascospores. m–o. Smooth-fusoid, brown ascospores. Scale bars: c = 100 mm, d–f, h–j = 50 mm, g = 25 m, k–o = 10 mm.

The genus *Kalmusia* is shown in the present study to be polyphyletic. *Kalmusia brevispora* and *K. scabrispora* form a robust clade with the type strain of *Didymocrea sadasivanii* (T.K.R. Reddy) Kowalski (1965: 405) and *Phaeodothis winteri* (Niessl) Aptroot (1995b: 358). Based on multigene phylogenetic analysis, *Phaeosphaeria brevispora* forms a well supported clade with *Kalmusia scabrispora*, thus *P. brevispora* was assigned to *Kalmusia* (as *K. brevispora*) (Zhang *et al*. 2009a). The present study indicates that both *K. scabrispora* and *K. brevispora* nest in Montagnulaceae, but are distinct from the generic type *K. ebuli*. Morphologically, *K. brevispora* and *K. scabrispora* share immersed ascomata forming under the epidermis or stems (culms) of monocotyledonous hosts, septate and filiform pseudoparaphyses, clavate asci with long pedicels, and symmetrically septate, pigmented, coarsely verrucose ascospores, and they may represent another genus of Montagnulaceae.

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