Record of *Nemania aureolutea* (*Xylariaceae*) from the southernmost region of Spain

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Abstract: A collection of *Nemania aureolutea*, a rarely recorded species with a rather north temperate known distribution, is reported for the first time, to our knowledge, from a Mediterranean region. The identification of the fungus is based on its morphological characterization, culture characteristics and comparison of the ITS sequence with those of the type collection. The slight morphological differences encountered in this collection are discussed and the peculiar environmental characteristics of the region it comes from are outlined.
Keywords: Andalusia, Ascomycota, Los Alcornocales Natural Park, ribosomal DNA, taxonomy, *Xylariales*.

Resumen: Una recolecta de *Nemania aureolutea*, una especie escasamente registrada, con una distribución conocida de zonas más bien templadas del norte, se cita por primera vez, según nuestro conocimiento, en una región mediterránea. La identificación del hongo se ha basado en su caracterización morfológica, las características de cultivo y la comparación de la secuencia ITS con la de la colección tipo. Se discuten las pequeñas diferencias morfológicas encontradas en esta colección y se reseñan las peculiares características ambientales de la región de donde proviene.
Palabras clave: Andalucía, Ascomycota, Parque Natural de Los Alcornocales, DNA ribosomal, taxonomía, *Xylariales*.

Résumé : une récolte de *Nemania aureolutea*, une espèce rarement signalée et dont la répartition connue est plutôt nord-tempérée, est signalée pour la première fois, à notre connaissance, d'une région méditerranéenne. L'identification du champignon s'appuie sur l'étude morphologique, ses caractéristiques en culture et la comparaison de sa séquence ITS avec celles de la récolte type. Les légères différences morphologiques que présente cette récolte sont discutées et l'écologie particulière de la région dont elle provient est présentée.
Mots-clés : ADN ribosomal, Andalousie, Ascomycota, Parc naturel de Los Alcornocales, taxinomie, *Xylariales*.

Introduction

Nemania aureolutea (L. Petrini & J.D. Rogers) Granmo was first recognized as a variety of *Hypoxylon aeneum* Nitschke by Petrini & Rogers (1986) as *H. aeneum* var. *aureoluteum* L. Petrini & J.D. Rogers. Its recognition as a distinct species was later made by Granmo *et al.* (1999), following the segregation of the genus *Nemania* S.F. Gray from *Hypoxylon* Bull. to accommodate the taxa allied to *H. serpens* (Pers. : Fr.) J. Kickx f., proposed by Pouzar (1985a, 1985b), now widely accepted and confirmed by phylogenetic data (Hsieh *et al*., 2010). However, Ju & Rogers (2002) preferred to keep it as a variety of *N. aenea* (Nitschke) Pouzar as *N. aenea var. aureolutea* (L. Petrini & J.D. Rogers) Y.-M. Ju & J.D. Rogers.

Nemania aureolutea is diagnosed by a subamyloid to inamyloid ascal apical apparatus when observed in Melzer's reagent, light brown ascospores 14–18 × 5.5–7 µm with an inconspicuous short germ slit and slow-growing orange colonies (Granmo *et al*., 1999). This set of characters deviating from *N. aenea* supports its status of a distinct species and this concept is followed here.

Since its recognition as a distinct taxon by Petrini & Rogers (1986), *N. aureolutea* has been predominantly reported from Northern and Central temperate Europe. For this reason, and on account of slight morphological differences, a collection of a large-spored *Nemania* sp. from the very south of Spain enjoying a Mediterranean climate proved difficult to identify and was first regarded as a potentially new taxon. This prompted a thorough morphological evaluation of this collection, coupled with in vitro isolation and sequencing of ITS regions of the nuclear ribosomal DNA (rDNA).

In the following we present our morphological, cultural and molecular phylogenetic results showing that this collection represents an unexpected occurrence of *N. aureolutea* in a Mediterranean environment. We also discuss the slightly deviating morphological differences of the fungus from more typical material encountered at higher latitudes and the peculiar ecological characteristics of the region where it was collected.

Materials and methods

The observations were carried out on living material in water and in rehydrated dry material in water or 1% SDS. Measurements of asci and ascospores were made in water and ascospores measurements processed with the free software Piximetre version 5.2 (http://ach.log.free.fr/Piximetre/). The amyloid reaction of the ascus apical apparatus was tested by adding a drop of Melzer's reagent or Lugol's solution to a water mount of perithecial contents. In case of a weak or absent reaction, Melzer's reagent is used after a short pretreatment in 3% KOH or directly in Lugol's solution. Microscopic observation of the asci and the paraphyses was carried out in water or after 1 min in 1% SDS and mounting in aqueous chlorazol black or in diluted India ink. Inconspicuous germ slits of ascospores were observed in chloral-lactophenol or preferably in PVA-lactophenol after 48h incubation. Measurements of stromata, asci and ascus apical apparati are recorded as height × width. Terminology and observation procedures follow Ju & Rogers (2002). The colour codes refer to Rayner's mycological chart (Rayner, 1970). Nomenclature follows MycoBank. Photomacrographs were taken with a Nikon Coolpix 995 digital camera either directly mounted on a stand or, for higher magnifications, through the eyepiece of an Olympus SZ60 stereomicroscope, by the means of a 30 mm diameter adapter (JF) or with an Olympus OM-D EM-1 digital camera with M. Zuiko Digital ED 60 mm F2.8 Macro lens (MAR). Photomicrographs were taken with the first camera mounted on the trinocular port of a Leitz Orthoplan microscope (JF) or with a Nikon D300 digital camera mounted on a trinocular Nikon Eclipse 50i microscope (MAR). The digitized photographs were processed with Adobe Photoshop Elements 10 (JF) and with Lightroom 5 (MAR) and the plates assembled with the first software. The specimen is deposited in LIP herbarium (University of Lille, France).

Cultures of the living specimen were made on PDA (Potato Dextrose Agar) with 5 mg/L of streptomycin in Petri dishes 5 cm diam. incubated at 25 °C.

DNA extraction, amplification, and sequencing. — Total DNA was extracted from pure culture blending a portion of them using

a micropestle in 600 µL CTAB buffer (CTAB 2%, NaCl 1.4 M, EDTA pH 8.0 20 mM, Tris-HCl pH 8.0 100 mM). The resulting mixture was incubated for 15 min at 65 ºC. A similar volume of chloroform:isoamylalcohol (24:1) was added and carefully mixed with the samples until their emulsion. It was then centrifuged for 10 min at 13.000 g, and the DNA in the supernatant was precipitated with a volume of isopropanol. After a new centrifugation of 15 min at the same speed, the pellet was washed in 70% cold ethanol, centrifuged again for 2 min and dried. It was finally resuspended in 200 µL ddH2O. PCR amplification was performed with the primers ITS1F and ITS4 (White *et al.*, 1990; Gardes & Bruns, 1993) for ITS, and LR0R and LR5 (Vilgalys & Hester, 1990) were used to amplify the 28S nLSU region. Chromatograms were checked searching for putative reading errors, and these were corrected.

Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 6 (Tamura *et al.*, 2013).

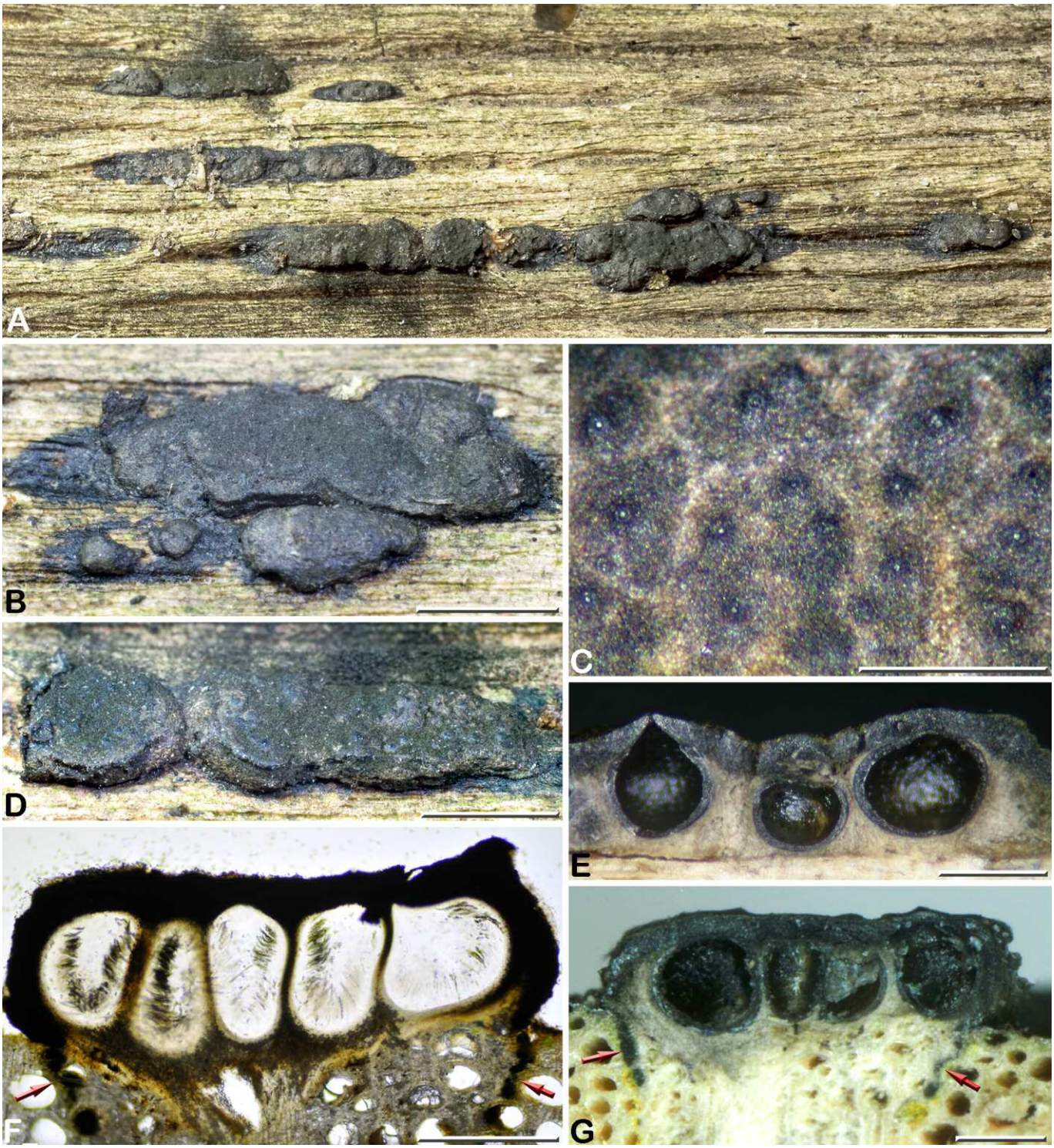

Plate 1 – *Nemania aureolutea*

MAR 101219-115. A: Habit of stromata on wood surface; B, D: Stromata showing abrupt margins and even surface, overlain by greenish ascospores deposits in D; C: Stromatal surface in close-up showing faintly papillate ostioles and a light greyish brown coating in shallow depressions between perithecial mounds; E: Stroma in longitudinal vertical section showing perithecia beneath a thick superficial crust and embedded in a greyish fibrous soft tissue; F: Stroma in transverse vertical section showing perithecia beneath a thick superficial crust with black stromatic marginal extensions in the wood (arrows), in water; G: Stroma in transverse vertical section showing perithecia beneath a thick superficial crust and embedded in a greyish fibrous soft tissue, with black stromatic marginal extensions in the wood (arrows). Scale bars: A = 10 mm; B, D = 2 mm; C, E-G = 0.5 mm.

Taxonomy

Nemania aureolutea (L. Petrini & J.D. Rogers) Granmo, *Sommerfeltia*, 27: 45 (1999). Plates 1–2, Fig. 1.

≡ *Hypoxylon aeneum* Nitschke var. *aureoluteum* L.E. Petrini & J.D. Rogers, *Mycotaxon*, 26: 413 (1986); *Nemania aenea* var. *aureolutea* (L.E. Petrini & J.D. Rogers) Y.-M. Ju & J.D. Rogers, *Nova Hedwigia*, 74 (1-2): 84 (2002).

Stromata effused-pulvinate, superficial, in linear rows along the grain of the wood, orbicular 0.7–2 mm diam. to ellipsoid-elongated 1.5–4 mm long × 1–2 mm wide × 0.6–0.75 mm thick, occasionally confluent, with steep margins frequently lined by a superficial blackening spreading on the substrate; perithecial contours not to faintly exposed; surface blackish, a carbonaceous crust 120–170 µm thick, extending downward in the wood at margins as a thin black line, overlain with a superficial light brownish grey pruinose coating remaining present in shallow depressions between perithecial mounds; interior light grey to brownish grey, soft, fibrous, embedding the perithecia, the underlying wood slightly bleached. **Perithecia** subglobose to slightly depressed-spherical, 0.4–0.6 mm diam. **Ostioles** black, obtusely papillate, occasionally apically truncate, 60–80 µm diam. at base. **Fresh spore deposit** on stromatal surface dark green.

Paraphyses copious, thin-walled, remotely septate, 5–6.5 µm wide at base, tapering to 1.5–1.8 µm above asci, minutely guttulate, embedded in mucilaginous material. **Asci** cylindrical to subclavate, with (6–)8 slightly overlapping uniseriately to irregularly biseriately arranged ascospores, the spore-bearing parts 66–94 × 11–13.8 µm, the stipes 45–70 µm long, with apical apparatus 3–4.2 × 2.3–2.7 µm (Me = 3.6 × 2.5 µm, N = 25), short-cylindrical to most often tubular, apically slightly flared and cupulate with a prominent rim, not blueing in Melzer's reagent, faintly blueing in Melzer's reagent after pretreatment in 3% KOH, blueing in Lugol's solution. **Ascospores** (13.6–)14.1–17.1(–18.4) × (4.4–)5.1–6.6(–6.9) µm, Q = (2.2–)2.4–3.1(–3.4), N = 120 (Me = 15.7 × 5.8 µm, Qe = 2.7), [(14.4–)15.7–19(–19.9) × (5.3–)5.7–7(–7.4) µm, Q = (2.2–)2.5–3(–3.1), N = 73 (Me = 17.3 × 6.4 µm, Qe = 2.7) when recorded in water from fresh material], narrowly ellipsoid, subequilateral, with broadly to narrowly rounded ends, occasionally slightly heteropolar with one end more narrowly rounded, unicellular, light olivaceous brown, with two large guttules, lacking a germ slit, without appendage or mucilaginous sheath; epispore smooth. **Asexual morph** on the natural substrate not observed.

Cultural characteristics: Colony 10–12 mm diam. at two weeks, pulvinate, subcircular in outline, salmon (41) with slightly scalloped margin surrounded by a halo 1.5–2 mm wide of luteous (12) pigment diffusing in the medium; reverse orange (7); 40 mm diam. at six weeks with an ellipsoid pulvinate central region 2 × 1.5 mm diam., with a slit-like central depression, pale salmon (41), overlain with a thin layer of white mycelium and conspicuous colourless exudation droplets, surrounded by an appressed, faintly zonate and scalloped marginal belt 1–1.4 mm wide of white to rosy buff (61) mycelium; reverse ochreous (44) at centre, buff (45)at periphery; medium unstained; odour fairly strong, acidulous, citrus-like. Asexual morph not produced after six weeks of incubation.

Specimen examined: Spain: Andalucía, Cádiz, Tarifa-Algeciras, Parque Natural de Los Alcornocales, Llanos del Juncal, 36º06'14.23" N, 5º32'23.99"W, 740 m, on dead decorticated wood of *Quercus canariensis* Willd. (*Fagaceae*), 10 Dec. 2019, *leg*. Miguel Á. Ribes, José Cuesta and Juan Antonio Valle, MAR 101219-115 (LIP), GenBank sequences: ITS = MW136058, LSU = MW136059.

Known distribution: Europe: Austria (Friebes, 2011), Czech Republic (Zíbarová & Kout, 2017), Denmark, Norway, U. K. (Granmo *et al.*, 1999), France (Fournier & Magni, 2002), Spain (Rubio Domínguez, 2012; this paper), Switzerland (Petrini & Rogers, 1986). North America: USA, Tennessee (Rogers *et al.*, 2008a); Hawaii (Rogers & Ju, 2012).

Comments

Because of its strongly carbonaceous stromata, asci with an inamyloid apical apparatus in Melzer's reagent and light brown subequilateral ascospores 15.7 × 5.8 µm on average lacking a germ slit, as well as an unusual geographical provenance, this collection proved difficult to equate to a known taxon. To rule out the possibility of the occurrence of a tropical/subtropical taxon, the literature reporting recently published tropical *Nemania* taxa was likewise consulted (Fournier *et al.*, 2018; Ju *et al.*, 2005; Rogers & Ju, 2002; Rogers *et al*., 2006; Rogers *et al*., 2008b).

Ascospore morphology was recalling that of species related to *N. aenea* including *N. aureolutea*, *N. aenea* var. *macrospora* (Miller) Y.-M. Ju & J.D. Rogers and *N. subaenea* Y.-M. Ju & J.D. Rogers. *Nemania aureolutea* appeared as the closest match on account of its ascus apical apparatus not bluing in Melzer's reagent; moreover, ascospores of *N. aenea* and *N. aenea* var. *macrospora* differ in having a fairly conspicuous germ slit; in *N. subaenea*, known to have a tropical distribution, the germ slit is predominantly on the more convex side (Tang *et al.*, 2007, as *N. plumbea* A.M.C. Tang, R. Jeewon & K.D. Hyde; Fournier *et al.*, 2018). *Nemania aureolutea* was therefore the most plausible name, which was confirmed by the slow-growing colony with vivid orange colour observed in culture (Plate 2), which is typical for this taxon. A further confirmation came from a phylogenetic comparison of the ITS sequence with those from the type collection from Switzerland used by Sánchez-Ballesteros *et al.* (2000) and Peláez *et al.* (2008), showing 100% similarity (Fig. 1). A LSU sequence was likewise generated but was not used because of the paucity of LSU sequences of *Nemania* spp. available for comparison.

In front of such a sound evidence, we can only interpret the thick carbonaceous stromatal crust observed in this collection as related to the environmental conditions, fairly different from those encountered in North and Central Europe.

After several unsuccessful attempts to make out a germ slit on ascospores of this collection after incubation in PVA-lactophenol, we repeated this operation on the herbarium specimens of *N. aureolutea* listed in Fournier & Magni (2002) (as *N. aenea* var. *aureolutea*). The results proved inconsistent, the germ slit being visible in a very low percentage of ascospores or apparently absent in some collections. Such a feature that is inconsistently made out cannot be retained as a significant differential character in the case of *N. aureolutea*.

The site of Los Alcornocales, where the fungus was collected, is located in the provinces of Cádiz and Málaga at the very south of Spain (SW of the Iberian Peninsula). The annual rainfalls are abundant (between 800 and 1400 mm), as well as the fogs regime, caused by the nearness to the Strait of Gibraltar. It has two bioclimatic belts: thermo (annual mean temperature 17–18 ºC) and mesomediterranean (14–16 ºC) with accused oceanity (Pérez Latorre *et al.*, 1999). The Natural Park has a Mediterranean climate, with maximum rainfall during winter and with at least two peaks of aridity during the summer, although at certain points the humidity in this season is directly and highly influencing the vegetation (Pérez Latorre *et al.*, 1999). According to Molero & Marfil (2017), Los Alcornocales is a thermo-mesomediterranean woodlands and microforest, specifically *Quercus suber* woodland. Molero & Marfil (2017) indicated: "The *Teucrio baetici-Quercetum suberis* is the cork oak woodland in the Algeciras and Aljibe Sector, occupying a large area and giving its name to the Los Alcornocales nature Park, a large woodland of *Quercus suber* which occurs throughout the thermo- and mesomediterranean thermotypes in ombroclimates ranging from subhumid to hyperhumid". This is a closed community with a dense shrubby stratum and epiphytes (*Polypodium cambricum*, *Davallia canariensis*), and contains *Olea sylvestris*, *Quercus coccifera*, *Phillyrea angustifolia*, *P. latifolia*, *Arbutus unedo*, *Viburnum tinus*, *Myr-*

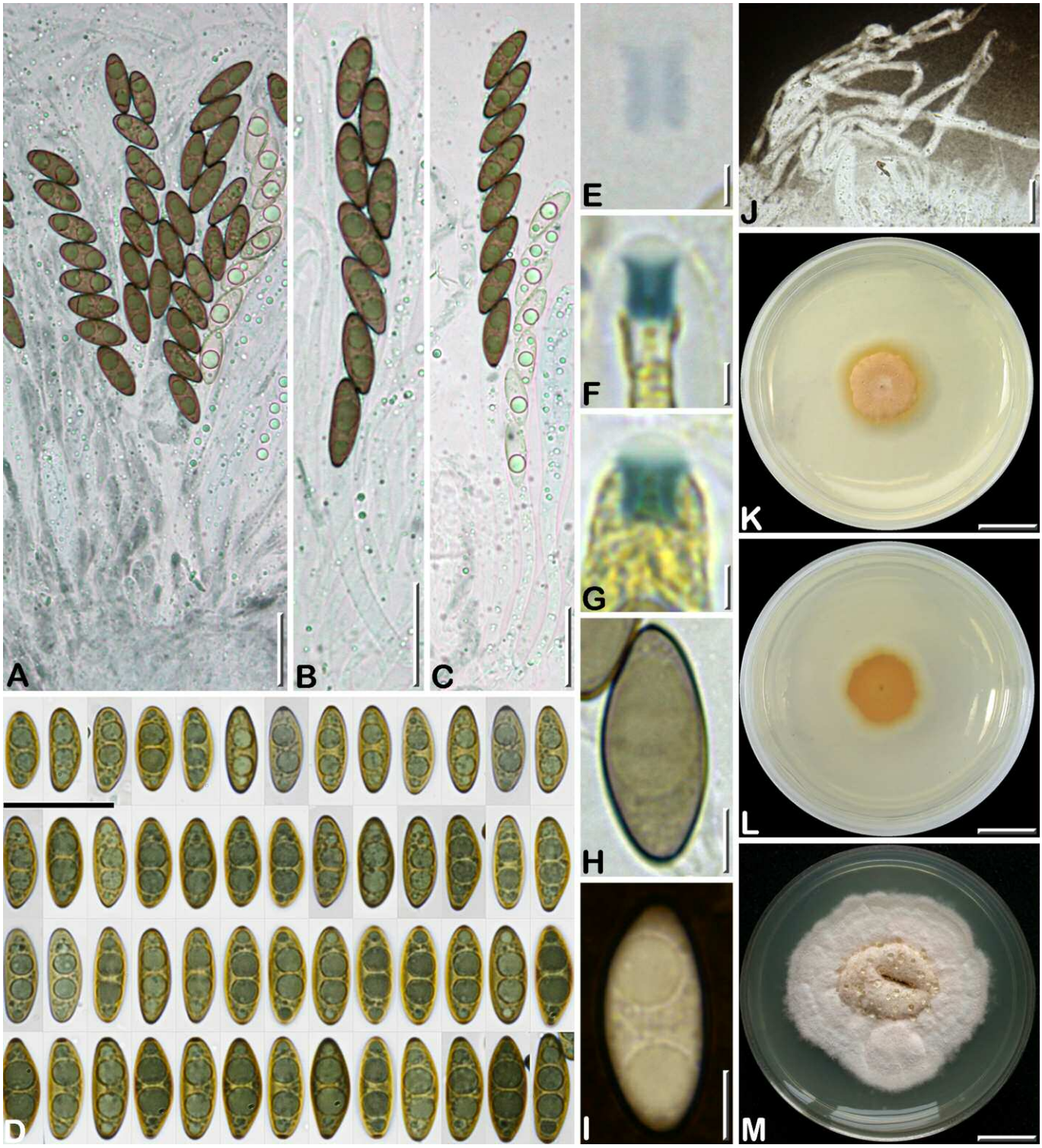

Plate 2 – *Nemania aureolutea*

MAR 101219-115. A-C: Asci with variously arranged ascospores, in chlorazol black; D: Ascospores, in water; E: Apical apparatus in Melzer's reagent after pretreatment in 3% KOH; F, G: Apical apparati in Lugol's solution; H: Ascospore in PVA-lactophenol, showing absence of germ slit; I: Ascospore in India ink, showing absence of appendage or mucilaginous sheath; J: Paraphyses at their base, in diluted India ink; K: Colony at two weeks; L: Reverse of the colony at two weeks; M: Colony at six weeks. Scale bars: A-D, J = 20 µm; E-G = 2 µm; H, I = 5 µm; K-M = 10 mm.

tus communis, *Erica arborea*, *Rubia agostinhoi*, *Teucrium baeticum*; and in the serial shrubland, *Teline linifolia*, *T. monspessulana*, *Cytisus baeticus*, *Adenocarpus telonensis*, *Stauracanthus boivinii*, *Genista tridens* and others. This fairly strong Atlantic influence contributes to the uniqueness of the climate of this region and likely accounts for the occurrence of unexpected fungal species like *N. aureolutea*.

By its occurrence in a region enjoying a Mediterranean climate, the known distribution of *N. aureolutea* in the North hemisphere is therefore significantly extended southwards since it can likewise be expected from some North African and South European regions lining the Mediterranean Sea. The record of *N. aureolutea* from Hawaii by Rogers & Ju (2012), even suggests a much wider distribution extending to tropical regions. We hope that this work will contribute to facilitate the identification of *N. aureolutea* and a better assessment of its distribution.

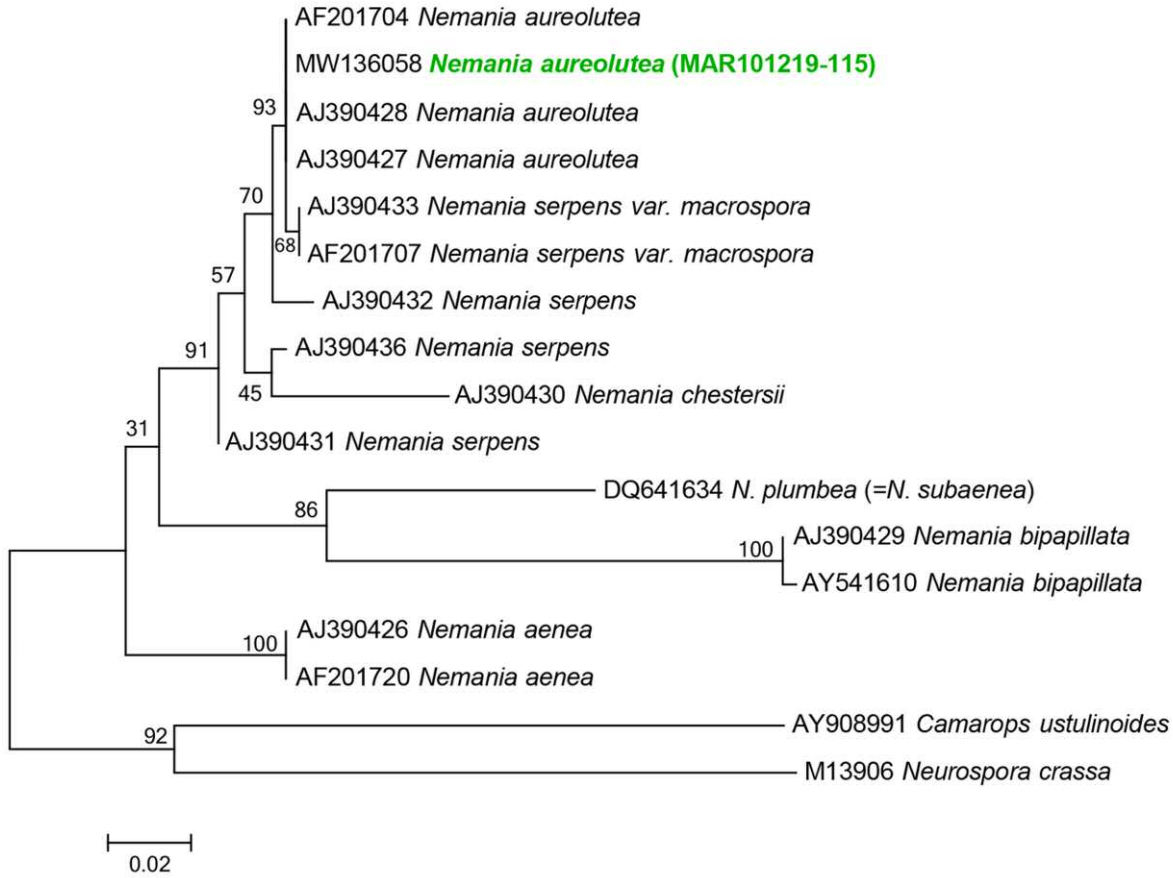

Fig. 1 – Maximum likelihood phylogeny (-lnL = 1370.78) of *Nemania* spp. inferred by using the method based on the Tamura-Nei model, from a 580 bp matrix of ITS sequences, rooted with *Neurospora crassa* and *Camarops ustulinoides*.

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