

Standard Paper

The crustose species of *Normandina* (Verrucariaceae)

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Abstract

Normandina acroglypta, as recently circumscribed, is shown to comprise two species: *N. acroglypta* s. str. with subsquamulose marginal areoles, two unidentified terpenoids by thin-layer chromatography, and growing over rock; and *N. chlorococca*, with convex marginal areoles, zeorin or no substances detected by thin-layer chromatography, and growing over bark. *Normandina dictyospora* is newly described from the Falkland Islands and is characterized by relatively large, submuriform ascospores, and no substances detected.

Key words: Falkland Islands, terpenoids

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Introduction

Normandina is a small genus of three currently accepted species: *N. acroglypta* (Norman) Aptroot, *N. pulchella* (Borrer) Nyl. and *N. simodensis* (Asahina) Aptroot (Aptroot 1991). Aptroot suggested that *Lauderlindsaya borneri* (Tul.) J. C. David & D. Hawksw. represented the perithecia of *N. pulchella*, and not a lichenicolous fungus, and confirmed the presence of zeorin for the first time in the family Verrucariaceae, in *N. pulchella* and *N. simodensis*. Muggia *et al.* (2010), in a multilocus analysis, confirmed that the genus was monophyletic and that *Lauderlindsaya* was a synonym. Molecular variation within *N. pulchella* showed no clear relation with geography, in samples originating from Europe and North America; *N. acroglypta* was nested within *N. pulchella*, and differed from *N. pulchella* only in a few positions of the mitochondrial marker. Frisch & Ohmura (2015) showed that *N. simodensis* was basal to *N. acroglypta* and *N. pulchella* in a multilocus analysis. In the sequences used, *N. acroglypta* and *N. pulchella* were recovered as separate clades within the genus but the authors reported conflicts between loci in some samples of these two species, which were excluded from the analysis. Lücking & Moncada (2017) published an mtSSU tree with *Normandina acroglypta*, *N. pulchella* and *N. simodensis*; in addition, three undescribed species morphologically similar to *N. pulchella* were recovered in material from North America and Colombia. A clade comprising a single sequence of *N. simodensis* and a single sequence of *Normandina* sp. from Norway was basal to the other species.

An unidentified sterile lichen producing subsquamulose areoles on dead mosses was collected several times in a survey of montane rocks by the author in 2018 on Helvellyn, Northern England. Molecular analysis suggested a position in Verrucariaceae, but the sample was unusual for that family in containing two terpenoids. Attempts to identify the material led to a reappraisal of crustose

species of *Normandina*, with the resurrection of *N. chlorococca* from synonymy with *N. acroglypta*. In addition, a collection from the Falkland Islands is described below as the new species *N. dictyospora*.

Materials and Methods

DNA was extracted from recently collected specimens, using the Qiagen DNeasy Plant Mini Kit; the manufacturer's instructions were followed except that water at 65 °C was used for the final elution. PCR amplification was carried out using Bioneer AccuPower PCR Premix 50 µl reaction tubes. The two internal transcribed spacer regions and the 5.8S region (ITS1-5.8S-ITS2) of the nuclear ribosomal gene, and part of the small subunit of the mitochondrial ribosomal DNA (mtSSU), were amplified using the primers ITS1F and ITS4, and mrSSU1 and mrSSU3R. PCR products were cleaned enzymatically using PureIT ExoZAP PCR CleanUp (Ampliqon). The PCR thermal cycling parameters followed Orange *et al.* (2020) for both gene regions. Sequencing was performed by The Sequencing Service, College of Life Sciences, University of Dundee (www.dnaseq.co.uk).

Sequences were assembled in BioEdit 7.0 (Hall 1999) and aligned using PRANK (Löytynoja & Goldman 2010) (<http://wasabiapp.org/software/prank/>) with the online interface at <https://www.ebi.ac.uk/goldman-srv/webprank/>. Gaps in the alignment were coded using FastGap (Borchsenius 2009).

Phylogenetic relationships and support values were investigated using maximum likelihood (ML) bootstrapping, as implemented in RAxML (Stamatakis 2006; Stamatakis *et al.* 2008), hosted on the CIPRES Science Gateway (Miller *et al.* 2010). Analyses with RAxML used rapid bootstrapping with 1000 iterations and the GTRGAMMA substitution model; a search for the best-scoring ML tree was carried out with the bootstrap analysis in a single run. The resulting tree was visualized using MEGA v. 4 (Tamura *et al.* 2007). Support values of ≥ 70% ML bootstrapping were regarded as significant. ITS and mtSSU sequences were analyzed separately and then concatenated for a further analysis. Sequences used in the analyses are shown in Table 1.

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Table 1. Sequences of *Normandina* used in analyses, with additional voucher information. Newly produced sequences are shown in bold.

Taxon	Region	Voucher	ITS	mtSSU
<i>Hydropunctaria maura</i>	-	-	JN638266	JN638296
<i>Normandina acroglypta</i>	Norway	BG L- 84863	-	GU121609
<i>N. acroglypta</i>	Norway	BG L-78601	-	GU121597
<i>N. acroglypta</i>	Norway	BG L-85187	-	GU121596
<i>N. acroglypta</i>	Austria	<i>Hafellner</i> 69200 (GZU)	-	GU121595
<i>N. acroglypta</i>	Scotland	<i>Orange</i> 21018 (NMW)	OM944030	OP151110
<i>N. acroglypta</i>	England	<i>Orange</i> 24085 (hb. A. Orange)	OM472106	OM453906
<i>N. acroglypta</i>	England	<i>Orange</i> 24113 (hb. A. Orange)	OM472107	-
<i>N. 'americana'</i>	Colombia	<i>Moncada & Lücking</i> 7745	MF070068	-
<i>N. 'americana'</i>	Colombia	<i>Moncada</i> 10556a	MF070069	-
<i>N. 'americana'</i>	Colombia	<i>Moncada</i> 10556b	MF070070	-
<i>N. 'americana'</i>	Colombia	<i>Moncada</i> 10570	MF070071	MF070074
<i>N. 'americana'</i>	Alaska	<i>Pérez-Ortega & Spribille</i> (BG)	-	GU121607
<i>N. 'americana'</i>	Alaska	<i>Pérez-Ortega & Spribille</i> (BG)	-	GU121608
<i>N. 'americana'</i>	California	TSB 38861	-	GU121598
<i>N. 'americana'</i>	Oregon	TSB 38874	-	GU121600
<i>N. 'americana'</i>	Oregon	TSB 38875	-	GU121601
<i>N. 'americana'</i>	California	TSB38860	-	GU121602
<i>N. chlorococca</i>	Norway	BG L-86463	-	GU121613
<i>N. chlorococca</i>	Ireland	<i>Douglass</i> s. n. (hb. J. R. Douglass)	ON944031	OP151111
<i>N. chlorococca</i>	England	<i>Orange</i> 24393 (hb. A. Orange)	OM472108	OP151109
<i>N. 'colombiensis'</i>	Colombia	<i>Moncada</i> 10521	-	MF070080
<i>N. 'colombiensis'</i>	Colombia	<i>Moncada</i> 10555	MF070067	MF070079
<i>N. 'colombiensis'</i>	Colombia	<i>Moncada</i> 10740a	-	MF070081
<i>N. 'colombiensis'</i>	Colombia	<i>Moncada</i> 10740b	-	MF070082
<i>N. 'aff. colombiensis'</i>	Colombia	<i>Moncada</i> 10567	-	MF070083
<i>N. dictyospora</i>	Falkland Islands	<i>Orange</i> 20131 (NMW)	OM472102	OM453905
<i>N. pulchella</i>	Japan	<i>Frisch</i> 12/Jp145 (TNS)	-	KF972460
<i>N. pulchella</i>	Colombia	<i>Moncada</i> 10497	-	MF070073
<i>N. pulchella</i>	Colombia	<i>Moncada</i> 10624a	-	MF070075
<i>N. pulchella</i>	Japan	<i>Ohmura</i> 7853 (TNS)	-	KF972459
<i>N. pulchella</i>	Norway	O-L-204993	MK812131	-
<i>N. pulchella</i>	Wales	<i>Orange</i> 22206 (NMW)	OM472103	OP151112
<i>N. pulchella</i>	Wales	<i>Orange</i> 23620 (NMW)	OM472105	OP151114
<i>N. pulchella</i>	Wales	<i>Orange</i> 25943 (hb. A. Orange)	OM472109	-
<i>N. pulchella</i>	USA	<i>Smith</i> CSW 01b	MF070066	-
<i>N. pulchella</i>	Hawaii	<i>Smith</i> CSW 01b1	-	MF070077
<i>N. pulchella</i>	Hawaii	<i>Smith</i> CSW 01b2	-	MF070078
<i>N. pulchella</i>	Slovenia	TSB 37499	-	GU121599
<i>N. pulchella</i>	Italy	TSB 38857	-	GU121610
<i>N. pulchella</i>	Italy	TSB 38858	-	GU121611
<i>N. pulchella</i>	Austria	TSB 38862	-	GU121603
<i>N. pulchella</i>	Austria	TSB 38863	-	GU121604
<i>N. pulchella</i>	Austria	TSB 38864	-	GU121605

(Continued)

Table 1. (Continued)

Taxon	Region	Voucher	ITS	mtSSU
<i>N. pulchella</i>	Austria	TSB 38865	-	GU121612
<i>N. pulchella</i>	Austria	TSB 38866	-	GU121606
<i>N. simodensis</i>	Japan	Ohmura 8475 (TNS)	-	KF972461
<i>Normandina</i> sp.	Brazil	Lücking 37506b	MF070065	-
<i>Normandina</i> sp.	Falkland Islands	Orange 22619 (NMW)	OM472104	OP151113
<i>Verrucula inconnexaria</i>	-	-	-	FJ225717

Thin-layer chromatography (TLC) was carried out using solvent system G (Orange *et al.* 2010). *Cladonia coccifera* s. lat. was used as a control for zeorin.

Spore sizes are cited as: (minimum–) mean minus one standard deviation – mean – mean plus one standard deviation (–maximum). The number of spores measured, and the number of specimens in which spores were measured, are shown in square brackets.

Results

Eighteen new sequences were obtained (Table 1).

A BLAST search of the mtSSU of the unidentified material from Helvellyn returned *Normandina* species and other *Verrucariaceae*, whereas the ITS sequence returned *Verrucaria* species. The morphology and the presence of terpenoids suggested a placement in *Normandina* and the sequences aligned well with other members of the genus.

Few accessions of *Normandina* are represented by both ITS and mtSSU sequences. However, the newly generated ITS and mtSSU trees recovered similar clades and showed no conflicts in topology (data not shown). The tree derived from the concatenated ITS and mtSSU sequences is shown in Fig. 1. There are two major clades: the first comprises *Normandina pulchella* s. lat., including the undescribed taxa *N. 'americana'* and *N. 'colombiensis'* proposed by Lücking & Moncada (2017); the second includes *N. acroglypta* s. lat. and a single collection of *N. simodensis*. Seven collections, from Great Britain, Norway and Austria, are recovered in a well-supported subclade considered here to represent *N. acroglypta* s. str. This is sister to a subclade comprising three collections from Great Britain, Ireland and Norway, considered to represent *N. chlorococca*, previously regarded as a synonym of *N. acroglypta*, the single collection of *N. simodensis*, and a collection from the Falkland Islands. The Falklands collection is basal to *N. chlorococca*; it is morphologically distinctive and is described below as the new species *N. dictyospora*. *Normandina simodensis* is basal to the clade comprising *N. chlorococca* and *N. dictyospora*.

Thin-layer chromatography of selected specimens is shown in Fig. 2. Newly sequenced material showed zeorin, or no substances, in two sequenced specimens of *Normandina chlorococca* respectively, and two terpenoids (neither zeorin) in three sequenced specimens of *N. acroglypta*. An additional ten specimens of unsequenced herbarium material contained either zeorin (in small amounts), no substances, or two terpenoids. Specimens with two terpenoids were associated with rock and the others with bark. Specimens containing two terpenoids and growing over rock were identified as *N. acroglypta* s. lat., by comparison with sequenced and type material, and specimens containing zeorin or with no substances and growing over bark were identified as *N. chlorococca* (see below).

The upper clade recovered within *Normandina acroglypta* s. lat. (Fig. 1) is referred to *N. chlorococca*, since two specimens which cluster here (Orange 24393 and Douglass s. n.) lack any trace of subsquamulose areoles, and are shown by TLC to contain a trace of zeorin or no substances, respectively. This is in accordance with the lectotype of *N. chlorococca*, which lacks subsquamulose areoles and contains no substances detectable by TLC. The lower clade is referred to *N. acroglypta* s. str., since three specimens that cluster here (Orange 21018, 24085 and 24113) have subsquamulose areoles and possess two terpenoids detected by TLC; this is in accordance with the morphology and chemistry of the syntype of *N. acroglypta* in O.

The Species

Normandina acroglypta (Norman) Aptroot

In Wirth, *Flechtenflora, Bestimmung und ökologische Kennzeichnung der Flechten Südwestdeutschlands und angrenzender Gebiete* (ed 2) (Stuttgart, Eugen Ulmer), 634 (1995).—*Thelidium acroglyptum* Norman, *Botaniska Notiser* 1, 154 (1867); type: Norway: Nordland, 'Ad Indyr parochia Gilderskaal Nordlandiae', 'Hab. supra muscos in rupibus Nordlandiae meridionalis', J. M. Norman (H-NYL 3654b!, O-L-1000! [thin-layer chromatography: 2 terpenoids], UPS—syntypes).

(Fig. 3C & D)

Thallus crustose, forming an uneven grey-green crust, but very young areoles have a subsquamulose appearance, being somewhat lobed at the margin, and either plane or slightly convex. The subsquamulose appearance is often ill-defined and was best seen in small sterile specimens from a montane site. The surface of the thallus locally gives rise to ill-defined soralia, comprising soredia 60–250 µm diam. In the few confirmed specimens seen, ascospores were hyaline, (23.5–)27.5–32.2–37(–43) × (5–)6–6.7–7.5(–8.5) µm, (3–)5–7-septate [22/3].

Chemistry. Two unidentified terpenoids, brown soon after heating then dull purple, UV+ orange-pink after heating (Fig. 2). Thallus K–, C–, PD–.

Ecology. On dead cushions of bryophytes, rarely lichens, over rock. The few confirmed specimens with habitat details are from lake shores, montane flushed faces, and damp shady stonework.

Notes. The syntype of *Thelidium acroglyptum* in O contained two terpenoids, and the thallus showed traces of subsquamulose areoles. The species differs from *N. chlorococca* and all other *Verrucariaceae*, as far as is known, by the presence of two



Fig. 1. Phylogenetic relationships of *Normandina* based on a maximum likelihood (ML) analysis of the ITS and mtSSU regions. The tree was rooted using *Hydropunctaria maura* and *Verrucula inconnexaria*. Significant ML bootstrap values are shown adjacent to each branch. Branches in bold indicate a support of ML bootstrap $\geq 70\%$. The names in inverted commas are provisional names used by Lücking & Moncada (2017).

terpenoids, neither of them zeorin. *Normandina chlorococca* differs by containing zeorin, or by lacking secondary substances; the young areoles lack a subsquamulose appearance and the species occurs over bark.

Additional specimens examined (all containing two terpenoids detected by TLC). **Great Britain:** *England:* **V.C. 69**, Westmorland, Helvellyn, Water Crag, NY33965.15415, 2018, *Orange* 24085 (hb. A. Orange); Helvellyn, Red Tarn, NY34784.15036, alt. 765 m, 2018, *Orange* 24113 (hb. A. Orange); *ibid.*, NY34600.15030, alt. 790 m, 2018, *Orange* 24122 (hb. A. Orange); Helvellyn, Brown Cove, crags on west side, NY33920.15563, alt. 765 m, 2018, *Orange* 24151 (hb. A. Orange).

Wales: **V.C. 42**, Breconshire, near Rhayader, below Caban-coch Reservoir, 22/925.644, 2006, *Orange* 16709 (NMW C.2005.001.604); **V.C. 49**, Caernarvonshire, N side of Snowdon, Llyn Glas, 23/619.558, 1997, *Gilbert & Giavarini* s. n. (NMW C97.28.4). *Scotland:* **V.C. 108**, West Sutherland, Loch Stack, 29/2868.4211, 2012, *Orange* 21018 (NMW C.2012.002.38).

Normandina chlorococca (Leighton) Orange comb. nov.

Mycobank No.: MB 846186

Verrucaria chlorococca Leighton, *The Lichen Flora of Great Britain and Ireland* (ed. 3), 484 (1879); type: Great Britain, England,

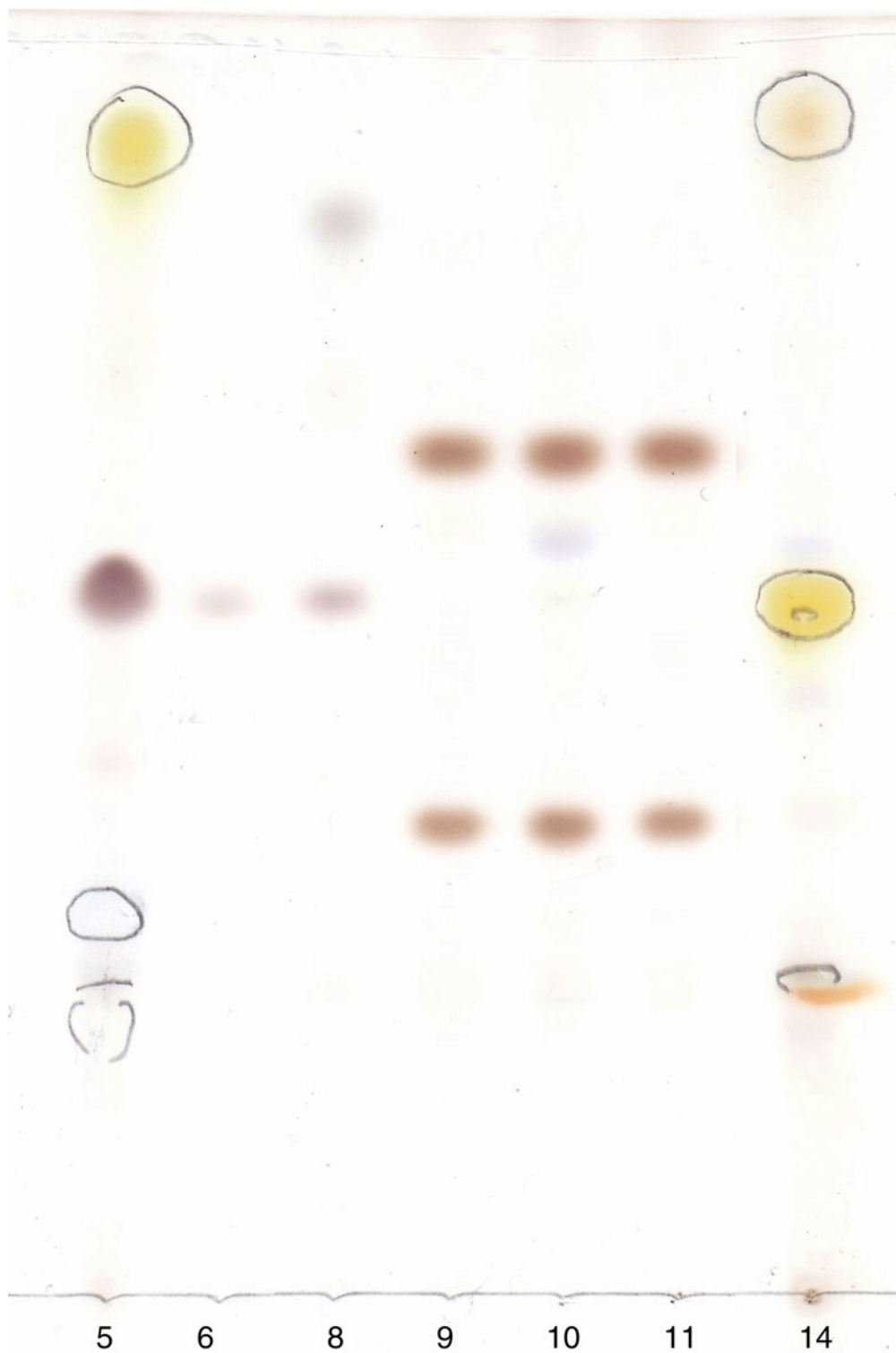


Fig. 2. Thin-layer chromatography plate in solvent G (some lanes omitted from figure); compounds are listed from bottom to top in each lane. Lane 5, *Cladonia coccifera* s. lat. (porphyrilic acid, unknown, zeorin, usnic acid). Lane 6, *Normandina chlorococca* (Orange 8866, zeorin). Lane 8, *Flakea papillata* (Orange 19425, zeorin, unknown terpenoid?). Lane 9, *N. acroglypta* (Gilbert & Giavarini NMW C.97.28.4, two terpenoids). Lane 10, *N. acroglypta* (Orange 16709, two terpenoids). Lane 11, *N. acroglypta* (Orange 21018, two terpenoids). Lane 14, controls (connorstictic acid, fumarprotocetraric acid, norstictic acid, atranorin). In colour online.

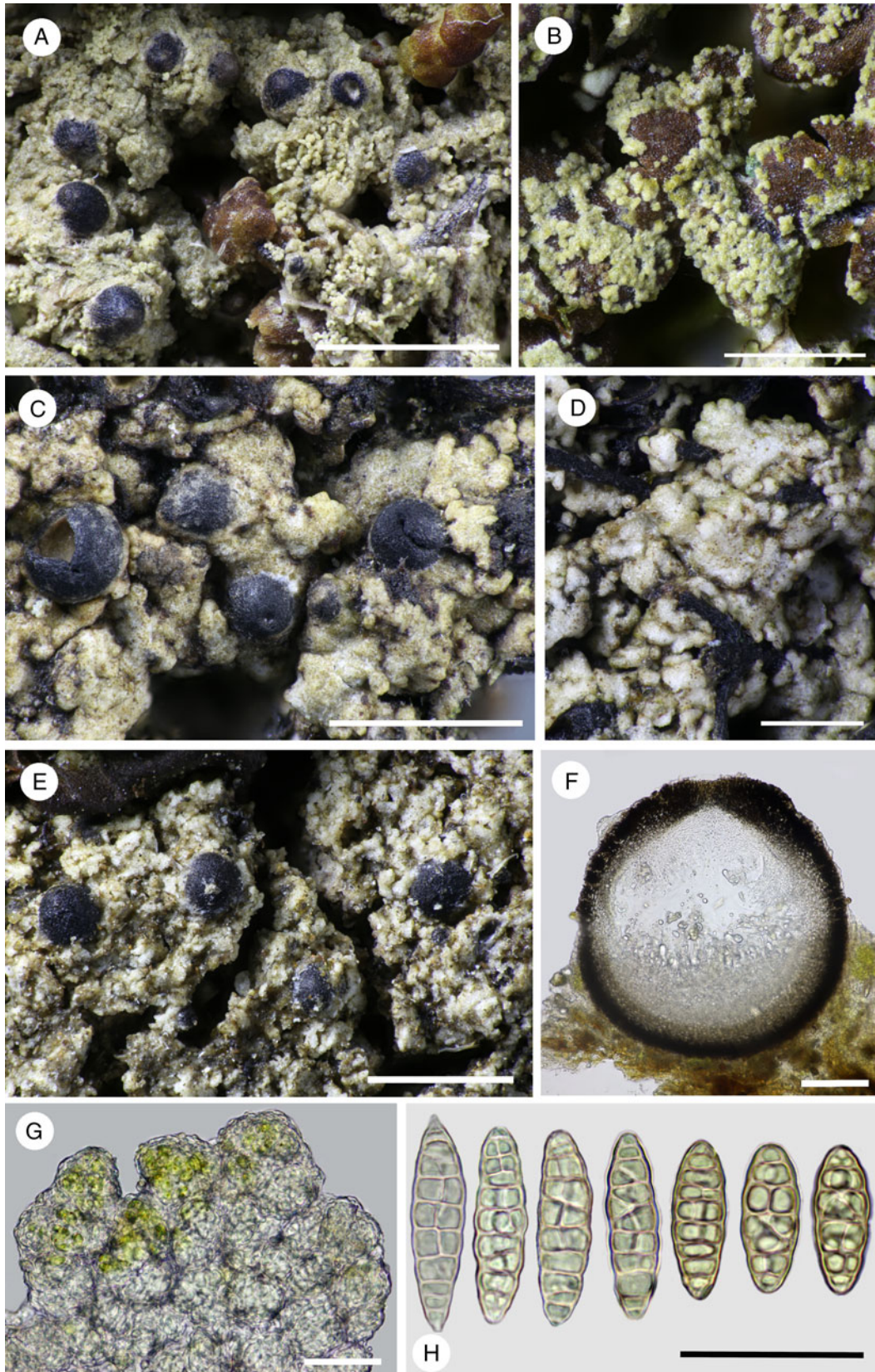


Fig. 3. Crustose species of *Normandina*. A, *N. chlorococca* (Giavarini, NMW C.2002.018.5), thallus with perithecia and soralia. B, *N. chlorococca* (Orange 24393), young areoles. C, *N. acroglypta* (Orange 21018). D, *N. acroglypta* (Orange 24151), marginal areoles. E, *N. dictyospora* (holotype), thallus with perithecia. F, *N. dictyospora* (holotype), section of perithecium. G, *N. dictyospora* (holotype), thallus tissue, lightly squashed to show goniocyst-like units. H, *N. dictyospora* (holotype), ascospores. Scales: A, C & E = 1 mm; B & D = 500 μ m; F & G = 100 μ m; H = 50 μ m. In colour online.

Oxfordshire, 'On ash near Stokenchurch, highest point of Chiltern Hills', February 1876, *C. Lorbalestier* (BM 001107462—lectotype! [thin-layer chromatography: no substances]).

(Fig. 3A & B)

Thallus crustose, forming an uneven grey-green crust, but very young areoles have the form of convex granules without any sub-squamulose appearance, and rapidly become irregular in shape and coalesce with other areoles. Ill-defined soralia often occur locally on the thallus. In the few confirmed specimens seen, ascospores were hyaline, (22.5–)27.5–31.7–36(–39.5) × (6–)6.5–7.4–8(–9) µm, 5–8-septate [34/4].

Chemistry. Zeorin in trace amounts, or no substances detected by TLC.

Ecology. On dead cushions of bryophytes, over bark of *Acer pseudoplatanus*, *Corylus avellana*, *Fraxinus excelsior*, *Populus tremula*, *Quercus*, *Salix* and *Ulmus*; evidently preferring base-rich bark.

Additional specimens examined (with TLC data). **Great Britain:** **England:** V.C. 5, South Somerset, S of Withypool, River Barle, SS8589.3381, 2019, *Orange* 24393 (hb. A. Orange) [trace of zeorin]; V.C. 23, Oxfordshire, on ash near Stokenchurch, [c. SU7696], February 1879, *C. Lorbalestier* (BM 001107462) [no substances]. **Wales:** V.C. 48, Merioneth, Nannau Estate, E side of Llyn Cynwch, 23/7385.3272, 2002, *Giavarini* s. n. (NMW C.2002.018.5); Llanbedr, Pentre Gwynfryn, 23/593.270, 2002, *Giavarini* s. n. (NMW C. 2002.018.6) [no substances]; V.C. 49, Caernarvonshire, Porthmadog, Prenteg, 23/5772.4088, 2002, *Orange* 14301 (NMW C.2005.001.693). **Scotland:** V.C. 73, Kirkcudbrightshire, Wood of Cree, NX38101.70894, 2018, *Douglass* s. n. (hb. J. R. Douglass) [no substances].—**Ireland:** V.C. H38, Down, Murlough National Nature Reserve, IJ4113.3512, 2020, *Douglass* s. n. (hb. J. R. Douglass) [no substances]; V.C. H39, Antrim, Glenshesk, Breen Oakwood, ID12172.33962, 2018, *Douglass* 2018-1235 (hb. J. R. Douglass) [trace of zeorin].—**Norway:** *Sogn og Fjordane:* Aurland, S side of Vassbygdvatnet, 60°51'N, 7°17'E, 1991, *Orange* 8866 (NMW C.95.19.22) [trace of zeorin].

Normandina dictyospora Orange sp. nov.

Mycobank No.: MB 846252

A crustose species of *Normandina* distinguished from other members of the genus by the submuriform ascospores.

Type: Falkland Islands, West Falkland, Port Howard, 51.6288°S, 59.53992°W, dead moss on top of low, mortared wall, 19 January 2011, *Orange* 20131 (NMW C.2011.015.227—holotype!).

(Fig. 3E–H)

Thallus crustose, grey-green, becoming whitish in the herbarium; areoles initially convex but rapidly becoming irregular in shape and coalescing into a coarsely and irregularly warty crust; vegetative propagules absent. Thallus in section without a true cortex, composed of goniocyst-like units 25–45 µm diam.

Perithecia 415–540 µm diam., 0.5–0.75-immersed in thallus, exposed part black, smooth. *Involucrellum* absent. *Exciple* 60–75 µm thick at sides and base, opaque in outer half, hyaline within, outer part dark reddish brown, K+ dark (greenish) grey. *Periphyses* 75 µm long. *Mature asci* not seen. *Ascospores*

narrowly ellipsoid, (34–)36.5–41.7–46.5(–50) × (12–)13–13.8–14.5 µm [19/1], hyaline or pale brown, submuriform with 3–7(–9) transverse septa, usually 0–1(–2) longitudinal septa per tier, with a total of 11–17(–20) cells visible in optical section.

Conidiomata not seen.


Chemistry. No substances detected by TLC.

Ecology. One collection from dead moss on top of a low wall adjacent to turf.

Notes. The new species differs from the other crustose species of *Normandina*, *N. acroglypta* and *N. chlorococca*, in the larger, submuriform ascospores. Although all the ascospores seen were outside asci, the submuriform condition is interpreted as normal and not due to the spores being overmature. No longitudinal septa have been observed in any collections of *N. acroglypta* or *N. chlorococca*. Although only one collection is known, the species is well characterized by morphology and is supported by molecular data, so is described here as new. Calcareous substrata are rare in the Falkland Islands, represented almost exclusively by bones and by built structures, so this is likely to be a rare species. This is the first species in the genus to have submuriform rather than septate ascospores.

Secondary lichen compounds are very rare in *Verrucariaceae*. Zeorin was reported for *Verrucariaceae* by Aptroot (1991), from *Normandina pulchella* and *N. simodensis*, and it occurs in small amounts in *N. chlorococca*. *Normandina acroglypta* is reported here to contain two terpenoids, neither of them zeorin. During the present study, small quantities of zeorin were detected in a specimen of *Flakea papillata* (Lesser Antilles, Martinique, *Orange* 19245, NMW C. 2010.001.125).

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