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## Phylogenetic placement of *Neorhamphoria Garethjonesii* gen. et sp. nov. (*Tubeufiales*, genus *incertae sedis*)

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### Abstract

*Neorhamphoria Garethjonesii* is introduced for a new genus and species with apothecial ascomata growing on dead wood of *Cotoneaster nummularius* (*Rosaceae*) in terrestrial habitats. *Neorhamphoria* is characterized by its dark apothecial ascomata, broad cellular pseudoparaphyses, with bitunicate, broad-clavate asci, and hyaline, muriform ascospores. Molecular phylogenetic analyses of combined LSU, SSU and TEF1 $\alpha$  sequence data indicate that *Neorhamphoria* belongs in Dothideomycetes and is related to *Tubeufiales*. Morphologically, *Neorhamphoria* differs from all genera of *Tubeufiales* and thus we treat it as a new genus in *Tubeufiales*, genus *incertae sedis*.

**Keywords** – apothecial ascomata – bitunicate asci – lignicolous, molecular phylogeny – muriform ascospores

### Introduction

Turkey has a very diverse flora, and the higher plants of Turkey have been well-studied, but the mycota has not been extensively investigated and most of the studies deal with macromycetes, generally the agaricoid fungi. Even though there has been no proper investigation of the Turkish micromycete mycota, the ascomycetous microfungi on higher plants of Turkey have been well-studied, but most of the studies deal with Erysiphales and a few with Diaporthales, Xylariales and other orders (Karaca 1961, Göbelez 1963, Baydar 1975, 1982, Güven & Tamer 1993). During the last 15 years, research on ascomycetous micromycetes in the country has intensified (Hüseyinov & Selçuk 2000, 2001, Mel'nik et al. 2004, Hüseyin & Yıldızbaş 2005, Hüseyin et al. 2006, 2009, 2016, Hüseyin et al. 2006, 2009, 2016, Selçuk et al. 2010, Bülbül et al. 2011, Bülbül & Hüseyin 2013, Selçuk & Hüseyin 2014).

This present taxon was collected during a trip to Kervansaray Mountain, Kırşehir Province, Central Anatolian Peninsula. This mountain lies in the Irano-Turanian phytogeographic region (Hamzaoğlu 1996). The species is somewhat reminiscent of *Rhamphoria* (Niessl 1876), with the type *R. delicatula* Niessl, from rotting wood of an unidentified plant from Brünn, Moravia, in the Czech Republic.

*Tubeufiales* comprises saprobic species commonly occurring on dead or decaying wood from diverse ecological distributions in terrestrial and aquatic habitats (Goh et al. 1998, Réblová & Barr 2000, Promputtha & Miller 2010, Boonmee et al. 2011, 2014a, b, Hyde et al. 2013, 2016, Rageshkumar & Sharma 2013). Currently, this order includes two families (*Tubeufiaceae* and *Weisneriomycetaceae*) and 23 genera of sexual and asexual morphs (Boonmee et al. 2014, Suetrong et al. 2014, Doilom et al. 2017). Its sexual morphs are characterized by light or dark, globose to subglobose, superficial ascomata, bitunicate asci, coloured or hyaline ascospores with transversely multi-septate, or muriform ascospores e.g. *Boerlagiomyces* (Crane et al. 1998, Doilom et al. 2017). Their asexual morphs are hyphomycetous, characterized by helicospore, chlamydosporous and phragmosporous conidia and they are commonly found in aquatic habitat. *Neorhamphoria garethjonesii* also shares some features with members of *Tubeufiales* in its saprobic lifestyle, its occurrence on woody substrates and in having muriform ascospores.

In this study, our blasts of LSU, SSU and TEF1 $\alpha$  sequence data indicated that this taxon belongs to Dothideomycetes with a loose relationship with tubeufiaceous species. Based on morphological characters and multigene phylogenetic analysis, *Neorhamphoria* is established as a new genus in *Tubeufiales*, genus *incertae sedis*.

## Materials & Methods

### Sample collection, morphological studies and isolation of fungi

The fungal specimen was collected in 2012 from Turkey. The identification of the host plant was made using the “Flora of Turkey and East Aegean Islands” (Davis 1967). Micro-morphological studies were carried out following the procedures outlined in Boonmee et al. (2014a, b). Type specimen and ex-type living culture are deposited Mae Fah Luang University Collections (MFLU and MFLUCC), Chiang Rai, Thailand and the Ahi Evran University, Arts and Sciences Faculty, Department of Biology, in Kırşehir Province of Turkey. Faces of fungi (FOF) and Index Fungorum (IF) numbers are provided as explained in Jayasiri et al. (2015) and Index Fungorum (Kirk 2016).

Single ascospore isolation was performed following the technique demonstrated in Chomnunti et al. (2014). Germinated ascospores were transferred to fresh malt extract agar (MEA, Difco Laboratories, Detroit, Michigan, USA) and grown at 28°C for one month. Culture morphological characters were examined after one month.

### Molecular procedures

Fungal colonies were scraped from surface mycelia after one month. DNA extraction, PCR amplification and sequences were performed under the conditions described in Hyde et al. (2016). The new sequences conducted in this study were blasted to check for related taxa in GenBank database ([www.ncbi.nlm.nih.gov/blast/](http://www.ncbi.nlm.nih.gov/blast/)), which indicated that this fungus belonged to Dothideomycetes.

### Phylogenetic analyses

Our new strain and closely related taxa from recent relevant publications (Boonmee et al. 2011, 2014a, b, Hyde et al. 2013, 2016, Suetrong et al. 2014, Wijayawardene et al. 2014,

Hongsanan et al. 2015, Doilom et al. 2017, Pratibha et al. 2015) were used in the phylogenetic analysis. Analyses of combined LSU, SSU and TEF1 $\alpha$  datasets were carried out to establish the placement of the new taxon. Multiple alignments were performed with the online program MAFFT v.7 (Kato & Standley 2013) and manually edited using BioEdit 7.0.5.3 (Hall 1999). Phylogeny online program “ALTER” was used to format fasta dataset for RAxML analysis (Glez-Peña et al. 2010). Maximum likelihood (ML) analysis was generated in online program “RAxML-HPC BlackBox tool” on the CIPRES 3.3 web portal (Miller et al. 2010), following the default setup. RAxML rapid bootstrapping and subsequent ML search used distinct model/data partitions with joint branch length optimization, executing 1,000 rapid bootstrap inferences and thereafter a thorough ML search. All free model parameters were estimated by RAxML and ML estimate of 25 per site rate categories. The final ML search was evaluated and optimized under GTRGAMMAI model.

**Table 1.** The new taxon used for phylogenetic analysis with GenBank accession numbers of LSU, SSU and TEF1 $\alpha$  sequence data. New sequences are in bold.

Species	Voucher	GenBank Accession number		
		LSU	SSU	TEF1 $\alpha$
<i>Acanthohelicospora pinicola</i>	MFLUCC10–0116	KF301534	KF301542	KF301555
<i>Acanthostigma chiangmaiensis</i>	MFLUCC10–0125	JN865197	JN865185	KF301560
<i>Aliquandostipite khaoyaiensis</i>	CBS 118232	GU301796	AF201453	GU349048
<i>Apiosporina collinsii</i>	CBS 118973	GU301798	GU296135	GU349057
<i>Boerlagiomyces macrospora</i>	MFLUCC12–0388	KU764712	KU712475	KU872750
<i>Botryosphaeria dothidea</i>	CBS 115476	DQ678051	DQ677998	DQ767637
<i>Botryosphaeria ribis</i>	CBS 115475	DQ678053	DQ678000	DQ677893
<i>Botryosphaeria stevensii</i>	CBS 431.82	DQ678064	DQ678012	DQ677907
<i>Chaetothyriothecium elegans</i>	CPC 21375	KF268420	–	–
<i>Chlamydotubeufia huaikangplaensis</i>	MFLUCC10–0926	JN865198	JN865186	–
<i>Chlamydotubeufia khunkornensis</i>	MFLUCC10–0118	JN865190	JN865178	KF301564
<i>Clavatispora thailandica</i>	MFLUCC10–0107	KF770458	KF770457	KF770459
<i>Dendrographa decolorans</i>	DUKE 0047570	AY548815	AY548809	DQ883725
<b><i>Neorhamphoria garethjonesii</i></b>	<b>MFLUCC16–0210</b>	<b>*****</b>	<b>*****</b>	<b>*****</b>
<i>Guignardia citricarpa</i>	CBS 102374	GU301815	GU296151	GU349053
<i>Helicoma khunkornensis</i>	MFLUCC10–0119	JN865191	JN865179	–
<i>Helicoma siamense</i>	MFLUCC12–0563	KU764713	KU712479	KU872751
<i>Helicoma siamense</i>	MFLUCC10–0120	JN865192	JN865180	KF301558
<i>Homortomyces combreti</i>	CPC 19808	JX517291	–	–
<i>Homortomyces tamaricis</i>	MFLUCC13–0441	KF537345	–	–
<i>Hysteropatella elliptica</i>	CBS 935.97	DQ767657	EF495114	DQ767640
<i>Jahnula aquatic</i>	R68-1	EF175655	EF175633	–
<i>Lichenocodium aeruginosum</i>	JL359 09	HQ174269	HQ174268	–
<i>Lichenocodium erodens</i>	JL363 09	HQ174267	HQ174266	–
<i>Lichenocodium lecanorae</i>	JL382 10	HQ174263	HQ174262	–
<i>Lichenocodium usneae</i>	JL352 09	HQ174265	HQ174264	–

Table 1 continued

Species	Voucher	GenBank Accession number		
		LSU	SSU	TEF1 $\alpha$
<i>Macrophomina phaseolina</i>	CBS 227.33	DQ678088	DQ678037	DQ677929
<i>Manglicola guatemalensis</i>	BCC 20079	FJ743449	FJ747443	–
<i>Manoharachariella tectonae</i>	MFLUCC12–0170	KU764705	–	KU872762
<i>Microthyrium microscopicum</i>	CBS 115976	GU301846	GU296175	GU349042
<i>Natipusilla bellaspora</i>	PE91-1b	JX474864	JX474869	–
<i>Natipusilla decorospora</i>	A236-1a	HM196369	HM196376	–
<i>Parawiesneriomyces zzygii</i>	CPC 26528	KX228339	–	–
<i>Patellaria atrata</i>	CBS 958.97	GU301855	GU296181	GU349038
<i>Phaeotrichum benjaminii</i>	CBS 541.72	AY004340	AY016348	DQ677892
<i>Pseudogliophragma indicum</i>	MTCC 11985	KM052851	KM052852	–
<i>Saccharata proteae</i>	CBS 115206	GU301869	GU296194	GU349030
<i>Sympoventuria capensis</i>	CBS 120136	DQ885906	KF156094	–
<i>Thaxteriellopsis lignicola</i>	MFLUCC15–0898	KU764711	KU712474	KU872749
<i>Trichodelitschia bisporula</i>	CBS 262.69	GU348996	GU349000	GU349020
<i>Trichodelitschia munkii</i>	Kruys 201 UPS	DQ384096	DQ384070	–
<i>Tubeufia Chiangmaiensis</i>	MFLUCC11–0514	KF301538	KF301543	KF301557
<i>Tubeufia javanica</i>	MFLUCC12–0545	KJ880036	KJ880035	KJ880037
<i>Tubeufia tectonae</i>	MFLUCC12–0392	KU764706	KU712460	KU872763
<i>Venturia inaequalis</i>	CBS 594.70	GU301879	NG_016539	GU349022
<i>Wiesneriomyces conjunctosporus</i>	BCC40633	KJ435455	KJ425442	–
<i>Wiesneriomyces laurinus</i>	BCC18609	KJ425459	KJ425443	–
<i>Zeloasporidium siamense</i>	IFRDCC2194	JQ036228	JQ036223	–

Bayesian command was generated using FaBox 1.14 (Villessen 2007). Bayesian posterior probability analysis was carried out under MrBayes 3.2.6 on the XSEDE at the CIPRES web portal (Ronquist & Huelsenbeck 2003). The parameter setting of 2 parallel runs, 4 chains, run for 4,000,000 generations, sample frequency every 1,000 generations and all other parameters were left as default. The 50% majority rule consensus tree was created from the remaining trees and illustrated in Treeview (Page 1996). The sequences obtained in this study are deposited in GenBank (Table 1). The final alignment was deposited in TreeBASE submission no. 20355 (<http://www.treebase.org>).

## Results

### Phylogenetic study

The dataset comprising 48 taxa with analysis of combined LSU, SSU and TEF1 $\alpha$  sequence data and indicated that the new taxon belonged in Dothideomycetes. The alignment had 1403 distinct alignment patterns and the ML tree received the best scoring tree with a final ln value of -22896.365251 as illustrated in Fig. 1. The new genus *Neorhamphoria* clustered close to the *Tubeufiales* clade with good support values (74% BS and >95 PP).

### Taxonomy

*Neorhamphoria* Boonmee, E. Hüseyin & F. Selçuk, **gen. nov.**

*Index Fungorum number:* 552704; *Facesoffungi number:* 02823

*Etymology* – The generic epithet ‘*Neorhamphoria*’ refers to its likeness to *Rhamphoria*.

*Saprobic* on dead wood in terrestrial habitat. Sexual morph: *Ascomata* apothecia, cup-shaped, superficial, solitary to grouped, black. *Receptacle* pulvinate, disc convex, disc and the margins are black. *Exciple peridium* consisting of thick-walled, pigmented, isodiametric cells. *Hymenium* upper part blackish brown and lower part is hyaline. *Hamathecium* comprising  $2 \times 3 \mu\text{m}$  wide, numerous, cylindrical, broad cellular pseudoparaphyses, anastomosed, constricted septate, hyaline, apical pigmented, exceeding asci in length, apices are glued together to develop epithecium. *Asci* 8-spored, bitunicate, saccate to broad-clavate, broadly rounded at apex, non-amyloid, with a short bifurcate pedicel or apedicellate. *Ascospores* overlapping 2–3-seriate, partially overlapping, obovoid or elliptic with broadly to narrow rounded ends, initially 1-septate at immature, becoming phragmosporous to muriform at maturity, 3-transversely septate, with 1–2-vertical septate, hyaline, smooth-walled. Asexual morph: Undetermined as did not sporulate in culture.

*Neorhamphoria garethjonesii* Boonmee, E. Hüseyin & F. Selçuk, **sp. nov.**

*Index Fungorum number:* 552703; *Facesoffungi number:* 02824

Figs 2–3

*Etymology* – The specific epithet ‘*garethjonesii*’ is name in honour of E.B. Gareth Jones in recognition of his contributions to mycology.

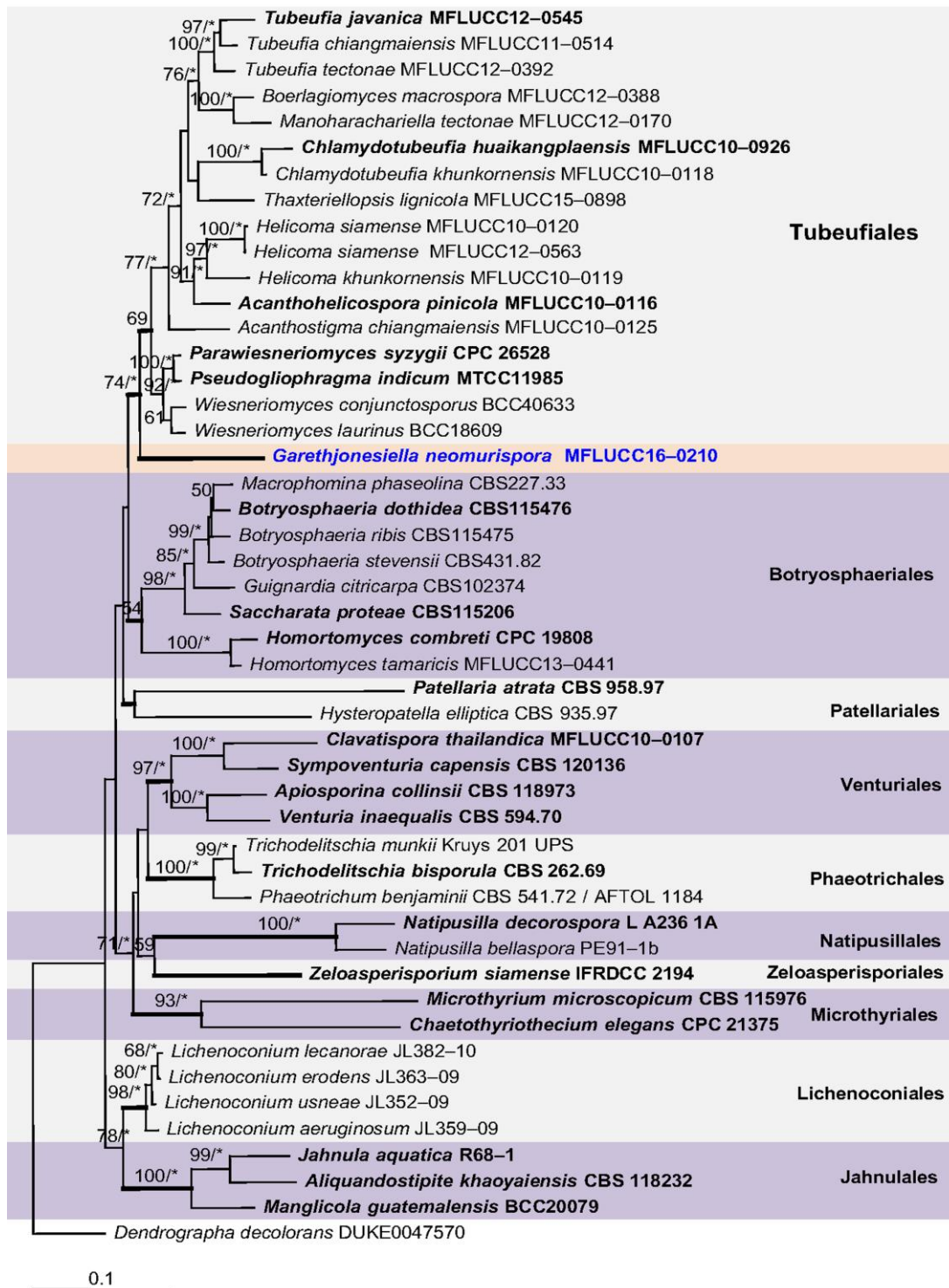
*Holotype* – MFLU16–2859

*Saprobic* on dead wood of *Cotoneaster nummularius* Fisch. & C. A. Mey., in terrestrial habitats. **Sexual morph:** *Ascomata* 179–199  $\mu\text{m}$  high  $\times$  163–322  $\mu\text{m}$  diam. ( $\bar{x}$  = 188.5  $\times$  257  $\mu\text{m}$ , n = 3), apothecia, cup-shaped, superficial, solitary to grouped, black. *Receptacle* pulvinate, disc convex, disc and the margins are black. *Exciple peridium* 21  $\times$  30  $\mu\text{m}$  wide consisting of thick-walled, pigmented, isodiametric cells. *Hymenium* upper part blackish brown and lower part is hyaline. *Hamathecium* comprising  $2 \times 3 \mu\text{m}$  wide, numerous, cylindrical, broad cellular pseudoparaphyses, anastomosed, constricted septate, hyaline, apical pigmented, exceeding asci in length, apices are glued together to develop epithecium. *Asci* 74–110  $\times$  21–29  $\mu\text{m}$  ( $\bar{x}$  = 88  $\times$  24.5  $\mu\text{m}$ , n = 20), 8-spored, bitunicate, saccate to broad-clavate, broadly rounded at apex, non-amyloid, with a short bifurcate pedicel or apedicellate. *Ascospores* 20–28  $\times$  9–13  $\mu\text{m}$  ( $\bar{x}$  = 25  $\times$  11  $\mu\text{m}$ , n = 20), overlapping 2–3-seriate, partially overlapping, obovoid or elliptic with broadly to narrow rounded ends, initially 1-septate at immature, becoming phragmosporous to muriform at maturity, 3-transversely septate, with 1–2-vertical septate, hyaline, smooth-walled. **Asexual morph:** Undetermined as did not sporulate in culture.

*Culture characteristics* – Ascospores germinating on MEA within 12 h and spores changed to brown with germ tubes produced from each cell. Colonies on MEA slow growing, reaching 5 mm diam. in 2 week at 28 °C, low convex, slightly effuse hairy, fimbriate edge, aerial mycelium radiating outwards, partially superficial, and partially immersed mycelium, cream to yellowish, not sporulate on culture at 30–45 days.

*Material examined* – TURKEY, Kırşehir Province, Boztepe district, mountain lighthouse forest, on dead wood of *Cotoneaster nummularius* Fisch. & C. A. Mey. (*Rosaceae*), 1370 m, 39°13'313'' N, 34°13'434'' E, 2 December 2012, E. Hüseyin (KE 201, **holotype**; MFLU16–2859, **isotype**) – ex-type living culture, MFLUCC16–0210.

*Notes* – The black apothecial ascomata, thin inner layer of bitunicate asci and muriform ascospores found in *Neorhamphoria garethjonesii* are similar to these characters in some genera (e.g. *Murangium* and *Tryblidaria*) of *Patellariaceae* (Yacharoen et al. 2015). *Neorhamphoria garethjonesii* differs from these genera in having hyaline muriform



**Fig. 1** Phylogram showing the best RAxML maximum likelihood tree (lnL = -22896.365251) generated from the combined multigene (LSU, SSU and TEF1 $\alpha$ ) analysis, with the GTRGAMMAI model, for showing the placement of the new genus *Neorhamphoria* and other closely related members of orders of Dothideomycetes. ML bootstrap values 1,000 repetitions with  $\geq 50\%$  (BS) are shown above the nodes and Bayesian posterior probabilities with  $\geq 0.95$  (PP) are marked with an asterisk (\*). The tree is rooted with *Dendrographa decolorans* (Turner & Borrer) Ertz & Tehler (*Roccellaceae*, *Arthoniales*). The new taxon in this study is highlighted in bold blue and all ex-type strains are in bold.



Fig. 2 *Neorhamphoria garethjonesii* (MFLU16–2859, holotype). a Type material and close up of ascoma. b Cross section of ascoma. c Peridium d Pseudoparaphyses e Immature ascus f,g Asci. h-j Asci and ascospores strained in Melzer's reagent. k, l Ascospores. Scale bars: a = 500  $\mu$ m, b = 200  $\mu$ m, c = 50  $\mu$ m, d = 5  $\mu$ m, e-i = 20  $\mu$ m, j-l = 10  $\mu$ m.

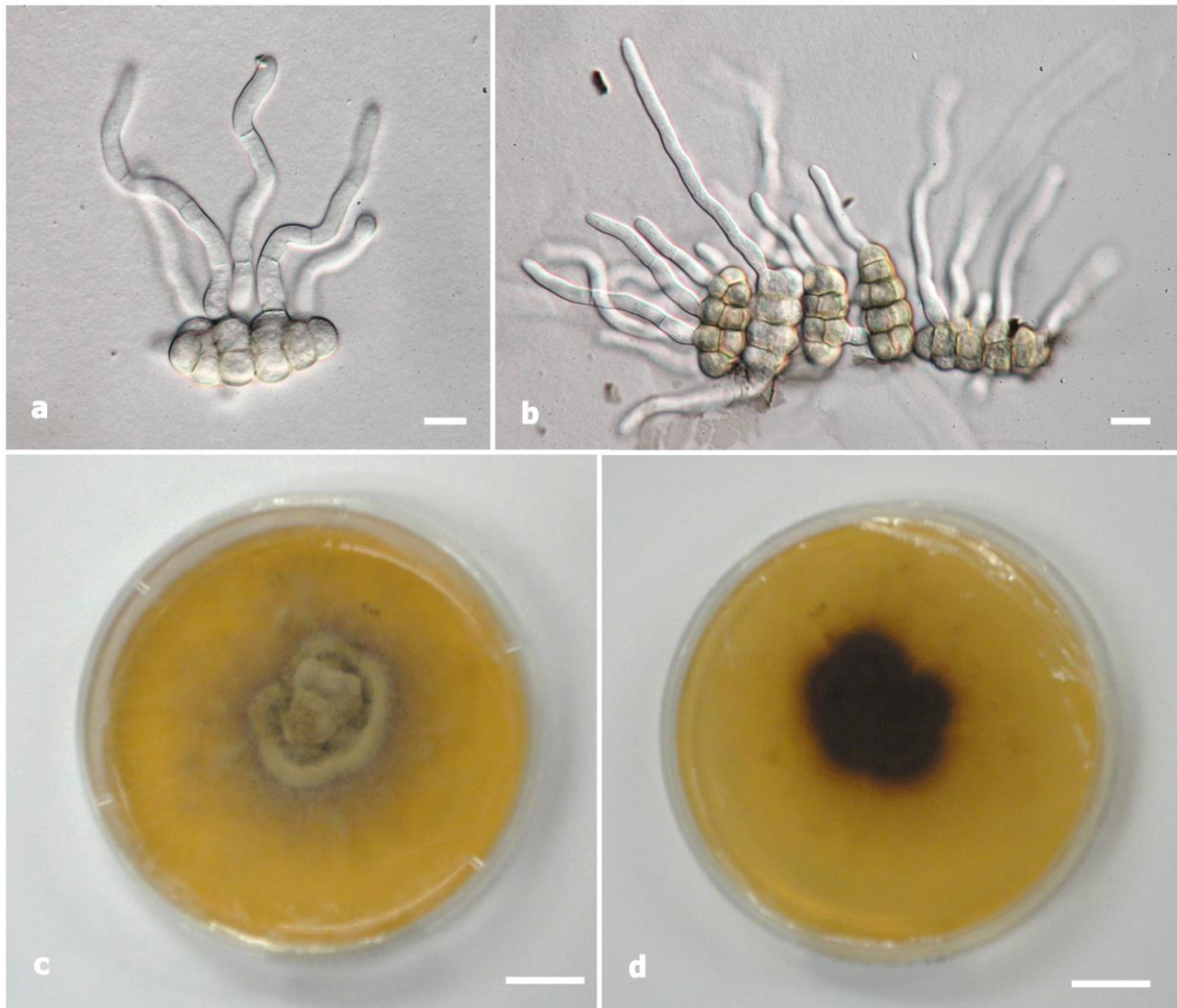


Fig. 3 *Neorhamphoria Garethjonesii* (MFLU16–2859, holotype). a, b Germinating ascospores. c, d Colonies cultured on MEA from surface and reverse at 8 weeks. Scale bars: a, b = 10  $\mu$ m, c, d = 10 mm.

ascospores with 3-transverse septa and 1–2 vertical septa. Multigene phylogenetic analysis (LSU, SSU and TEF1 $\alpha$ ) shows that *N. Garethjonesii* formed a clade at the base of *Tubeufiaceae*, while the clade of *Patellariaceae* is distant from *Neorhamphoria Garethjonesii* (Fig. 1). According to overall morphological characteristics, the taxon given as *N. Garethjonesii* differs from all tubeufiaceous genera. Therefore, we introduce this new genus to accommodate a monotypic species *N. Garethjonesii* which we treat in Dothideomycetes genera *incertae sedis*.

## Discussion

*Neorhamphoria Garethjonesii* was first identified as a species of *Rhamphoria* as it appears to have unitunicate asci. However, a blast search and analysis of LSU, SSU and TEF1 $\alpha$  sequence data showed that it is related to dothideomycetous taxa. The blasted LSU and SSU sequences showed the new taxon to be loosely related to species of *Chlamydotubeufia*, *Helicoma*, *Helicosporium*, *Tubeufia* of *Tubeufiales* with 95% to 96% similarity, while TEF $\alpha$  sequence data showed it to be related to species of *Alternaria*, *Bipolaris*, *Neofusicoccum* and a *Pleosporaceae* sp. with 91% similarity. Analysis of LSU sequence data, placed *Neorhamphoria* close to *Trypetheliales* in a lineage basal to



*Patellariales*, but this lacked good statistical support. The taxon was distantly related to *Tubeufiales* (tree not shown). Analyses of combined LSU, SSU and TEF1 $\alpha$  sequence data showed that *Neorhamphoria* clustered with *Tubeufiales* (Fig. 1). This genus is probably a basal genus or family related to *Tubeufiales*, however this cannot be confirmed until sequence data and more related taxa are available for study. *Neorhamphoria* is therefore treated in *Tubeufiales*, genus *incertae sedis*.

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