Hans-Otto Baral and Teppo Rämä[a],*

# Morphological update on *Calycina marina* (Pezizellaceae, Helotiales, Leotiomycetes), a new combination for *Laetinaevia marina*

**Abstract:** *Laetinaevia marina* is a frequent ascomycetous fungus that is widely distributed on the seashores of Scandinavia and Great Britain. It grows on fronds of Fucales (Phaeophyceae) that are washed ashore and lie decaying at the high tide mark or further up on the beach. It is one of the few described marine discomycetes. In the current generic concept it is regarded as a member of Naevioideae (Dermateaceae or Mollisiaceae), while it has previously been placed in the genus *Orbilia* (Orbiliaceae). We studied fresh collections, in live and dead state, to provide a morphological update on the species and to elucidate its systematic placement using molecular systematics. The hemiamyloid reaction of the apical ring, the absence of croziers, and the faintly refractive vacuolar bodies of the terminal cells of living paraphyses are reported here for the first time. A thin gel sheath surrounding the ascospores was confirmed to exist. Based on morphological characters, ribosomal RNA and protein coding gene sequences published for this species here for the first time, we show that *L. marina* is distinct from the genera and families it was previously placed in, and propose the new combination *Calycina marina*, despite its erumpent, sessile, pulvinate and rather immarginate apothecia. Furthermore, we present additional observations on the ecology, phenology, and distribution of the species.

**Keywords:** algicolous fungi; discomycetous fungi; marine fungi; morphology; phylogeny.

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## Introduction

Discomycetes form a morphological group of filamentous ascomycetes that possess cup-like or discoid fruiting structures (apothecia) in which sexual spores are produced. Among the 424 known marine ascomycetous fungi, there are only nine non-lichenized discomycetous species in the genera *Dactylospora* Körb., *Gloniella* Sacc., *Lachnum* Retz., *Laetinaevia* Nannf., *Patellaria* Fr. and *Vibrissea* Fr. (Suetrong and Jones 2006, Jones et al. 2009, Pang et al. 2014). The number of marine non-lichenized discomycetes is low compared to the number of non-lichenized discomycetes in the terrestrial environment (including freshwater habitats) where these fungi thrive with >5000 species estimated (Baral, in Jaklitsch et al. 2015). The low number of marine discomycetes may be due to several reasons, such as sensitivity of the exposed apothecia to grazing marine animals, submersion in saline water and wave action (Suetrong and Jones 2006).

One of these marine discomycetes is *Laetinaevia marina* (Boyd) Spooner, a microfungus that fruits on decaying seaweed. This is a rather frequent species and found along seashores of Scandinavia and Great Britain. It grows on fronds of the genera *Ascophyllum* and *Fucus* (Fucales, Phaeophyceae) which are washed ashore and are degraded by microorganisms. D.A. Boyd made the first collections of *L. marina* in ~1888 at West Kilbride (North Ayrshire, Scotland) on *Fucus*. Based on Boyd's material, Phillips wrote a manuscript that includes the first morphological description of the species (Smith 1909, Kirk and Spooner 1984). In this unpublished manuscript, the species was called "*Calloria marina* Phil.". Boyd, however, placed the fungus in *Orbilia* Fr. obviously because of the tiny orange apothecia and paraphyses with clavate heads (Smith 1909). He had also observed the fungus on "*Ascophyllum*, *Halydris* &c. (=etc.)" (Smith 1909) at different sites in the west of Scotland ("Strathclyde") in autumn and spring, and assumed that it occurs throughout the year, wherever decaying seaweed is abundant.

Eriksson (1973) collected the species in April and June to August in 1962–1969 in Denmark (Sjælland), Norway

(Nordland and Troms) and Sweden (Skåne and Bohuslän) on *Ascophyllum nodosum*, *Fucus serratus* and *Fucus* sp. He retained its systematic placement in *Orbilia* and concluded that it is widely distributed in Scandinavia. Kirk and Spooner (1984) studied Boyd's preserved Scottish collections, including some collected by W.B. Grove from Wales (Gwynedd) on *Ascophyllum nodosum* and *Fucus vesiculosus* in spring and autumn 1907–1919, but did not mention any more recent records. Spooner removed the species from Orbiliaceae and transferred it tentatively to *Laetinaevia* (Naevioideae, Dermateaceae) based on morphological characters (Kirk and Spooner 1984). Since the type material could not be traced, he designated a lectotype (Arran, Brodick, Invercloy, *Ascophyllum nodosum*, Apr. 1908, D.A. Boyd) that is preserved in Kew.

The studies above do not include information about morphological characters that can only be observed when living cells are examined. These state-dependent characters include characteristics of the vacuoles of paraphyses, lipid bodies and nuclei in the ascospores, and measurements of turgescent cells. Furthermore, two herbarium-stable characters were previously neglected. They concern the type of iodine reaction of the apical ring and the mode of ascus formation at the ascogenous hyphae.

The fungus was also not subjected to phylogenetic analyses based on DNA sequences, and hence the systematic placement and appropriate genus of the species remain unverified. For the present paper, we studied fresh, living collections from Portsmouth, UK, and Central and Northern Norway. Our aims were to provide a morphological revision of the species and study its systematic placement based on DNA extracted from freshly collected material. We have obtained DNA sequences from four gene regions for the first time for this species and describe the ecology, phenology and distribution of the species.

# Materials and methods

## Morphological studies and culturing

Fresh algal thalli with apothecia were studied under a dissecting microscope (Leica MZ16, Leica Microsystems, Wetzlar, Germany). Sections were mounted in tap water. Lugol's solution of high concentration (IKI=1% $I_2$, 3% KI, 100 ml water) was applied for staining ascus walls, and aqueous Cresyl Blue (CRB) for staining gel and vacuoles. The microscope slides were examined with a Zeiss Standard 14 (Carl Zeiss, Oberkochen, Germany) and Leica DM2500 compound microscope (Leica Microsystems, Wetzlar, Germany). Pictures were taken with Nikon Coolpix

E4500 (Nikon Corporation, Tokyo, Japan), Olympus Colorview I and UC50 microscope cameras (Olympus Soft Imaging Solutions, Münster, Germany). Voucher specimens are preserved in the herbarium of Tromsø University Museum (TROM) and private herbarium of H.B.

One of the voucher specimens, T. Rämä 3180A from Portsmouth (TROM-F26103), was used in culturing of the fungus with the intention of revealing its asexual state. Using sterile techniques, a fragment of fertile hymenium from a single fruiting body was transferred onto a sterile slide containing a drop of distilled water. The suspension was agitated and blown on a malt extract agar plate that was prepared using 4 g of malt extract agar (Jensen & Co, Nittedal, Norway) from grocery store in 1 l of filtered and autoclaved seawater (0.2SeaMEA). The plate also contained the antibiotic streptomycin (25 mg $l^1$) (Biochrom AG, Berlin, Germany) and tetracycline (10 mg $l^1$) (Calbiochem, part of Merck, Darmstadt, Germany). After germination of the spores at +20°C during the first 24 h, the isolate was cultured in the dark at +5°C. The fungus grew slowly in culture, and did not produce an asexual state.

## Molecular work and phylogenetic analyses

We extracted DNA of *Laetinaevia marina* voucher specimens and the culture isolate using a modified cetyltrimethyl ammonium bromide (CTAB) extraction protocol (Murray and Thompson 1980) and amplified the target loci according to Mysterud et al. (2007) and Johnson et al. (2009) with slight modifications. PCR amplifications were performed on a PTC-0200 DNA engine (MJ Research, Waltham, MA, USA) and GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA) using the primer pairs ITS5–ITS4 (White et al. 1990), LR0R–LR5 (Vilgalys and Hester 1990, Rehner and Samuels 1994), NS1–NS24 (White et al. 1990, Gargas and Taylor 1992) and RPB1Ac–RPB1Cr (Stiller and Hall 1997, Matheny et al. 2002, both with unpublished modifications by Giho Sung). The primers were used in concentrations of 0.5 μM (0.6 μM for RPB1) together with Illustra PuReTaq Ready-To-Go PCR beads (GE Healthcare, Buckinghamshire, UK) in a reaction volume of 25 μl. The cyclic PCR program consisted of an initial 3-min denaturation step at 94°C, followed by 40 cycles of 30 s at 94°C, 30 s at 52°C (annealing) and 1 min at 72°C (synthesis), and termination with a 10-min elongation step at 72°C. The PCR products were cleaned using 0.25 units of ExoSAP-IT (Affymetrix, Santa Clara, CA, USA) for 6 μl of PCR product. Sequencing reactions were performed on an Applied Biosystems 3130xl Genetic Analyzer (Foster City, CA, USA) in BigDye Terminator sequencing buffer using PCR primers as sequencing primers and the BigDye Cycle Sequencing kit v3.1 (Applied Biosystems, Carlsbad, CA, USA).

DNA sequences of *Calloria urticae* (Pers.) J. Schröt. *ex* Rehm [=*C. neglecta* (Lib.) B. Hein] and *Laetinaevia carneoflavida* (Rehm) Nannf. *ex* B. Hein were provided by Guy Marson (pers. comm., unofficial collaborator at Musée National d'Histoire Naturelle, Paris, France). Crushed apothecia were subjected to direct PCR amplification using the Phire Plant Direct PCR Master Mix kit (F-160 kit, Thermo Scientific, Waltham, MA, USA). Two primers, SF16a and LR17b, were developed (Marson et al., unpublished) to amplify approximately 2500 base pair long sequences of nuclear ribosomal DNA that included the internal transcribed spacer 1 (ITS1), the 5.8S gene, the internal transcribed spacer 2 (ITS2), and the 28S regions. PCRs were performed in an iCycler (Bio-Rad, Hercules, CA, USA) in a total volume of 20 µl that contained 1× Phire Plant Direct PCR Master Mix and 0.5 µM of each primer. Finally, 1 µl of the fungal DNA from the extraction supernatant was added to the reaction tube. Cycling conditions consisted of an initial denaturation at 98°C for 5 min, followed by 40 cycles with denaturation at 98°C for 7 s, annealing at 59.9°C for 8 s and 40 s of elongation at 72°C, and a final elongation at 72° for 3 min. PCR products were purified with the Agencourt AMPure XP system (Beckman Coulter, Brea, CA, USA). The whole region was sequenced using seven sequencing reactions that generated partially overlapping fragments. The forward primers used were SF16a, 5.8SR and LR3R, and the reverse primers LR17b , LR5, LR1 and 5.8S (Vilgalys and Hester 1990). Sequencing was performed using the Big Dye Terminator chemistry (Applied Biosystems, Carlsbad, CA, USA) on an ABI 3730 capillary DNA automated sequencer (Applied Biosystems, Carlsbad, CA, USA).

Sequenced forward and reverse strands were assembled and manually checked using Geneious® v. 7.1.7 and v. 7.1.4 (Biomatters Ltd., Auckland, New Zealand). The alignments were made using the G- and E-INS-i algorithm in MAFFT v. 7.0.7 (Katoh and Standley 2013) followed by manual adjustment and removal of ambiguously aligned sites in Geneious. A three-gene dataset was constructed to determine the higher order placement of *L. marina* in the Ascomycota tree of life. It contained SSU, LSU and RPB1 sequences of 27 taxa (Table 1). Long insertions and ambiguously aligned positions were removed from the alignments of individual genes. Preliminary analyses for each gene were run in MrBayes v. 3.2.4 (Ronquist et al. 2012) using the same settings as the final analyses. The resulting gene trees showed no incongruence, hence the alignments were concatenated and the final analyses run in MrBayes. The analysis was run using three partitions (by gene), each with the same model settings that were unlinked across the partitions. Based on results from

jModeltest v. 2.1.6 (Guindon and Gascuel 2003, Darriba et al. 2012), a GTR model with gamma-distributed rate variation across sites and a proportion of invariable sites included was used in combination with Dirichlet distributed substitution rate and state frequency priors. *Neolecta irregularis* (Peck) Korf *et* J.K. Rogers (Taphrinomycotina) was used as the outgroup taxon and three subruns, each with four chains, were run for 20 million generations, after which the standard deviation of split frequencies was <0.01 and the Markov chain Monte Carlo (mcmc) had reached stationarity as inspected in Tracer v. 1.6 (Rambaut et al. 2014). First 5000 trees of the resulting 20,001 ones were discarded as burnin, and the remaining trees were used to calculate the posterior probability (PP) support values for the nodes of a consensus tree produced using the 50% majority rule option.

The ITS dataset was set up to examine the systematic placement of *L. marina* within the order Helotiales. It contained sequences belonging to 26 fungal isolates and voucher specimens (Table 1) including the outgroup taxon *Mollisia cinerea* (Batsch) P. Karst., and had 451 sites of which 216 bp were unique. Two different versions of the ITS analysis were conducted using MrBayes (Ronquist et al. 2012). First, the analysis was run using a GTR model with gamma-distributed rate variation across sites and the same priors as above. In the second version, a TIM2ef+G substitution model with equal nucleotide frequencies and fixed substitution rates was specified, as suggested by jModeltest (Guindon and Gascuel 2003, Darriba et al. 2012) based on Bayesian information criteria and decision theory performance. Both versions were run and summarized in the same way as the three-gene dataset. Both produced similar consensus trees, with only small differences in posterior probability support values for some of the nodes, and the tree produced with TIM2ef+G model is shown in the results.

The sequences generated for this study are deposited in GenBank (Benson et al. 2014) under accession numbers KT185666–KT185667 and KT185669–KT185677. All sequences used in this study are listed in Table 1.

## Results

### Systematics

The combined three-gene analysis showed that *Laetinaevia marina* is related to *Calycina citrina* (Hedw.) Gray [≡ *Bisporella citrina* (Batsch) Korf *et* S.E. Carp.] in the Helotiales (PP=1), whereas sequences of Orbiliomycetes are distant (Figure 1). The ITS analysis placed *L. marina*

**Table 1:** Species, isolates and GenBank accession numbers of the ascomycete sequences used in the phylogenetic analyses.

| Species | Isolate | Three-gene dataset | | | ITS dataset |
|---|---|---|---|---|---|
| | | SSU | LSU | RPB1 | |
| *Ascocoryne sarcoides* | Aftol 1834 | FJ176830 | FJ176886 | – | – |
| *Brunnipila fuscescens* | FC-2197 | – | – | – | AB481254 |
| *Brunnipila fuscescens* | KUS-F52031 | – | JN086695 | – | – |
| *Bulgaria inquinans* | Aftol 916 | DQ471008 | DQ470960 | DQ471152 | – |
| *Calloria urticae* | G.M. 2015-04-12 #5 | – | – | – | **KT185667** |
| *Calycellina punctata* | Cantrell GA18 | – | – | – | U57494 |
| *Calycina citrina*[a] | F14046 (UBC) | – | – | – | AF335454 |
| *C. citrina*[a] | Andy 9/27/03 | – | AY789385 | – | AY789386 |
| *C. citrina*[a] | Aftol 1301 | FJ176815 | FJ176871 | – | – |
| *C. citrina* | F118000 | – | – | – | KC412005 |
| *C. claroflava* | F132983 | – | – | – | KC412006 |
| *C. herbarum* | KUS-F52362 | – | – | – | JN033407 |
| *C. herbarum* | KUS-F51458 | – | – | – | JN033390 |
| *C. lactea* | H.B. 7224 | – | – | – | KC412007 |
| *C. languida* | F116600 | – | – | – | KC412003 |
| *C. languida* | F116599 | – | – | – | KC412002 |
| *C. marina* | TROM-F26093 | **KT185672** | **KT185670** | – | **KT185677** |
| *C. marina* | TROM-F26098 | – | – | – | **KT185676** |
| *C. marina* | TROM-F26101 | **KT185673** | **KT185671** | **KT185669** | **KT185674** |
| *C. marina* | TROM-F26103 | – | – | – | **KT185675** |
| *C. populina*[b] | CBS 247.62 | – | – | – | JN033382 |
| *Capronia munkii* | Aftol 656 | EF413603 | EF413604 | EF413605 | – |
| *C. pilosella* | Aftol 657 | DQ823106 | DQ823099 | DQ840554 | – |
| *Chalara dualis* | ICMP 14950 | – | – | – | EF029209 |
| *C. pseudoaffinis* | CCF 3979 | – | – | – | FR667224 |
| *Chloroscypha enterochroma* | Aftol 67 | AY544700 | AY544656 | – | – |
| *Cladonia stipitata* | Aftol 1657 | DQ973003 | DQ973026 | DQ973050 | – |
| *Debaryomyces hansenii* | JCM 1990 | JN940470 | JN940504 | JN992543 | – |
| *Dermea acerina* | Aftol 941 | DQ247809 | DQ247801 | DQ471164 | – |
| *Heyderia abietis* | HMAS71954 | AY789295 | AY789296 | – | – |
| *Hyalopeziza pygmaea* | KUS-F51564 | – | JN086713 | – | JN033410 |
| *Hymenoscyphus fructigenus* | M159 | EU940081 | EU940157 | – | – |
| *Hyphodiscus hymeniophilus* | TNS-F31802 | – | AB546950 | – | AB546951 |
| *Laetinaevia carneoflavida* | G.M. 2014-07-25 | – | – | – | **KT185666** |
| *Mollisia cinerea* | CBS 128349 | – | – | – | JF514855 |
| *M. cinerea* | Aftol 76 | DQ470990 | DQ470942 | DQ471122 | – |
| *Mollisina uncinata* | TNS-F38901 | – | JN086757 | – | JN033457 |
| *Naevala perexigua*[c] | CBS 115920 | – | – | – | AY853229 |
| *Neofabraea malicorticis* | Aftol 149 | AY544706 | AY544662 | DQ471124 | – |
| *Neolecta irregularis* | Aftol 1363 | DQ842040 | DQ470986 | – | – |
| *Orbilia auricolor* | Aftol 906 | DQ471001 | DQ470953 | – | – |
| *O. vinosa* | Aftol 905 | DQ471000 | DQ470952 | DQ471145 | – |
| *Peziza vesiculosa* | Aftol 507 | DQ470995 | DQ470948 | DQ471140 | – |
| *Phialina lachnobrachyoides* | KUS-F52576 | – | – | – | JN033424 |
| *Pilidium acerinum* | BPI 843554 | AY487090 | AY487089 | – | – |
| *Sclerotinia sclerotiorum* | Aftol 928 | DQ471013 | DQ470965 | – | – |
| *Trichoglossum hirsutum* | Aftol 64 | AY544697 | AY544653 | DQ471119 | – |

[a]As *Bisporella citrina*.
[b]As *Calycellina populina*.
[c]As *Naevala minutissima*.
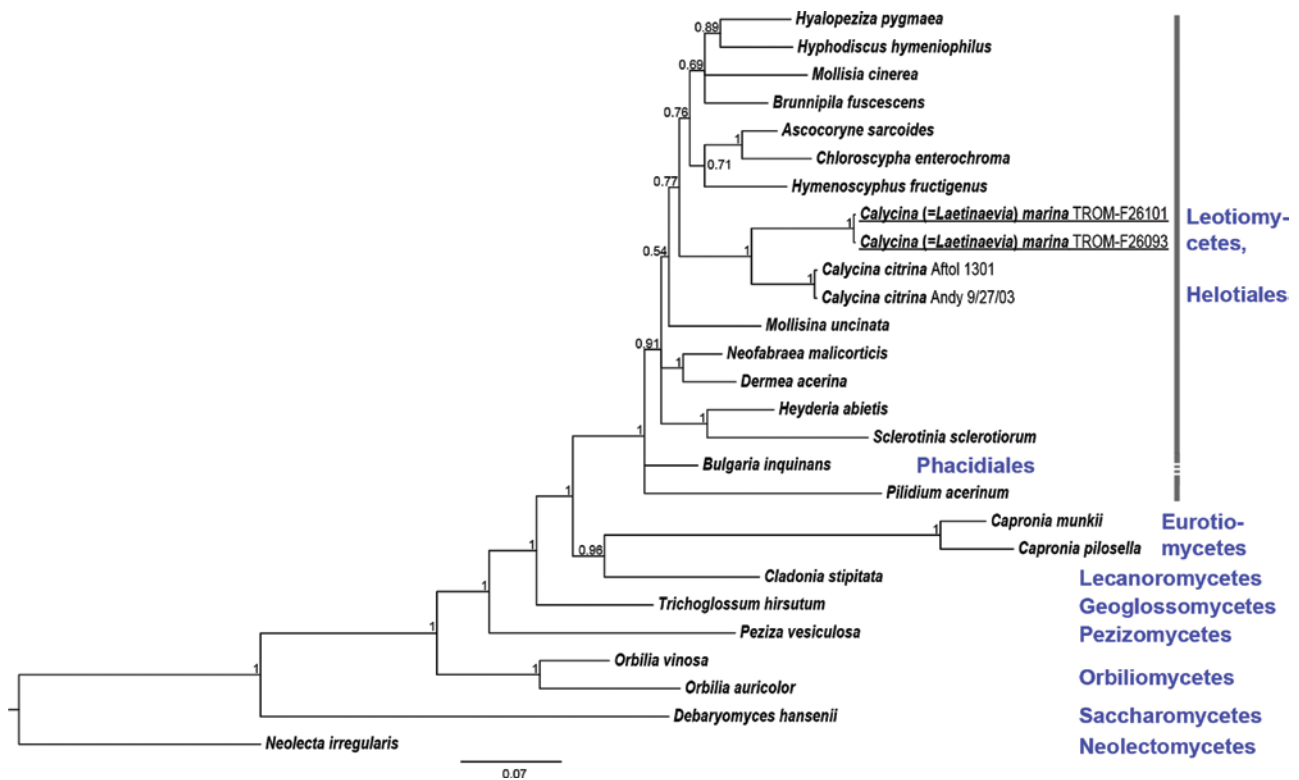The sequences generated for this study are in bold face.

**Figure 1:** Fifty percent majority rule consensus tree from the combined three-gene analysis (SSU, LSU, RPB1) showing the placement of *Calycina marina* within the phylum Ascomycota. The log-likelihood of the tree was ln(L)=-16,075.01. Node support is given as posterior probabilities at the nodes, the scale bar shows estimated substitutions per site, and strain or isolate identifiers follow taxon names. The new sequences generated for this study are underlined.

sequences closest to *Calycina claroflava* (Grev.) Kuntze (Figure 2, PP=0.96). This result was also obtained in several preliminary runs using slightly different sets of taxa and run options (results not shown). *Calycina herbarum* (Pers.) Gray (type of *Calycina* Nees *ex* Gray) forms a sister taxon to this clade (PP=0.99) which contains also *Calycina populina* (Fuckel) Kuntze and *Calycina lactea* (Sacc.) Baral et al. Together with further species placed in *Calycina*, and with those assigned to the genera *Mollisina* Höhn. *ex* Weese, *Phialina* Höhn., and *Calycellina* Höhn., this group of fungi represents the family Pezizellaceae (PP=0.99) as recently resurrected by Baral (in Jaklitsch et al. 2015).

Three Naevioideae species, *Calloria urticae*, *Laetinaevia carneoflavida*, and *Naevala perexigua* (Roberge *ex* Desm.) K. *et* L. Holm [=*Naevala minutissima* (Auersw.) B. Hein], were placed basal to Pezizellaceae in our analysis (Figure 2). Taxa that were found by us to show morphological similarities with *L. marina*, i.e. *Cyathicula* De Not. (Helotiaceae) in the apical ring type and *Corticifraga* D. Hawksw. *et* R. Sant. (incertae sedis) in excipular characters, were not closely related with it due to high genetic distance between aligned sequences (results not shown).

## Taxonomy

### *Calycina marina* (Boyd) Rämä *et* Baral, comb. nov. (Figures 3 and 4) MB812843
**Basionym:** *Orbilia marina* Boyd, in Smith, Trans. Br. Mycol. Soc. 3: 113, pl. 6 fig. 1 (1909) [1908], as *"Orbilia marina* (Phil.) Boyd"

[≡ *Orbilia marina* (W. Phillips) Boyd, in Elliott et al. (ed.), Fauna, Flora & Geology of the Clyde Area: 69 (1901) nom. nud. Art. 38.1(a)]

≡ *Laetinaevia marina* (Boyd) Spooner, in Kirk and Spooner, Kew Bull. 38: 568 (1984)

**Typification: Holotype:** unknown. **Lectotype:** K(M) 33419.

Explanation of symbols: *=microscopic characters of living cells, †=microscopic characters of dead cells, numbers in {} refer to the number of collections, in which a specific character was observed.

**Apothecia** moist or rehydrated 0.25−0.65 mm diam., 0.13−0.16 mm thick (receptacle 0.09−0.11 mm), pulvinate,
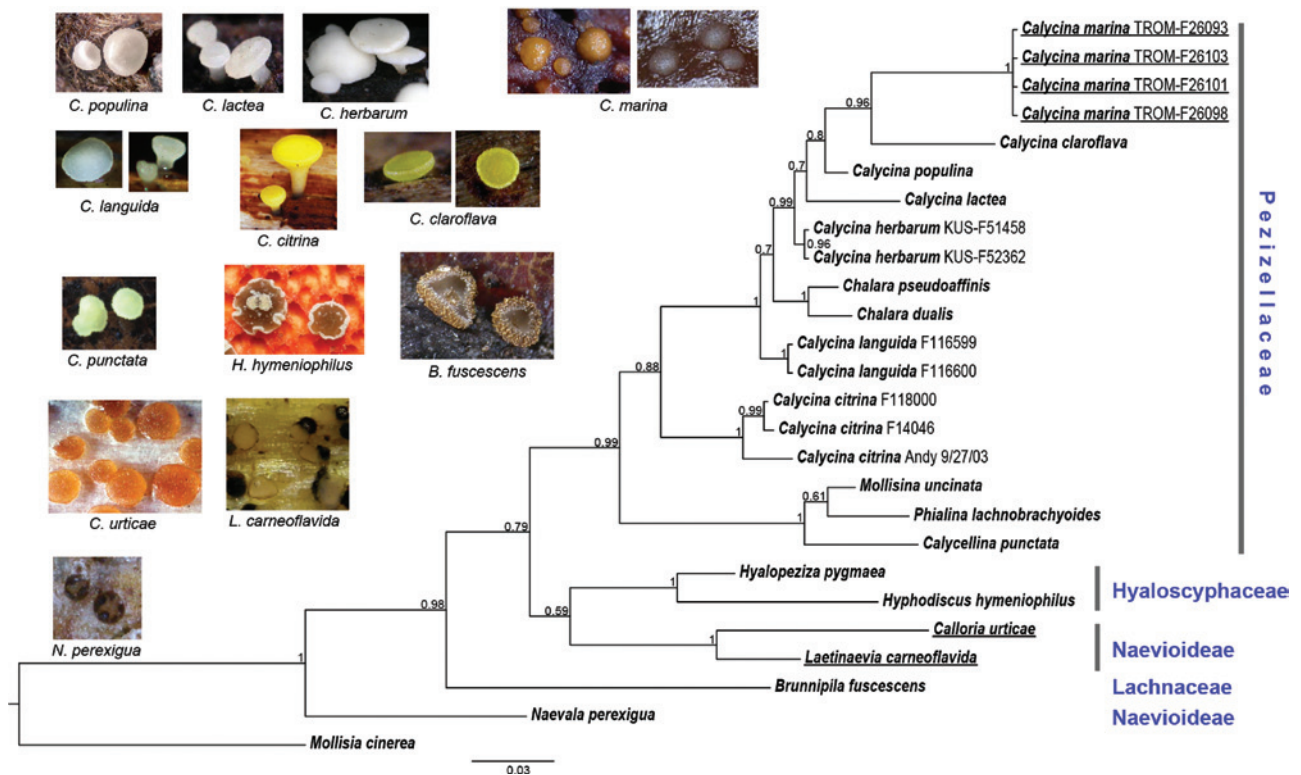
**Figure 2:** Fifty percent majority rule consensus tree from the Bayesian ITS analysis showing placement of *C. marina* within the Pezizellaceae (Helotiales) and its relationship to members of Naevioideae (Nannfeldt 1932). The log-likelihood of the tree was ln(L)=-2912.00. Node support is given as posterior probabilities at the nodes, the scale bar shows estimated substitutions per site and strain or isolate identifiers follow taxon names. The new sequences generated for this study are underlined. Clade annotations in coloured font are based on Nannfeldt (1932) and Hein (1976). Origin of photos: *Brunnipila fuscescens*: H.O.B. (H.B. 8156a), *Calycina citrina*: P. Thompson, *C. claroflava*: H.O.B. (H.B. 7666), *C. herbarum*: H.O.B. (H.B. 8947), *C. lactea*: H.O.B. (H.B. 9861), *C. languida*: B. Perić (XII.2014), *C. marina*: H.O.B. (H.B. 5792) and T.R. (T.R. 3160B), *C. populina*: E. Rubio (ERD-6306), *Calycellina punctata*: P. Thompson (19.X.2009), *Hyphodiscus hymeniophilus*: S. Blaser (XI.2014), *Laetinaevia carneoflavida*: G. Marson (G.M. 2014-07-25), *Naevala perexigua*: H.O.B. (H.B. 9308a).

slightly to strongly translucent, round, slightly gelatinous, scattered or often subgregarious to gregarious; disc whitish cream, light to bright yellowish-orange-ochraceous, or pale to dark greyish-brownish-alutaceous, flat at first, soon slightly to strongly convex, margin eventually indistinct, not protruding, smooth; sessile, erumpent from beneath cortex of thallus; dry flat to medium convex, ± concolorous. **Asci** *(50–)58–87(–96)×(6.5–)7–8.5(–9.7) μm {3}, †(38–)43–60(–76)×(5.5–)6–7.5(–8) μm {3}, (4–)8-spored, spores *obliquely biseriate († uniseriate below), pars sporifera *(26–)30–40(–55) μm long {3}, living mature asci protruding up to 15–20 μm beyond paraphyses but some also ~10 μm shorter than those, †5–10 μm shorter than paraphyses; **apex** (*/†) slightly conical, apical ring (†) staining medium to strongly dirty blue in IKI at low concentration, at high concentration dirty red-brown (hemiamyloid, type RB) {3}, immature 1.7×1.1 μm (height×width), becoming 1.2×1.5 μm when mature, protruding into ascoplasm by forming a small apical chamber, distally not extending up to ectotunica, ~*Conchatium*-type, deeply pure blue in IKI

when KOH-pretreated {1}, contracted to 0.5–0.8×1–1.4 μm, immature with thick lateral wall (Figure 4w lower left); **base** narrowed, arising from simple septa without basal protuberances {5}; entire wall surface slightly to distinctly lilac in CRB, especially towards apex. **Ascospores** *(7–)8–13(–15)×(2.8–)3.3–4(–4.5) μm {4}, exceptionally *4–5.5 μm wide {1}, †(5.5–)7–11(–12.5)×(2.5–)2.8–3.3 μm {2}, subcylindrical to subfusoid or ellipsoid, homopolar or only very slightly heteropolar, ends obtuse, straight to slightly inequilateral; containing ~1–3 lipid bodies 0.3–0.5(–1) μm diam. in each half or near the ends, lipid content 0.5–1, uninucleate (seen in living spores in IKI), lacking glycogen; wall ± negative in CRB, sometimes with sheath; overmature spores 1-septate, up to 16 μm long. **Paraphyses** apically slightly to rather strongly clavate-capitate, rarely ellipsoid-fusoid or moniliform-lageniform, hyaline; terminal cell *(7–)8–17(–21)×(3–)3.5–6(–8.3) μm {3}, †(3–)4–5,3 μm wide {1}, covered by a very faintly refractive, thick, hyaline gel staining deep lilac in CRB; terminal cell (*) containing ± large and globose, faintly refractive, hyaline
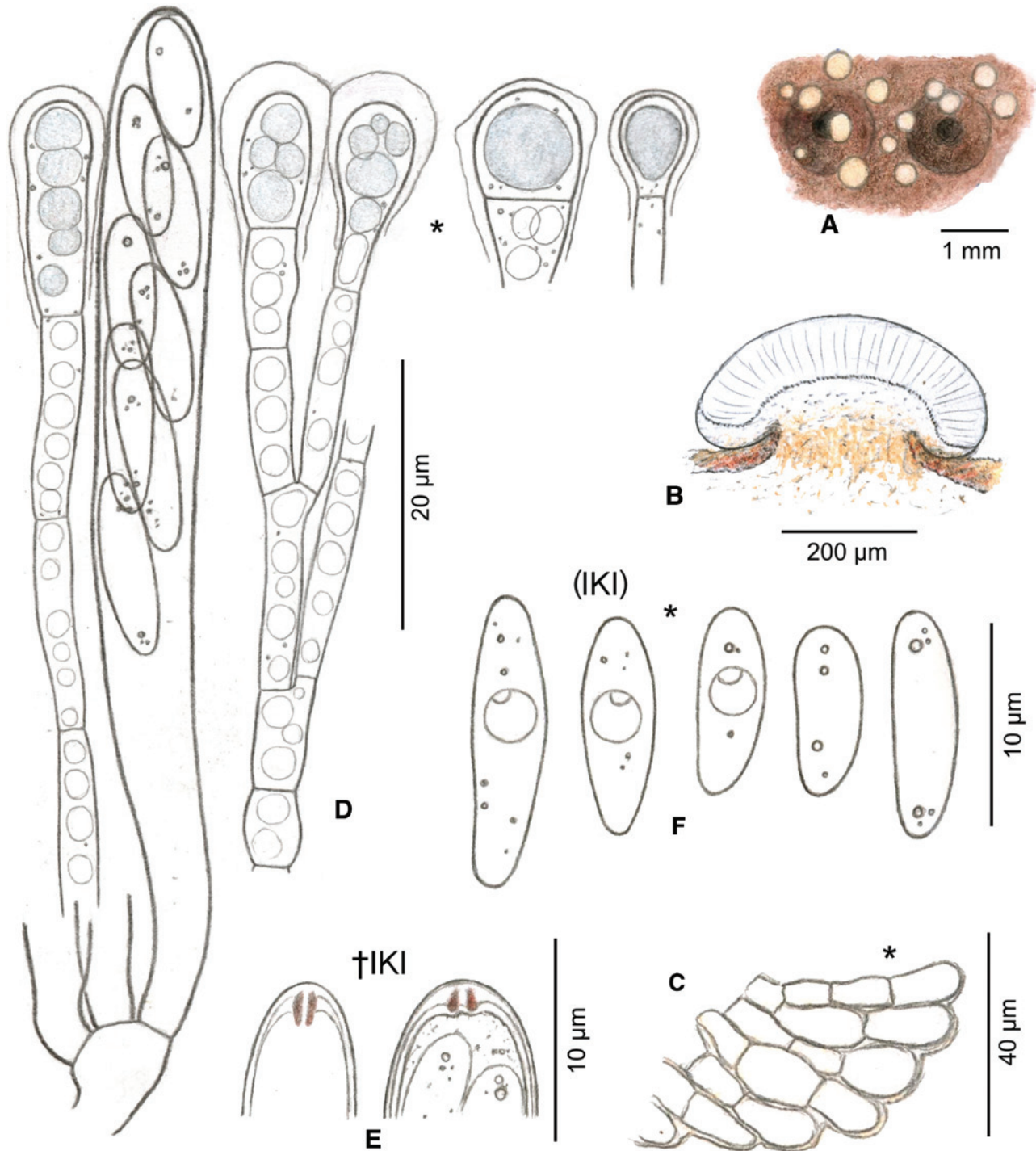
**Figure 3:** *Calycina marina* (H.B. 5792). (A) A swarm of apothecia on thallus of *Fucus serratus* (with conceptacles and their ostiole openings); (B) Median section of apothecium; (C) Median section of ectal excipulum near margin; (D) Ascus and paraphyses; (E) Ascus apices (immature and mature), with hemiamyloid (dirty red) apical rings; (F) Ascospores (three left with central nucleus and nucleolus visible when adding IKI). All elements in living state, except for (E).

vacuolar bodies that stain deep (blackish) red-brown in IKI and pale turquoise in CRB {1} (but not seen in other collections), (†) with many minute, light orange drops (in $H_2O$); lower cells *9–18×1.8–3(–4) µm {3}, with non-refractive vacuoles; branched at base, rarely at uppermost septum or even near apex. **Medullary excipulum** pale to light yellow-ochre, ~30–50 µm thick, of slightly gelatinized, more or less irregularly oriented, dense textura angularis-prismatica, indistinctly delimited from ectal excipulum, basal part deep orange-ochre, emerging from inner parts
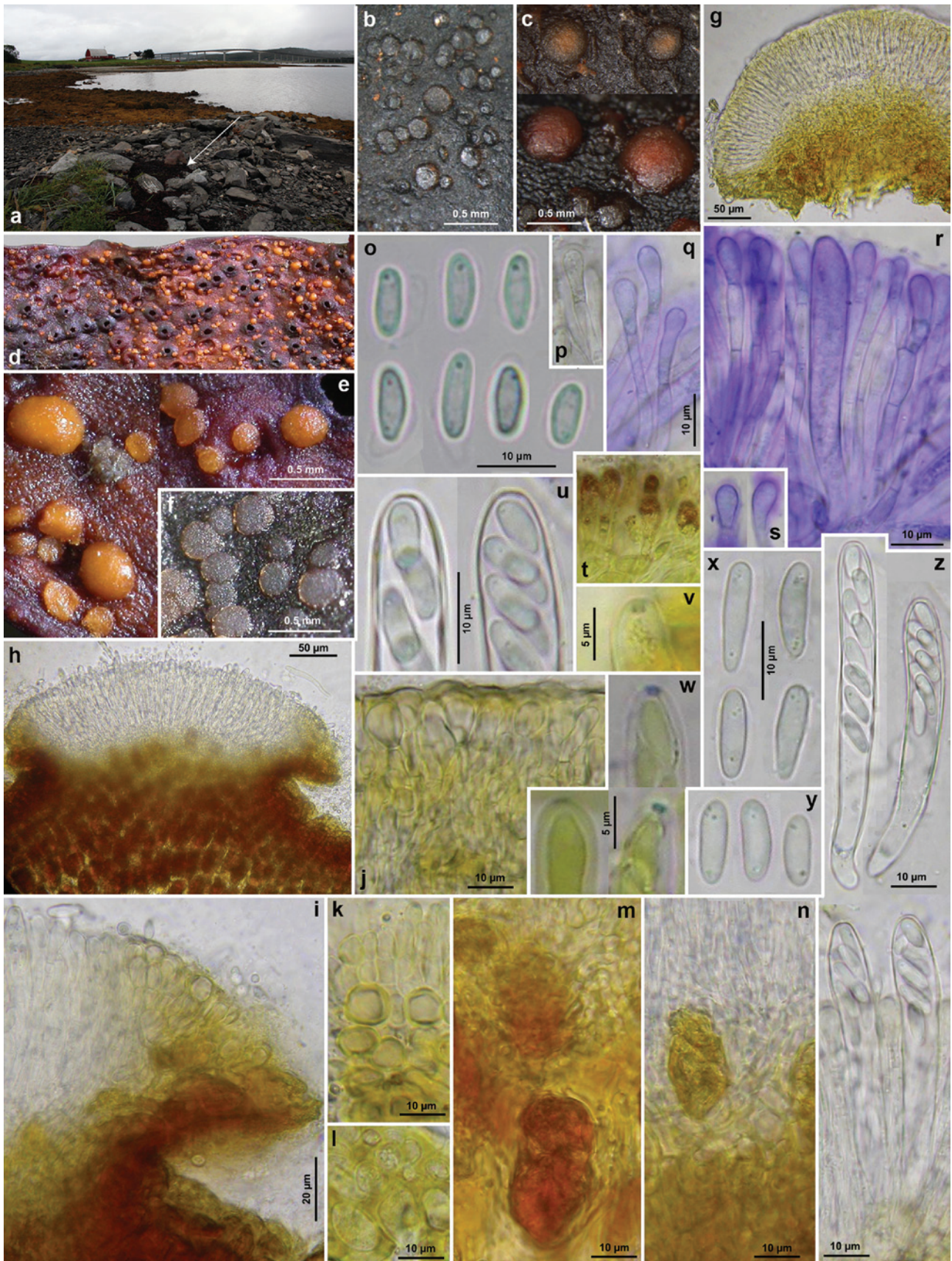
530 —— H.-O. Baral and T. Rämä: *Calycina marina* comb. nov.

DE GRUYTER

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**Figure 4:** *Calycina marina* (a) Habitat of T.R. 3166 on *Ascophyllum nodosum*, the exact locality is marked with an arrow. (b) Dry apothecia. (c–f) Rehydrated apothecia. (g–h) Median sections through apothecia in 5–10% KOH (g) and $H_2O$ (h). (i) Enlargement of (h), showing marginal excipulum in $H_2O$. (j–l) Marginal excipulum in internal view in squash mount in $H_2O$ (j) and in external view showing thick-walled (gelatinized) cells of ectal excipulum in KOH (k–l). (m) Median section of basal part of ectal excipulum, enclosing host tissue in $H_2O$. (n) Central part of medullary excipulum in $H_2O$. (o, x–y) Ascospores in $H_2O$. (p–t) Paraphyses in $H_2O$ (p), CRB (q–s) and IKI (t). (u) Apices of mature asci in $H_2O$. (v) Apex of immature ascus in IKI. (w) Apices of immature and mature asci in KOH+IKI. (z) Mature asci in $H_2O$. All microscopic photos (except g, k–l, v–w) in living state; d–e were taken in May 2015, 18 years after being collected. Specimens examined: a, f, h–j, m–n, t–v, x, z: T.R. 3166 (H.B. 9909); b, o: T.R. 3173; c: T.R. 3192 (above) and T.R. 3193, d–e, g, k–l: H.B. 5792; p–s, y: T.R. 3167 (H.B. 9910); w: UME 25785 (H.B. 9953).

of thallus by an upwards oriented, strongly gelatinized textura intricata-porrecta. **Ectal excipulum** light to bright yellow-ochre near base, of ± strongly gelatinized textura prismatica-globulosa oriented at a 30–45° angle to the surface from base to margin, ~20–50 µm thick at base and lower flanks, cells *8–17×3–11 µm {2}; 30–50 µm thick near margin, subhyaline to light yellow, slightly gelatinized, marginal cortical cells *7.5–13.5(–16)×4.5–8(–11) µm {2}; gel slightly to medium refractive, covering also cortical cells; cells containing a few minute lipid bodies; crystals absent. **Anchoring hyphae** in thallus rather abundant, hyaline to pale yellow, undulating, †2–3.5 µm wide, walls 0.2–0.5(–1) µm thick {1}. **Asexual state:** unknown. **Cultural characteristics:** Colonies on 0.2SeaMEA slow growing, 11 mm in diam. after 3 months of growth at 5°C in dark, margin filiform, white to creamish white; mycelium on agar surface and immersed, vegetative hyphae 2.5–4.0 µm wide; no conidiophores or conidia observed.

**Habitat:** At high tide mark and in the splash zone, on lower and upper face of stranded, decaying thalli of *Ascophyllum nodosum* {8}, *Fucus serratus* {1}, *F. vesiculosus* {3} lying on sea shore. **Assoc.:** sclerotia of a *Typhula* (hyaline to ochraceous, sterile, spherical bodies 0.25–0.4 mm diam.). **Desiccation tolerance:** after 3 days in herbarium many ± immature asci and lower cells of paraphyses still viable. **Phenology:** Year-round (see also Eriksson 1973, Kirk and Spooner 1984).

**Specimens examined: England, Hampshire,** Portsmouth, Eastney, entrance to Portsmouth Harbor, on decaying *Ascophyllum nodosum* above or at high tide mark, 23.I.2015, T. Rämä 3180A (TROM-F26103), 3181 (TROM-F26105), 3182 (TROM-F26106); **Scotland, Aberdeenshire,** Catterline Bay, Catterline pier, on decaying *A. nodosum* above high tide mark, 4.VI.2015, T. Rämä 3195 (TROM-F26122); **Norway, Sør-Trøndelag,** 9 km WNW of Trondheim, 1 km E of Flakk, on thallus of *Fucus serratus*, 3.V.1997, O. Eriksson (H.B. 5792); **Nordland,** Nesna, on decaying *A. nodosum*, VIII.1968, O.E. Eriksson 3850 (UME 25785, H.B. 9953); **Troms,** Håkøya, Haugåsstrand, on decaying *A. nodosum* in splash zone, 21.VI.2014, T. Rämä 3160B (TROM-F26101); at high tide mark, 5.V.2015 T. Rämä 3192 (TROM-F26112); T. Rämä 3193 (TROM-F26113); N shore near Vika, on decaying *F. vesiculosus* at high tide mark after spring tide, 19.VIII.2014, T. Rämä 3170 (H.B. 9908); T. Rämä 3171 (H.B. 9910); Kvaløya, Finnland, on decaying *A. nodosum* at high tide mark after spring tide, 16.VIII.2014, T. Rämä 3166 (H.B. 9909); T. Rämä 3167; same site, on decaying *A. nodosum* at high tide mark after spring tide, 8.IX.2014, T. Rämä 3172 (TROM-F26092), 3173 (TROM-F26098); Nordfjordbotn, on decaying *A. nodosum* in splash zone, 20.VI.2013, T. Rämä 3149A (TROM-F26093, TROM-F26094) and E.B.G Jones; Tromsø, Tromsøya, Lanes, on decaying *A. nodosum* in splash zone, 9.VI.2011, T. Rämä 3142 (TROM-F26095); Tromsø University Museum, on decaying *A. nodosum* in splash zone, 26.II.2015, T. Rämä 3190 (TROM-F26091).

# Discussion

## Morphology

The results of our morphological studies on *Calycina marina* agree largely with the original description of *Orbilia marina* and further amendments to it provided by Eriksson (1973) and Kirk and Spooner (1984). The original description by Phillips includes apothecia 0.25 mm diam., "scattered, erumpent, sessile, at first concave, then plane or convex, thin, transparent when moist, circular, varying from alutaceous to pale orange". The "elliptic-oblong" spores are given as rather short and wide (8–10×4 µm) compared to the present data, but the provided illustration shows variation in spore width, suggesting that 3 µm wide spores also occurred. Eriksson (1973) discovered the species in Scandinavia, confirmed Boyd's description and amended it by apothecia up to 0.4 mm diam. (dry), growing in swarms, their basal parts composed of scleroplectenchymatous hyphae, branched paraphyses with swollen heads imbedded in gelatinous sheaths, asci with an apical ring though not clearly positive in iodine, and ascospores surrounded by a thin gelatinous sheath.

In the lack of American records, Kohlmeyer and Kohlmeyer (1979) examined authentic material in Herb. K.

The authors mainly repeated Eriksson's description and added a spore size of 7.5–10×4–5 µm and, like Eriksson, they thought that the ascus apical rings were inamyloid. This negative result is probably a methodological one: they used Melzer's reagent without KOH-pretreatment, which suppresses hemiamyloid reactions (see Baral 1987, 2009).

Spooner (in Kirk and Spooner 1984) described the apical ring as "rather shallow, outlined blue in Melzer's reagent", and the spores as 9–11(–13)×2.5–3.5 µm, "narrowly ellipsoid, often slightly clavate, inequilateral, with 2 small polar guttules, occasionally developing a median septum". The ectal excipulum is said to be formed of subhyaline to very pale brown subangular cells at the base (4–7×2–3.5 µm), with slightly thickened, refractive walls, 10–12 µm thick at the flanks, here of parallel, 2 µm wide hyphae, at the margin with broader, clavate cells of 8–12×4–7 µm.

In our study, we observed even larger apothecia, up to 0.5–0.65 mm in diameter when moist, and a clear iodine reaction in the ascus apex in contrast to Eriksson (1973) and Kohlmeyer and Kohlmeyer (1979). We find a spore shape that agrees well with Eriksson (1973) and Kirk and Spooner (1984), being straight or slightly inequilateral, partly fusoid and with obtuse ends. A median septum, as reported by Kirk and Spooner (1984), was occasionally seen by us in overmature spores, but never inside living asci. According to Eriksson's (1973) and our observation, a gelatinous sheath surrounds the ascospores (Figure 4). However, this sheath was not seen in every collection studied by us, and might be more difficult to demonstrate in herbarium material.

A rather large variation in ascus and spore dimensions was noted among our collections. Ascus length and width did not even overlap when comparing measurements from some of the living specimens. Extraordinarily wide spores of 8–10×4–5.5 µm were seen in some apothecia of T.R. 3170 (H.B. 9908), while other apothecia of the same sample showed the typical narrow spores of (7–)8–11(–12)×3.2–4.2(–4.5) µm. Another collection (T.R. 3166, H.B. 9909) contained rather long spores of (8–)10–13(–15)×3.3–4.3 µm. Two further measurements underline this variation: (7–)9–13(–14)×(2.8–)3.2–3.8 µm in the sample from which the drawing is derived (H.B. 5792), and 9–12×3.5–4 µm in T.R. 3171 (H.B. 9910). A rather large variation was also noted in paraphysis width, both at the apex and in its lower parts.

## Generic placement

*Laetinaevia marina* is here transferred to *Calycina* mainly based on the molecular data. Macroscopically it shows, however, little similarity with members of that genus as it deviates in erumpent, sessile, cushion-like, alutaceous to light ochre-orange pigmented apothecia, though the colour varies considerably among populations and includes also whitish-cream when fresh. Due to their translucence in the hydrated state, the apothecia may take on the dark color of the host tissue beneath. In ascus characters, a certain morphological similarity exists with *Calycina*, particularly the apical ring, though this structure is more reminiscent of the *Conchatium*-type. However, paraphyses with short-celled, inflated terminal cells with gel caps are unknown in *Calycina*, and short, hair-like cells on the ectal excipulum, typical of that genus, were not observed here. Drought tolerance is a further feature that was not observed in species of *Calycina*, and obviously originates from the harsh environment at the seashore.

Spooner (in Kirk and Spooner 1984) removed this discomycete from Orbiliaceae because of the excipular structure, erumpent apothecia, amyloid apical rings, and eventually septate spores. However, Spooner was unaware that some of these features do indeed occur in that family (excipulum of elongate prismatic cells, erumpent apothecia, septate spores). Instead, he saw a relationship with the Naevioideae, at that time a subfamily of Dermateaceae, and tentatively transferred the species to *Laetinaevia*, mainly because of the clavate marginal cells. Spooner also compared the genera *Calloria* Fr., in which the species was originally placed by W. Phillips, and *Gloeopeziza* Zukal.

Our results do not corroborate a placement in *Laetinaevia*. The ascus apex resembles more that of *Cyathicula cyathoidea* (Bull.) Thüm. (*Conchatium*-type, Figures 3E, 4v; Triebel and Baral 1996, Figure 5), whereas typical *Laetinaevia* species have a "T-shaped" ring of the *Pezicula*-type. The apical cell of each paraphysis contains a few large, globose, slightly refractive vacuolar bodies (Figure 3D) which stain pale turquoise in CRB and deep blackish red-brown in IKI, features so far not seen in personally studied *Laetinaevia* species but typical of *Calycina* species. However, these vacuolar bodies could not be seen in *Ascophyllum* samples when mounted in water or CRB (Figures 4p–s), although a distinct red-brown reaction of the vacuoles in IKI was noted (Figure 4t). Concerning the earlier placement in *Orbilia*, nothing conforms with a relationship with the Orbiliomycetes, apart from the ascus wall surface that stains lilac in CRB (Figure 4r). The amyloid ring is the main argument against such placement, and also a spore body, typical of *Orbilia*, is absent from the living spores.

Sequences are largely lacking for taxa placed in the Naevioideae. However, our phylogenetic analyses demonstrate *C. marina* as being related, yet distant, to the only three fungi of the Naevioideae as defined by Nannfeldt

(1932) and Hein (1976) for which ITS sequences were available, and for which the family name Calloriaceae could be applied: *Calloria urticae*, *Laetinaevia carneoflavida*, and *Naevala perexigua*. It was a surprise for us that *C. marina* turned out to be phylogenetically closely associated with species of *Calycina* and their *Chalara* asexual states, instead of *Corticifraga* which is morphologically more similar. The phylogenetic analysis based on the ITS barcoding region (Schoch et al. 2012) placed it close to the type of *Calycina*, *C. herbarum*. However, we could not resolve the relationships between the studied *Calycina* species and the *Chalara* anamorphs present in GenBank due to some unsupported nodes.

Based on our results, the name *Calycina populina* should be used instead of *Calycellina populina* (Fuckel) Höhn., since the type species *Calycellina punctata* (Fr.) Lowen *et* Dumont is placed in a separate clade with *Mollisina uncinata* Arendh. *et* R. Sharma and *Phialina lachnobrachyoides* (Raitv.) Huhtinen. There are many *Calycina* species on herbaceous, foliose or woody substrates that remain to be sequenced, and hence, can change our view on the systematics of this genus.

In the anticipation of future molecular work based on freshly collected and documented material, we place this marine discomycete in *Calycina*, to which it is molecularly closely affiliated.

## Ecology

*Calycina marina* is the only species now recognized in *Calycina* that grows on seaweed. It can be found on brown seaweed but has clearly been overlooked, due to its unexpected habitat and the paucity of mycologists recognising it. This fungus can be detected on dead, decaying algal material, especially *Ascophyllum nodosum* thalli, found on the shore at high tide mark or above. The algal material that supports the fruiting of *C. marina* is in the early to middle stages of the decay process. It seems that the fungus fruits rather quickly after the algae are washed ashore which indicates a colonization from marine sources, suggesting that the fungus is already present as an endophyte in or on the algae when the material is washed ashore.

There are presently 41 records listed for Scotland and England in the checklist of fungi of the British Isles (http://www.fieldmycology.net/FRDBI/FRDBI.asp). The fungus is easily found in Tromsø in Northern Norway, and is presumably common in the temperate and arctic waters along the whole Western European coast up to the White Sea, where it is known to be common (Tchesunov

et al. 2008). Twelve specimens from Scandinavia are preserved in UME and UPS, including three from brackish water of the Baltic Sea (Skåne and Gotland) with salinity of 7–7.5 (http://tangbloggen.com/2012/01/24/ostersjon/). It is unknown whether the fungus can exist and produce fruiting bodies on *Fucus vesiculosus* and the endemic *F. radicans* in the eastern and northern parts of the Baltic Sea with extremely low salinity (3–7).

Boyd found the fungus fruiting in April–May and August–September in Scotland (Smith 1909), and Eriksson (1973) in April, June and July in Scandinavia. We recorded fruiting bodies also in January, February, May and November, and there are specimens in K collected in December, and observations in the British database from October. Consequently, March is the only month in which fruiting bodies have yet to be collected. Based on this, we can confirm Boyd's assumption (in Smith 1909) of the year-round fruiting in suitable habitats.

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# Bionotes

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