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First collection of the asexual state of *Trichaleurina javanica* from nature and the placement of *Kumanasamuha*

Iturriaga T[1*], Raudabaugh DB[2], Karakehian JM[3,4], Miller AN[3], Hodge KT[1] and Pfister DH[5]

[1]*School of Integrative Plant Science, Cornell University, Ithaca, New York, USA*
[2]*Department of Environmental Engineering, Duke University, Durham, North Carolina, USA*
[3]*Illinois Natural History Survey, University of Illinois Urbana-Champaign, Champaign, Illinois, USA*
[4]*Department of Plant Biology, University of Illinois Urbana-Champaign, Champaign, Illinois, USA*
[5]*Department of Organismic and Evolutionary Biology, Harvard University, Cambridge, Massachusetts, USA*

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Abstract

Ascomata of *Trichaleurina javanica* (Pezizomycetes) are encountered frequently in nature in tropical Asia. Its anamorphic state has been described previously as similar to *Kumanasamuha*. Our study describes the unusual anamorphic fungal specimen, MOZ170, collected from Gorongosa National Park, Mozambique. The fungal strain MOZ170 is identified using ribosomal DNA sequence data, its morphology is described, and morphological differences between the naturally growing anamorph and *in vitro* derived culture are compared. Phylogenetic placement of *Kumanasamuha sundara* was also determined using available data. The internal transcribed spacer (ITS) and partial large ribosomal subunit (LSU) were sequenced. Phylogenetic analyses of LSU supported MOZ170 as the anamorph of *T. javanica*, and revealed the proper placement of the type species of *Kumanasamuha*, i.e., *K. sundara*, within the Dothideomycetes. MOZ170 is characterized by its dark conidiophores growing in tufts, and conidia with curved, appressed crests and ridges. The comparison between naturally growing and *in vitro* grown cultures showed that the *in vitro* cultured anamorph had larger conidiogenous cells, larger conidia, and longer and more numerous lateral fertile branches compared to the fungus in nature. The present report represents the first anamorph collected from nature for this genus and one of the few natural collections of the anamorphic state within Chorioactidaceae with the exception of those of *Desmazierella* species.

Key words – Chorioactidaceae – Gorongosa National Park – Mozambique

Introduction

A brown fungal mat consisting of a large mass of superficial hyphae and conidiophores was collected during a field expedition in Mozambique. Through morphological and phylogenetic analyses, the fungal mat (MOZ170) was determined to represent the anamorphic state of *Trichaleurina javanica*. This teleomorph is a large gelatinous cup fungus found frequently across tropical Asia, but to our knowledge, the anamorphic state has not been collected in nature. In the past, the genus *Trichaleurina* largely was overlooked within the Pezizomycetes, but phylogenetic evidence now places it in the Chorioactidaceae (Carbone et al. 2013b). Its current placement is supported by morphological features of the apothecia, particularly the presence of prominent

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Corresponding Author: Teresa Iturriaga – e-mail – ti14@cornell.edu

brown, ornamented hairs on the receptacle and the ascospore ornamentation. Genera within the Chorioactidaceae are found typically on decomposed wood, leaf litter and other woody substrates (Pfister et al. 2008, Nagao et al. 2009, Carbone et al. 2013b).

Currently, four species of *Trichaleurina* are described and documented. The anamorphic states have been reported rarely in nature and cultures have typically been derived from ascospores (Carbone et al. 2013a ,b). In the case of species of *Chorioactis*, the type genus of the family, and *Trichaleurina* anamorphic states previously have been referred to as the genus *Kumanasamuha*. The anamorphic state of *Chorioactis geaster* was described as *K. geaster* (Nagao et al. 2009). Peterson et al. (2004) suggested this anamorphic state of *C. geaster* had an affinity with *Conoplea*. Carbone et al. (2013a) concluded that the anamorphic state of *T. javanica* was morphologically similar to *K. sundara*, the type species of the genus *Kumanasamuha*. Ribosomal DNA sequence data became available recently for *K. sundara* (GenBank MH878368, CBS 488.73 culture strain) (Vu et al. 2019) allowing comparison and placement at higher taxonomic levels.

The aims of this study were to 1) provide the first description of the anamorphic state of *T. javanica* from naturally occurring substrate and compare it with the morphology of *in vitro* culture, 2) characterize the growth conditions that allowed these observations, and 3) properly place the genus *Kumanasamuha* in a phylogenetic framework. This study is important in highlighting morphological differences between the natural and *in vitro* cultured strains and enhances the knowledge of the anamorphic states in the Chorioactidaceae and their biology.

Materials & methods

Collection and isolation

The hyphal mat of MOZ170 was collected naturally growing on wood in a Miombo woodland in Gorongosa National Park (S 19.00241° E 034.19784°, elevation 154 m), Mozambique on May 23, 2016. The vegetation of this location is dominated by trees of *Brachystegia boehmii* Taub., *B. spiciformis* Benth. and *Julbernardia globiflora* (Benth.) Troupin. In the field, the fungus was found growing on the surface of a fallen, decorticated and decaying, large-diameter tree trunk of unknown identity. Small portions of the wood on which the fungus was growing were removed using a sturdy knife, packed in a paper bag and brought to the Edward O. Wilson Biodiversity Laboratory at the Chitengo base camp. Cultures were obtained by transferring small portions of the hyphal mat using a flame-sterilized fine needle onto 60 mm Petri dishes with 2% water agar containing 0.25 g $^{-L}$ chloramphenicol. Inoculated plates were sealed with Parafilm and incubated at ambient temperature (~ 30°C). After two weeks, Petri plates were examined and actively growing hyphae were transferred to freshly prepared Petri plates containing malt extract agar, sealed with Parafilm, and incubated at 20°C for three months under 12 h light and 12 h darkness.

Morphological study

Microscopic examination was conducted using an Olympus BX52 microscope with differential interference contrast (DIC) and equipped with an Olympus QColor 3 digital camera. Images were processed in Adobe Photoshop 7.0; measurements were conducted using NIH ImageJ 1.63. Colors are given for material rehydrated in tap water unless otherwise specified. The microscopic study was conducted on material mounted in tap water. All structures were measured in a straight line between the apex and basal points, or between the two widest points (Iturriaga & Korf 1990). Thirty measurements were recorded for each examined structure unless otherwise noted. Macroscopic images were taken with a Cannon EOS 50 Mark II.

DNA extraction, amplification and sequencing

DNA was extracted using an E.Z.N.A.® High Performance Fungal DNA Kit (Omega Bio-tek) according to the manufacturer's instructions. PCR was completed on a Bio-Rad PTC 200 thermal cycler. The total reaction volume was 25 µL (12.5 µL GoTaq® Green Master Mix, 1 µL of each 10 µM primer ITS1F and LR3 (Gardes & Bruns 1993, Vilgalys & Hester 1990), 3 µL DNA

template, and 7.5 µL DNA-free water). The following thermal cycle parameters were used: initial denaturation at 94 °C for 2 minutes, followed by 30 cycles of 94°C for 30 seconds, 55°C for 45 seconds, 72°C for 1 minute with a final extension step of 72°C for 10 minutes. Gel electrophoresis (1% TBE agarose gel stained with ethidium bromide) was used to verify the presence of a PCR product before purification using aWizard® SV Gel and PCR Clean-Up System (Promega). A BigDye® Terminator 3.1 cycle sequencing kit (Applied Biosystems Inc.) was used to sequence the entire ITS and partial LSU regions in both directions using the ITS5, ITS4, LROR, LR3 (Rehner & Samuels 1994, White et al. 1990) primers on an Applied Biosystems 3730XL high-throughput capillary sequencer. Identity was confirmed through BLASTn analysis using the NCBI database. The resulting sequence has been deposited into GenBank (MW488268).

Phylogenetic analysis

Phylogenetic analyses were completed using LSU sequences obtained from GenBank (Table 1). Sequences were aligned in PASTA (Mirarab et al. 2015) with the following settings: aligner (MAFFT), merger (Muscle), tree estimator (FastTree), model (GTR+G20), max.subproblem (default size), decomposition (centroid), and iteration limit (3). The best alignment was evaluated in SeaView (Gouy et al. 2010), and Gblocks (Castresana 2000) was used to eliminate poorly aligned positions and divergent regions using the following options: allow smaller final blocks, allow gap positions within the final blocks, allow less strict flanking positions. A maximum likelihood analysis was completed using RAxML Black Box (bootstrap 1000 iterations), and Bayesian analysis was conducted using MrBayes 3.2.2 on XSEDE (3.2.6). Both analyses were conducted using the CIPRES Science Gateway (Miller et al. 2010). Bayesian analysis was conducted using the GTR + I + G model with four rate classes and four independent chains, and ran for 10 million generations with 25% burn-in. Nodes with ≥ 70% bootstrap support and ≥ 95% Bayesian posterior probability were considered significantly supported (Alfaro et al. 2003).

Table 1 LSU sequences used for the phylogenetic analysis of MOZ170 and placement of *K. sundara* (CBS 488.73 culture strain)

Species ID	GenBank accession no.	References
Acarospora cervina	AY640941	Reeb et al. 2004
Acarospora laqueata	AY640943	Reeb et al. 2004
Arthrobotrys musiformis	AY261149	Li et al. unpublished
Arthrobotrys botryospora	AY261146	Li et al. unpublished
Arthrobotrys vermicola	AY261143	Li et al. unpublished
Ascosphaera apis	FJ358275	Gueidan et al. 2008
Ascosphaera colubrina	FJ358276	Gueidan et al. 2008
Ascosphaera larvis	FJ358277	Gueidan et al. 2008
Chorioactis geaster	AY307942	Peterson et al. 2004
Coniochaeta decumbens	NG_067257	Weber et al. 2002
Coniochaeta discoidea	AY346297	Huhndorf et al. 2004
Desmazierella acicola	AY945854	Pfister et al. 2008
Dothidea insculpta	AY640949	Reeb et al. 2004
Dothidea ribesia	AY016360	Lumbsch & Lindermuth 2001
Dothiora cannabinae	NG_068997	Spatafora et al. 2006
Geoglossum nigritum	AY544650	Lutzoni et al. 2004
Kumanasamuha sundara	MH878368	Vu et al. 2019
Monilinia fructicola	AY544683	Lutzoni et al. 2004
Monilinia laxa	AY544670	Lutzoni et al. 2004
Neournula pouchetii	KT968655	Peterson et al. 2004
Peziza lobulata	AY500548	Hansen et al. 2005
Peziza praetervisa	U40618	Norman & Egger 1996
Peziza vesiculosa	DQ470948	Spatafora et al. 2006
Pseudosarcosoma latahense	FJ176860 TYPE	Schoch et al. 2009

Table 1 Continued.

Species ID	GenBank accession no.	References
	EU652391	Gordon unpublished
Roccella boergesernii	AY548814	Lutzoni et al. 2004
Roccella canariensis	AY779328	Lumbsch et al. 2005
Russula pseudociliata	NG_064397	Buyck et al. unpublished
Sclerophora coniophaea	JX000094	Prieto et al. 2012
Sclerophora farinacea	JX000095	Prieto et al. 2012
Trichaleurina javanica	KF418269	Carbone et al. 2013b
	KF418270	Carbone et al. 2013b
	KF418271	Carbone et al. 2013b
***Trichaleurina javanica* MOZ170**	**MW488268**	**Current study**
Trichaleurina tenuispora	KF418259 TYPE	Carbone et al. 2013b
	KF418265	Carbone et al. 2013b
Trichoglossum hirsutum	AY640976	Reeb et al. 2004
Wolfina aurantiopsis	KC306743	Agnello et al. 2013

Culture and specimen preservation

The specimen voucher for MOZ170 was divided into two parts and deposited at the Cornell University Plant Pathology Herbarium (CUP-70728) and the Illinois Natural History Survey Fungarium (ILLS00121425). Cultures were deposited at the CBS-KNAW collections (CBS-147018). Cultures were dried at 45°C for 10 h and deposited at the Illinois Natural History Survey Fungarium (ILLS00121425), Cornell University Plant Pathology Herbarium (CUP-70728), and at the Farlow Herbarium (HUH-11406).

Results

Taxonomy

Trichaleurina javanica (Rehm) M. Carbone, Agnello & P. Alvarado, Ascomycete.org. 5: 6. 2013.

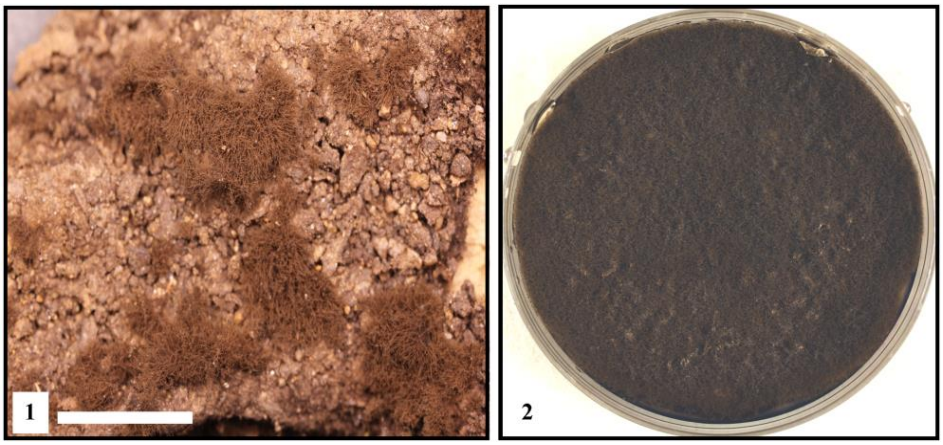
Basionym – *Sarcosoma javanicum* Rehm, in Hennings, Hedwigia 32: 226. 1893.

Synonymous names proposed by previous workers: *Trichaleurina polytricha* Rehm, *Urnula philippinarum* Rehm, *Sarcosoma decaryi* Pat., *Sarcosoma novoguineense* Ramsb. and perhaps *Bulgaria celebica* Henn. See Carbone et al. 2013a, LeGal 1953.

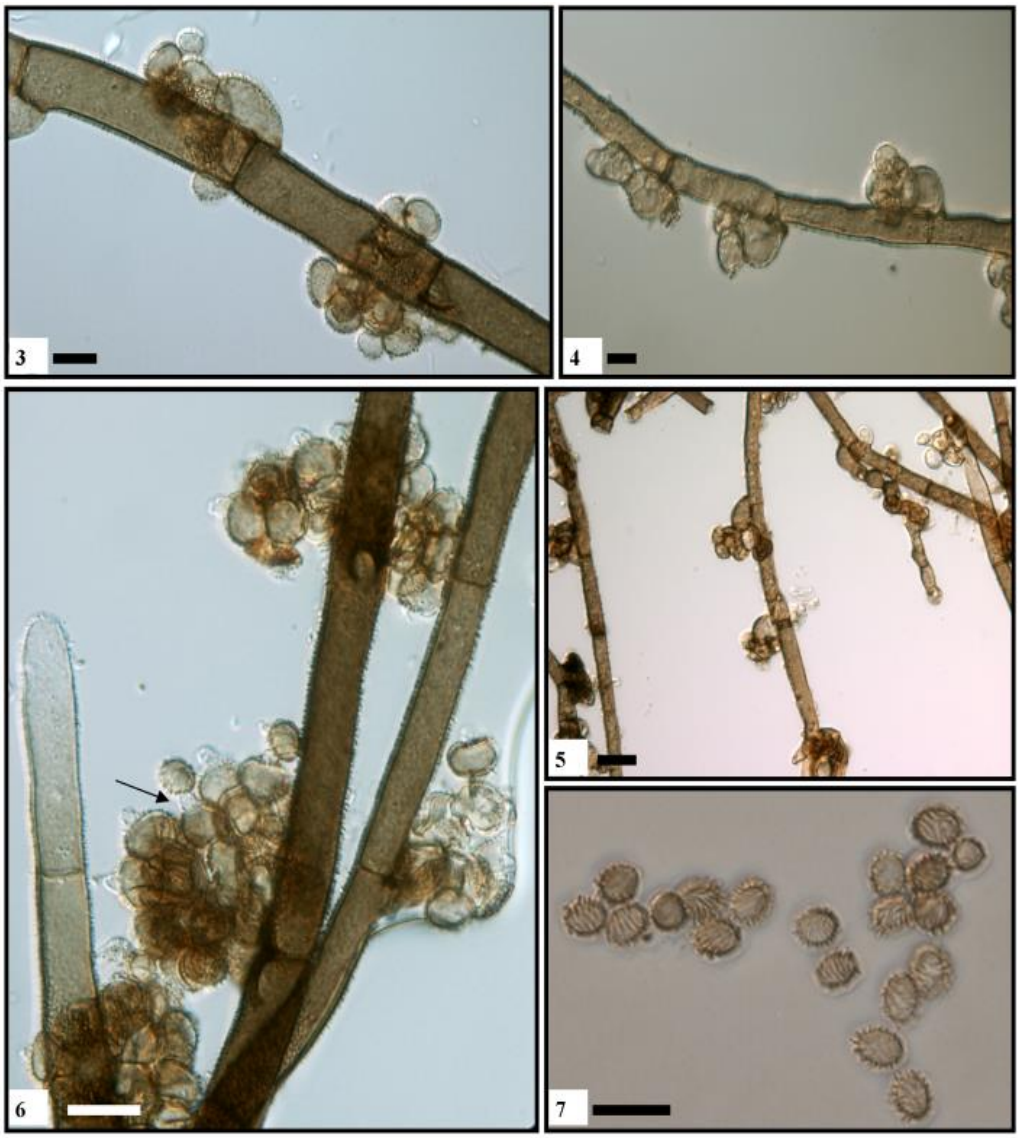
For description of the teleomorph see Carbone et al. 2013a.

Anamorphic state

Conidiophores arising from a mat of blackish-brown tufted hyphae on decomposed wood (Fig. 1) and from the *in vitro* culture (Fig. 2). Conidiophores erect, 300-800 µm long, cylindrical, septate, branched, brown, 9.0-10.7 µm wide at mid-point of its length, tapering slightly towards the apex where 6.0-7.0 µm wide, terminal cell hyaline; cell walls of the conidiophores 0.5-1.0 µm thick, finely rugose to verrucose with warts/spines. Fertile branches produced laterally at most septa (Fig. 3), curved, short, arising from a small lateral protrusion of the conidiophore, appressed to it, brown, 14.5-19 x 5.0-10 µm, with a broad point of attachment of 4-5 µm diam, wall verrucose. Plane of the fertile branches in relation to the conidiophore varies. Fertile branches produce cells from two areas (Figs 3–5), at approx. ~ 90° angle to each other, cells 4.8-5.0 x 5.2-8.6 µm. Conidiogenous cells produced as clustered or periclinal buds on the fertile lateral branches (Fig. 3). The whorl of conidiogenous cells continues to bud polyblastically for several generations (Figs 5, 6). Conidiogenous cells spherical to subspherical, brown, with spiny/warted wall, (3.3-)5.3-7.5 x 5.5-6.0 µm, with a hyaline apical peg 1.6-2.3 x 1.9-2.6 µm. Solitary conidia are produced from these pegs (Fig. 6). Conidia 3.0-6.0 x 4.0-5.0 µm diam., pale brown, ellipsoid to subspherical, ornamented with 1-2 µm spines when young; spines later elongate into crests and ridges (Fig. 7).



Figs 1–**2** – Macromorphology of *Trichaleurina javanica* MOZ170. 1 On wood substrate covered with a fine layer of soil. 2 Growth on malt extract agar in 60 mm Petri plate. Scale bar: 1 = 2 mm.



Figs 3–**7** – Micromorphology of *Trichaleurina javanica* MOZ170. 3 Whorl cells from *T. javanica* from naturally growing population. 4, 5 Whorl cells from *T. javanica* grown on MEA culture media. 6 Denticle cell with budding spore (arrow) on MEA media. 7 Conidia grown on MEA media. Scale bars: 4, 5, 7, 8 = 10 µm, 6 = 20 µm.

Fig. 8 – Phylogram of the RAxML maximum likelihood analysis using LSU nrDNA sequences. Bootstrap values ≥ 70% are shown above or below the branches. Thickened branches indicate Bayesian posterior probabilities ≥ 95%. MOZ170 groups with *T. javanica* within the Chorioactidaceae and *K. sundara* MH878368 is outside the Chorioactidaceae. Red asterisks indicate taxa of interest. The outgroup is *Russula pseudociliata*, a member of the Basidiomycota.

Comparisons of *T. javanica* within natural and in vitro culture

The morphology of MOZ170 from nature and *in vitro* grown cultures show a significant difference in their morphology. Fertile lateral branches are longer (15-22 µm vs. 14.4-19 µm) and more numerous, the conidiogenous cells are wider (7-10 µm vs. 5-6.5 µm), and the conidia are spherical, 3-5 µm diam in the field-collected material as opposed to broadly ellipsoid, 5-7 x 4-6 µm in the *in vitro* grown cultures (Table 2).

Table 2 Morphological differences among anamorphs of *Kumanasamuha*, *Trichaleurina javanica* and *T. tenuispora*

Species	Source	Length of main conidiophores	Length of fertile branches	Conidiogenous cells	Conidia*
K. sundara	Rao & Rao 1964	160–1150 µm	10–45 µm	Globose, 5–12 µm	Ellipsoid, 5–7 × 4–6 µm
K. arakuensis	Carbone et al. 2013b	Up to 2000 µm	15-33 µm, non-septate	Flask-shaped, 9–12 × 6–12 µm	± spherical, 4–6 µm

Table 2 Continued.

Species	Source	Length of main conidiophores	Length of fertile branches	Conidiogenous cells	Conidia*
K. kalakadensis	Carbone et al. 2013b	Over 2000 µm	Up to 125 µm	Globose, 6.7–9.8 µm	Oval to subspherical, 3.4–5.0 µm
T. javanica (MOZ170, from MEA culture)	current study	300–800 µm	15–22 x 5-10 µm	Globose, 7–10 µm	Ellipsoid, 5–7 × 4–6 µm
T. javanica (MOZ170, from nature)	current study	400-950 µm	14.4-19 x 5-10 µm	Globose, 5-6.5 µm	Spherical, 3-5 um
T. tenuispora	Carbone et al. 2013b	500–1500 µm	20–75 µm	Globose, 8–10 µm	Ellipsoid, 5–7 × 4–5 µm

* Description based on Carbone et al. 2013b

Phylogenetic analyses and ITS sequence comparison

Both maximum likelihood and Bayesian analyses of the 28S LSU region supports isolate MOZ170 as being *Trichaleurina javanica*, but *Kumanasamuha sundara* strain CBS 488.73 was placed outside the Chorioactidaceae (Fig. 8). The MOZ170 ITS sequence best matched *Trichaleurina javanica* isolate HK022 voucher HKAS 88981 (97% query coverage with 98% identity (MG871291). GenBank BLASTn indicated that the *Kumanasamuha sundara* LSU sequence based on the ex-type culture was 99% similar to *Nigrograna mycophila* (KX650553), which is in the Dothideomycetes.

Discussion

Although several genera in the family Chorioactidaceae have limited distributions in temperate regions (Pfister et al. 2008), the genus *Trichaleurina* is thought to occur across tropical Asia, Africa, India, Madagascar and the islands of the Indian Ocean (Carbone et al. 2013b, LeGal 1953, MyCoPortal 2020). In most cases, the apothecia of Chorioactidaceae are collected on decaying wood or conifer debris. Anamorphic states of species within the Chorioactidaceae have been collected in nature or derived in culture for species of *Desmazierella*, anamorphic state verticicladium-like, (Martinovic et al. 2016); *Chorioactis*, anamorphic state Kumanasamuha-like (Peterson et al. 2004, Nagao et al. 2009); and *Pseudosarcosoma*, anamorphic state verticicladium-like (Paden & Tylutki 1969). The anamorph of *C. geaster* was collected in nature in Japan (Peterson et al. 2004, Nagao et al. 2009) alongside the teleomorph. In addition, Nagao et al. (2009) observed a few conidial mats, presumed to be *C. geaster*, during a field survey. In the instances where anamorphic states have been observed in the family, the hyphae, conidiogenous apparati and conidia are brown or nearly black as are the ascomatal hairs, and are ornamented with warts and spines.

Regarding MOZ170, there was no evidence suggesting the co-occurrence of teleomorph and anamorph. No apothecia of *T. javanica* were encountered during our three-week field survey in Gorongosa National Park. This may be due to the dry conditions we encountered, or fruiting may occur in another season. We found no evidence in the literature that the anamorph of *T. javanica* has been previously encountered in nature. Carbone et al. (2013a) suggested that anamorphs in their study were obtained from single ascospore isolation from ascomata. Based on these cultures, Carbone et al. (2013a) indicated the asexual states of both *T. javanica* and *T. tenuispora* appeared Kumanasamuha-like, but differed morphologically from the type specimen of *K. sundara*.

Kumanasamuha sundara, the type species of the genus, is an anamorphic fungus originally collected in India (Rao & Rao 1964). It resembles anamorphs within the Chorioactidaceae. Carbone et al. (2013a) noted key differences between *K. sundara* and anamorphs of *Trichaleurina* spp., but at that time no molecular data were available for *K. sundara* to investigate relationships. Recently,

Vu et al. (2019) provided sequence data for *K. sundara,* strain CBS 488.73, and suggested taxonomic thresholds for filamentous fungal identification at genus (98.2%), family (96.2%), order (94.7%) and class (92.7%) similarity, based on LSU barcodes. Our phylogenetic analyses place the *K sundara* strain outside the Chorioactidaceae. BLASTn analysis suggests it belongs within the genus *Nigrograna* (Dothideomycetes) based on 99% LSU sequence similarity to *Nigrograna mycophila.* In addition, phylogenetic analysis supports MOZ170 as the anamorphic state of *T. javanica*.

Conditions in culture may alter the morphological features that are detected in field collections. In the present study, morphological comparison of a natural versus *in vitro* cultured strain of MOZ170 showed that *in vitro* features were generally larger than those from field collections with different shaped conidiogenous cells and conidia. Rich growth medium such as MEA and high humidity may be responsible, but such differences also may be age-dependent. These differences reinforce the importance of using both morphological and molecular data for species determination and verification (Lücking et al. 2020).

Beyond the taxonomic and life history information presented here, we note that members of the genus *Trichaleurina* are also studied for their medicinal properties. Sogan et al. (2018) determined that ascomata of *T. celebica* (? = *T. javanica*) contain teratogenic compounds. Providing the information that this fungus can be cultivated might prove important in studies of toxicity and chemical compound discovery. LeGal (1953) reported that the copious gel of *T. javanica* (as *Sarcosoma javanicum*), known in Madagascar under the common name Ranomatonantibary, meaning "tears of an old woman," was used to treat ophthalmia among the Betsimisarakas people.

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Accessibility of data

Alignment file and phylogenetic trees are available upon request.

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