Published in final edited form as:
Mycologia. 2011 ; 103(1): 209–218. doi:10.3852/10-178.

Nectria eustromatica sp. nov., an exceptional species with a hypocreaceous stroma

Walter M. Jaklitsch[1] and **Hermann Voglmayr**
Faculty Centre of Biodiversity, University of Vienna, Rennweg 14, A-1030 Vienna, Austria

Abstract

A new species with remarkable morphology, *Nectria eustromatica*, is described, based on morphology of the teleomorph and anamorph, ecology and molecular phylogenetic analyses. *Nectria eustromatica* is characterized by sphaeroid perithecia immersed in pseudoparenchymatous stromata formed singly or collectively on a subiculum. Despite its deviating teleomorph morphology, it is placed within *Nectria* sensu stricto in phylogenetic analyses of a combined dataset of LSU, ITS, *rpb2* and *tef1* sequences with high internal support. *Nectria eustromatica* has been collected specifically on *Hippocrepis (Coronilla) emerus* in southern Europe. The anamorph of *N. eustromatica* shares morphological traits with the genera *Stilbella* and *Tubercularia* but produces non-phialidic macroconidia in addition to phialoconidia.

Keywords

Ascomycetes; *Hypocrea*; ITS; LSU; morphology; *Nectria*; phylogenetic markers; *rpb2*; sequence analysis; *Stilbella*; *Stilbocrea*; *tef1*; *Tubercularia*

INTRODUCTION

Ascomata and stromata of the Hypocreales have usually light or bright colors (Rossman et al. 1999). Exceptions for example are *Hypocrea lixii* Pat. (Jaklitsch 2009) or *H. schweinitzii* (Fr.) Sacc. and similar species (Samuels et al. 1998) of the Hypocreaceae. In the Nectriaceae the stroma when present is typically a hypostroma, a loose or compact, pros- or pseudoparenchymatous layer or pillow, which gives rise to more or less free, superficial perithecia on its top, usually with clearly discernible perithecial contours, even when tightly associated in clusters. In genera of the Bionectriaceae such as *Stilbocrea* Pat. (Rossman et al. 1999) ascomata are immersed in light-colored, prosenchymatous stromata.

Intense searches for *Hypocrea* teleomorphs in Europe have revealed a fungus that superficially resembles representatives of the Hypocreaceae or other stromatic ascomycetes because of its dark brown to nearly black stromata. However the centrum morphology of this fungus is nectriaceous. This fungus is described here as a new species of *Nectria* (Fr.) Fr.

[1]Corresponding author. walter.jaklitsch@univie.ac.at.

Europe PMC Funders Author Manuscripts

MATERIALS AND METHODS

Isolates and specimens

Taxon names and accession numbers of gene sequences included in this study are provided (Table I); data on isolates sequenced in the present study also are provided (Table II). Representative isolates have been deposited at the Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands (CBS). Specimens have been deposited in the Herbarium of the Institute of Botany, University of Vienna (WU).

Ascospore isolates were prepared as described by Jaklitsch (2009). Cultures were grown in 9 cm diam Petri dishes either in the dark at 15 C, in daylight or with alternating 12 h cool white fluorescent light and 12 h darkness at 20–25 C on oatmeal agar (OA, Sigma), 2% malt extract agar (MEA), potato dextrose agar (PDA), low nutrient agar (SNA) and cornmeal dextrose agar (CMD, Jaklitsch 2009).

Morphological observations

Conidiation structures were examined, measured and photographed on a compound microscope from cultures grown on SNA, PDA, OA or MEA after mounting in 3% KOH. Dry stromata were rehydrated overnight with water vapor in a closed glass chamber at room temperature, treated briefly with 3% KOH, embedded in Tissue-Tek O.C.T. Compound 4583 (Sakura Finetek Europe B.V., Zoeterwoude, the Netherlands) and sectioned 10 μm thick with a freezing microtome. Sections were measured and photographed in lactic acid or in 50% glycerol or 3% KOH where noted. Asci and ascospores were measured in separate preparations in 3% KOH. (See Jaklitsch [2009] for the terminology of stromatal traits.) Measurements are reported as maxima and minima in parentheses and the mean plus and minus the standard deviation of a number of measurements given in parentheses. Nomarski differential interference contrast (DIC) was used for observations and measurements. Images were recorded with the Nikon Coolpix 4500 or DS-U2 digital cameras. Measurements were made with NIS-Elements D 3.0 software.

DNA extraction, PCR amplifications and sequencing

Mycelium for DNA extraction was grown in liquid malt extract culture, harvested, freeze-dried and ground according to Voglmayr and Jaklitsch (2008). Genomic DNA was extracted with the modified CTAB method described in Riethmüller et al. (2002). A 1.6 kb fragment containing partial SSU, ITS1, 5.8S, ITS2 and partial LSU was amplified with the primer pair V9G (de Hoog and Gerrits van den Ende 1998) and LR5 (Vilgalys and Hester 1990). A 1.1 kb fragment of RNA Polymerase II subunit B (*rpb2*) was amplified with the primer pair fRPB2-5f and fRPB2-7cr (Liu et al. 1999). A 1.3 kb fragment of the *tef1* gene encoding translation elongation factor 1 alpha was amplified with the primer pair EF1728F (Chaverri and Samuels 2003) and TEF1LLErev (Jaklitsch et al. 2005). This fragment includes the fourth and the fifth intron and a part of the last large exon. PCR products were purified by an enzymatic PCR cleanup (Werle et al. 1994) as described in Voglmayr and Jaklitsch (2008). DNA was cycle sequenced with the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit 3.1 (Applied Biosystems, Warrington, UK) and an automated DNA sequencer (ABI 3730xl Genetic Analyzer, Applied Biosystems) with the same primers as in PCR; in addition for the SSU-ITS-LSU fragment primers LR3 (Vilgalys and Hester 1990), ITS4 (White et al. 1990) and F5.8Sr (5′TGCGTTCAAARATTCGATG 3′) were used as internal sequencing primers. Due to abrupt signal loss in some *Nectria* species with GC-rich sequence regions in the ITS, it sometimes was necessary to use F5.8Sf (5′CAACAACGGATCTCTTGGYTC 3′) and ITS5 (White et al. 1990) as additional internal sequencing primers. (All sequences generated in this study are listed in Table I.)

Molecular phylogenetic analyses

For the phylogenetic analyses representative LSU, ITS, *tef1* and *rpb2* sequences of Hypocreales were selected from GenBank according to a BLAST query that revealed a high sequence homology of LSU sequences of the new species to *Nectria* sensu stricto (Table I). However because only few sequences were available for *Nectria* the dataset was complemented with some representative *Nectria* species collected by the authors or obtained from CBS (Table II). The final matrix contained sequences from 23 taxa, including *Hypocrea rufa* and *Sphaerostilbella aureonitens* as outgroups. All alignments were produced with Muscle 3.6 (Edgar 2004). A combined dataset of LSU, ITS, *rpb2* and *tef1* sequences was used for the analyses. After the exclusion of leading and trailing gap regions and of ambiguously aligned positions in the ITS and *tef1* alignments, the combined matrix contained 3645 characters (viz. 834 nucleotides from the LSU, 563 from the ITS1-5.8S-ITS2 region, 1110 nucleotides from *rpb2* and 1138 nucleotides from *tef1*). Incongruence between the different gene regions included in the multigene analyses was evaluated by comparison of maximum parsimony (MP) bootstrap trees of the individual gene regions, which were calculated with the same parameters as for the combined analysis given below. Incongruence receiving MP bootstrap support under 70% was considered low, posing no major obstacle for the combined analyses. Maximum parsimony (MP) analyses were performed with PAUP* 4.0 b10 (Swofford 2002) with 1000 replicates of heuristic search with random addition of sequences and subsequent TBR branch swapping (MULTREES option in effect, COLLAPSE = MAXBRLEN, steepest descent option not in effect). All molecular characters were unordered and given equal weight. Analyses were performed with gaps treated as missing data. Bootstrap analysis with 1000 replicates was performed in the same way but using 10 rounds of random sequence addition and subsequent TBR branch swapping during each bootstrap replicate. For maximum likelihood (ML) and Bayesian analyses first the appropriate models of sequence substitution were selected with Modeltest 3.6 (Posada and Crandall 1998) with the Akaike information criterion (AIC). These nucleotide substitution models were revealed by Modeltest: for *rpb2* the general time reversible model was chosen, additionally assuming a proportion of invariant sites with gamma-distributed substitution rates of the remaining sites (GTR + I + G); for LSU the ITS and *tef1* matrices the model of Tamura and Nei (1993) was selected, additionally assuming a proportion of invariant sites with gamma-distributed substitution rates of the remaining sites (TRN + I + G). Because the latter model could not be implemented in ML and Bayesian analyses the GTR + I + G model was used for all sequence regions of the combined matrix. For the analyses partitioned substitution models were implemented for each gene. For ML analyses 200 rounds of random addition of sequences as well as 200 bootstrap replicates were computed with RAxML 7.0.4 (Stamatakis 2006) with the GTRMIXI and GTRCAT algorithms respectively. GTRCAT efficiently approximates the GTR + G model; GTRMIXI uses GTRCAT during heuristic search, but the full GTR + I + G model for the final likelihood computation. Best rearrangement settings were estimated by RAxML during tree search. Bayesian analyses were performed with MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001), implementing the GTR + I + G model. Three parallel runs of four incrementally heated simultaneous Markov chains were performed over 1 000 000 generations from which every 100th tree was sampled in each run. The first 200 trees were discarded, and a 90% majority rule consensus of the remaining trees was computed to obtain estimates for the probabilities that groups are monophyletic given the sequence data (posterior probabilities). The multiple sequence alignment file has been deposited in TreeBASE and is available at http://purl.org/phylo/treebase/phylows/study/TB2:S10590.

RESULTS

Phylogenetic considerations

Of the 3645 characters of the combined matrix 888 were parsimony informative (LSU: 107, ITS: 92, *rpb2*: 470, *tef1*: 219). MP analyses revealed one most parsimonious tree of length 3826 (not shown). The best ML tree (lnL = –21091.24) (Fig. 1) is similar to the MP tree except for minor differences in topology concerning *Nectria berolinensis, N. lamyi* and *N. aurantiaca*. Tree topologies of the Bayesian analyses were the same as in the ML tree. The three Bayesian runs revealed almost identical posterior probabilities. MP bootstrap support above 70%, ML bootstrap support above 70% and Bayesian posterior probabilities above 90% are illustrated (Fig. 1) at first, second and third position above or below the branches respectively. Comparison of the MP bootstrap trees from the individual genes revealed similar topologies but little backbone resolution. Differences in topology were characterized by low bootstrap support (below 65%), indicating only minor incongruence among individual gene regions. In addition MP bootstrap support was mostly higher in the combined analysis than the highest support value obtained in the single gene analyses (data not shown).

Phylogenetic analyses place *Nectria eustromatica* in the *Nectria* sensu stricto clade with high support (Fig. 1). Sister-group relationship to *N. pseudotrichia* is highly supported in all analyses. *Nectria* is revealed as polyphyletic because it forms two distinct, highly supported clades: *Nectria* sensu stricto, represented in our analyses by *N. cinnabarina, N. aurantiaca*, *N. pseudotrichia* and *N. eustromatica*, and "Pleonectria" containing the remaining Nectria species that were included in the analyses (Fig. 1).

TAXONOMY

Nectria eustromatica Jaklitsch & Voglmayr, sp. nov. Figs. 2, 3

MycoBank MB518506

Etymology—*Eustromatica* addresses the true, pseudoparenchymatous stroma.

Stromata 0.4–1.8 mm diam, pulvinata, atrofusca, pseudoparenchymatosa, 1–15 perithecia includentia. Asci (94–)100–125(−138) × (21–)24–37(−41) μm, bi- ad octospori, ellipsoidei, clavati vel saccati, unitunicati, sine apparatu apicali. Ascosporae (24–)29–37(−43) × (8–)9–12(−15) μm, biseriatae, bicellulares, oblongae vel fabiformes, hyalinae ad luteae.

Stromata (0.4–)0.7–1.4(−1.8) × (0.4–)0.5–1.1(−1.6) mm, 0.3–0.7(−0.9) mm thick (n = 50), solitary, scattered or in fascicles of up to seven, sometimes laterally fused, erumpent from bark, on a hyphal hypostroma, dark brown, orange-brown, dark gray to black, pseudoparenchymatous, with a soft consistency when fresh; pulvinate to semiglobose, nearly globose when uniperitheciate; outline variable, circular, oblong or often angular. Sides similar to the surface or lighter, dull gray to orange-brown, smooth, glabrous or slightly downy. Stroma surface convex, smooth or tubercular due to variably projecting perithecial contours, glabrous, but often appearing downy or slightly velutinous when young; when mature with 1–15 flat or convex, shiny, tarry, black ostiolar dots, (39–)57–108(−200) μm diam (n = 60), with circular, oblong or angular outline. Stroma interior lighter than the surface, orange-brown, brighter orange in 3% KOH. Stromata hydrophobic, difficult to moisten by vapor, black in water; in 3% KOH exterior unchanged, no pigment dissolved.

Stroma anatomy—Cortical layer (17–)21–45(−58) μm thick (n = 40), consisting of 1–3(−4) layers of coarse, distinct, globose or angular, dark brown to dark reddish brown cells,

(9–)13–26(−34) × (7–)9–19 (−29) μm (n = 35) in section, with walls 0.5–2.5 μm thick, forming a textura angularis. Surface lacking hairs, but with erect cells or cell groups forming warts, causing the rough or velvety appearance of stromata under lower magnifications. Subcortical tissue reaching to the base of perithecia and sometimes to the stroma surface, comprising a t. angularis of roundish, angular, isodiametric to oblong cells, (7–)12–27(−39) × (6–)8–15(−24) μm (n = 35) in vertical section, (7–) 10–17(−19) × (5–)7–12(−16) μm (n = 30) in cross section, with walls 0.5–1 μm thick, yellow, orange-brown in glycerol, KOH and water; between widely spaced perithecia replaced by wide, vertically oriented hyphae. Subperithecial tissue comprising a t. epidermoidea of thin-walled, yellow cells (6–)7–19(−30) × (4–)5–12(−15) μm (n = 35), denser and darker yellow than the subcortical tissue. Basal tissue a t. intricata of thin- and thick-walled hyphae (3–)4–6(−8) μm wide (n = 35), in lower layers connecting several stromata as a subiculum, similarly pigmented or somewhat darker than subperithecial tissue. Perithecia (300–)350–460 (−490) μm high including ostioles and (235–)270–345(−380) μm wide (n = 22), sphaeroid. Peridium (17–)23–41(−50) μm thick at the base (n = 18) and (12–)21–32(−38) μm (n = 18) at the sides, yellow; at the sides of closely appressed perithecia distinct, of elongate, compressed refractive yellow cells, otherwise usually indistinctly differentiated from the surrounding pseudoparenchymatous tissue, lined inside by narrow, often collapsed, hyaline cells, 2.5–5(−8) μm wide. Ostioles (120–)125–148(−167) μm long, even with the surface or projecting 10–40(−52) μm, (24–) 26–42(−52) μm wide at the apex inside and (90–)104–144(−180) μm outside (n = 18, 18), including vertical to converging, elongate, subclavate, dark brown apical cells. Ostiole contents turning yellow in 3% KOH, filled with periphyses merging downward into acute, nearly lanceolate apical paraphyses in apical regions of the perithecia. True paraphyses absent. Asci (94–)100–125(−138) × (21–)24–37(−41) μm (n = 30), clavate, ellipsoidal or saccate, with a minute stipe or acute base, without a differentiated apical structure; entirely filled with (2–)4–8 ascospores in biseriate arrangement. Ascospores (24–)29–37(−43) × (8–)9–12 (−15) μm, l/w (1.7–)2.6–3.6(−4.5) (n = 60), hyaline to yellowish, first unicellular, falcate or sigmoid with acute ends when immature, becoming bicellular, straight or slightly curved, allantoid to bean-shaped, with broadly rounded ends, thick-walled (ca. 1 μm); septum central, not constricted; perispore thin, hyaline, delicately verruculose in 3% KOH when old.

Cultures and anamorph—*Nectria eustromatica* grows on all agar media tested, fastest on PDA and OA. It sporulates on all media except CMD. Macroconidia were found only on sporodochia on MEA and OA.

On SNA at 20 ± 2 C colony colorless, thin, circular, margin ill defined. No pigment, no distinct odor formed. After 2 wk conidia produced in minute wet heads; later in white granules consisting of compact, dense aggregates of conidiophores. Conidia only rarely > 10 μm long.

On OA at 20 ± 2 C colony whitish, pale rosy to pale brown with gray margin; odor unpleasant, acidic; after 4–5 wk plate covered by mycelium. In 1–6 wk numerous white fluffy tufts appearing in the colony center, spreading across the colony. Tufts partly turning into sporodochia within several weeks; conidiophores becoming fertile after 5–6 wk.

On MEA after 4 mo at 15 C colony colorless, without a distinct odor; conidia produced in mucous, carrot-colored drops on sporodochia; macroconidia produced within 1 y. At 20–25 C colony white to yellowish, with short spiny aerial hyphae; white tufts appearing after approximately 1 mo. After 2.5–3 mo minute white tufts and pale brownish sporodochia up to ca. 4 mm diam present.

On PDA at 20–25 C growth fast, after 4–5 wk plate covered by mycelium. Colony whitish to dull yellow, lobed. Conidiation after 5 wk in mostly roundish, white shrubs, fluffy tufts, spots or on sporodochia.

Sporodochia 0.3–3.5(−4) mm diam, compact, pulvinate, semiglobose, ellipsoidal to subglobose, white, pale reddish brown, to carrot-colored (on MEA), pseudoparenchymatous, with a loose white tomentum of aerial hyphae and conidiophores on surfaces. Conidiophores either solitary on surface hyphae, in minute white tufts or densely aggregated on the surface of sporodochia; erect, more or less fan- or broom-shaped, consisting of a straight main axis mostly 3–5 μm wide, attenuated upward to 2–3.5 μm terminally, cylindrical or with widening to 6–8 μm in age, smooth, with age becoming thick-walled with outer wall swelling in KOH; rarely with small rounded warts. Main axis unbranched or with monochasial, 1- to few-celled branches 3–4.5 μm wide, at several levels, loosely and asymmetrically arranged at acute angles, rarely perpendicular; branches rarely paired or verticillate. Phialides terminal on branches of similar width, solitary or in groups of two, divergent, rarely parallel, (7–)10–20(−37) × (2.0–)2.2–2.7(−3.0) μm, l/w (3.0–)4.2–8.0(−12.8) (n = 60; from PDA and SNA), cylindrical, straight or slightly curved, sometimes slightly constricted at the base. Conidia numerous, amassing in colorless, brownish to carrot-colored, turbid drops up to 0.4 mm diam, (5.5–)6.8–9.5(−13.8) × (2.0–)2.5–2.8(−3.5) μm, l/w (2.0–)2.6–3.7(−5.1) (n = 78; from MEA, PDA and SNA; with highest variability in size on SNA); hyaline, unicellular, oblong to cylindrical, straight or slightly curved, smooth, eguttulate or with inconspicuous minute guttules, often with a distinct, truncate abscission scar. Non-phialidic macroconidia produced on the same conidiophores in basal regions or terminally on long narrow hyphae 2–3.5 μm wide; macroconidia formed solitarily, (20–)27–38(−54) × (8.7–)9.7–12.5 (−14.8) μm, l/w (1.5–)2.4–3.5(−4.1) (n = 55, from MEA and OA), hyaline, oblong, cylindrical or narrowly ellipsoidal, straight or curved, smooth, with walls 0.8–1.7 μm thick, eguttulate, without a scar.

Habitat—on recently dead standing branches/trunks of *Hippocrepis* (*Coronilla*) *emerus*.

Distribution—Southern Europe, collected in Croatia and Italy.

Holotype—CROATIA, PRIMORSKO-GORANSKA, Opatija, Mošcenička Draga, village area, on dead twigs of *Hippocrepis emerus*, soc. *Cucurbitaria coronillae* and some immersed pyrenomycetes, 29 Mar 2007, *W. Jaklitsch* & *H. Voglmayr, W.J. 3079* (WU 30194; culture NC = CBS 121896).

Additional material examined—ITALY, LAZIO, Bagnaia, Prov. Viterbo, at Villa Lante, on *Hippocrepis emerus*, 28 Jul 2009, *W. Jaklitsch, H. Voglmayr* & *W. Gams* (WU 30195; culture NC1 = CBS 125578).

DISCUSSION

Nectria eustromatica is a member of *Nectria* sensu stricto (Fig. 1). For a long time the genus *Nectria* was conceived as fungi forming bright-colored, superficial perithecial ascomata with one-septate hyaline ascospores in unitunicate asci and devoid of true paraphyses. Scolecosporous taxa were classified in *Scoleconectria* Seaver (see Booth 1959) and *Ophionectria* Sacc. (Rossman 1977), those with muriform ascospores in *Pleonectria* Sacc. or *Thyronectria* Sacc. (Seeler 1940, Booth 1959). Later ascomatal wall structure and anamorphs were given superior significance in the definition of genera by Samuels and Rossman (1979). Rossman (1983) added phragmosporous taxa and later (Rossman 1989) dictyosporous taxa to the genus *Nectria* when she defined the *Nectria cinnabarina* group. The species of this group are characterized by large, often warted, often collabent, more or

less red, KOH+ ascomata with a two-layered wall, sometimes covered by yellow or greenish scurf, aggregated in cespitose clusters on an often well developed, pseudoparenchymatous hypostroma, and by anamorphs placed in the form genera *Tubercularia* Tode, *Stilbella* Lindau, *Gyrostroma* Naumov or *Zythiostroma* Höhn. ex Falck. This group was refined by Rossman et al. (1999) and was known as *Nectria* sensu stricto until Hirooka et al. (2009) determined that *Nectria* is paraphyletic and falls into two major clades; thus *Nectria* sensu stricto becomes restricted to species with *Tubercularia* anamorphs centering around the type species of *Nectria, N. cinnabarina*. Species with the pycnidial *Gyrostroma* and *Zythiostroma* anamorphs are included in the second principal clade, here called "*Pleonectria*".

Nectria eustromatica is a member of *Nectria* sensu stricto according to the most recent circumscription. All other species of *Nectria* sensu stricto differ from *N. eustromatica* by discrete perithecia. Perithecia of *Nectria cinnabarina* sometimes may be laterally fused, as shown by Seifert (1985, p 100), and have thick-walled clavate ostiolar cells similar to those of *N. eustromatica*, as shown by the same author. Our fungus may be interpreted as a result of a development of the thick pseudoparenchymatous wall of *N. cinnabarina* to a compact stroma, as an upward extension of the pseudoparenchymatous hypostroma that merged with the outer parts of the perithecial wall. *Nectria pseudotrichia*, which, according to the phylogenetic analysis, is a sister species of *N. eustromatica*, differs by muriform ascospores and the stipitate, synnematous anamorph *Tubercularia lateritia* (Berk.) Seifert from *N. eustromatica*. Also *Nectria aurantiaca* differs by a similar synnematous anamorph. No anamorph of *N. eustromatica* has been seen in nature. The anamorph of *N. eustromatica* formed in culture is assignable to either *Tubercularia* or *Stilbella* devoid of synnemata. Conidiophores of *N. eustromatica* with repeatedly monochasial branching are similar to those of the *Nectria aurantiaca* and *N. pseudotrichia* anamorphs (Seifert 1985, p 105, p 123, FIG. 39d; Booth 1959, p 30), whereas conidiophores of *Tubercularia vulgaris*, the anamorph of *N. cinnabarina*, are acropleurogenous in contrast to those of our fungus, which has branches of almost equal length as the elements of the axis. The formation of macroconidia has not been reported for any species currently known to belong to *Nectria* sensu stricto. They are produced later than the phialoconidia, often only after several months, while the latter are formed during a long period, usually still at times when macroconidia appear. The macroconidia, albeit being distinctly larger, resemble phialoconidia of *N. aurantiaca* (Booth 1959). Interestingly, *catalinensis* C.E. Lima, described from *Gleditsia* in Argentina (Lima et al. 1988), forms similar but smaller macroconidia (21–24.5 × 11.5–15 μm) in culture. *Nectria catalinensis, N. balansae* Speg. and *N. sordida* Speg. are also similar to *N. eustromatica* in forming pseudoparenchymatous stromata and ascospores of similar size. However stromata of these species, all described from Argentina, are red or reddish-brown and have a tubercular surface due to partly projecting perithecia. *Nectria catalinensis* and *N. balansae* differ from *N. eustromatica* also in striate ascospores, and *N. sordida* in distinctly smaller perithecia (Lima et al. 1988, Samuels and Brayford 1994). The phylogenetic placement of *N. catalinensis* and *N. sordida* is unknown, while *N. balansae* is shown to be only distantly related to *N. eustromatica* phylogenetically, based on a LSU sequence. The ascospores of *N. eustromatica*, although wider, are similar to those of the Indian *Peethambara sundara* Subram. & Bhat in length and shape (Rossman et al. 1999). However the latter fungus has free, yellow perithecia and belongs to the Bionectriaceae. Its anamorph produces large bicellular conidia on synnemata and belongs to genus *Didymostilbe* Henn. (Seifert 1985).

Stromata of *Nectria eustromatica* occur specifically on stems of *Hippocrepis emerus*, mostly on basal parts, maturing from there. Immature stromata of *Cucurbitaria coronillae* (Fr.) Sacc., which usually occur in large numbers on the same branches, superficially resemble those of *N. eustromatica*. However a cross section of a *N. eustromatica* stroma exposing the orange or orange-brown interior reveals its true nature. The superficial similarity of these

two fungi is probably the reason why *N. eustromatica* apparently has escaped notice. The overall appearance is that of a dothidealean fungus. No hint toward a description of this fungus in such a genus has been found, except for *Dothidea coluteae* Berk. & M.A. Curtis, described from twigs of a *Colutea* sp. in Pennsylvania, USA. The holotype (K 164150) of this fungus was examined and found to be a typical *Dothidea*, similar to *D. sambuci*, with bicellular, yellowish brown ascospores, 17–23 × 6.5–9.5 μm, of slightly unequal cells, in bitunicate asci.

Acknowledgments

We thank Walter Gams for hospitality and excursion support in Italy; the fungarium curators of K, B. Spooner and B. Aguirre-Hudson for loan of type material, CBS for cultures of *Nectria aurantiaca* and *N. pseudotrichia*, EU SYNTHESYS for a grant (GB-TAF-4625) to HV for a research stay at Royal Botanic Gardens Kew enabling him to collect *Nectria aquifolii* and the Austrian Research Fund (FWF; projects P19143, P22081) for financial support.

LITERATURE CITED

Booth C. Studies of Pyrenomycetes IV. *Nectria* I. Mycol Pap. 1959; 73:1–115.

Chaverri P, Samuels GJ. Hypocrea/Trichoderma (Ascomycota, Hypocreales, Hypocreaceae): species with green ascospores. Stud Mycol. 2004; 48:1–116. 2003.

de Hoog GS, Gerrits van den Ende AHG. Molecular diagnostics of clinical strains of filamentous Basidio-mycetes. Mycoses. 1998; 41:183–189. doi:10.1111/j.1439-0507.1998.tb00321.x. [PubMed: 9715630]

Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 2004; 32:1792–1797. doi:10.1093/nar/gkh340. [PubMed: 15034147]

Hirooka Y, Rossman AY, Chaverri P. Systematics of the genus *Nectria* based on a six-gene phylogeny [MSA meeting 2009 abstract]. Inoculum. 2009; 60:22.

Huelsenbeck JP, Ronquist F. MrBayes: Bayesian inference of phylogenetic trees. Bioinformatics. 2001; 17:754–755. doi:10.1093/bioinformatics/17.8.754. [PubMed: 11524383]

Jaklitsch WM. European species of *Hypocrea* I. The green-spored species. Stud Mycol. 2009; 63:1–91. doi:10.3114/sim.2009.63.01. [PubMed: 19826500]

Jaklitsch WM, Komon M, Kubicek CP, Druzhinina IS. *Hypocrea voglmayrii* sp. nov. from the Austrian Alps represents a new phylogenetic clade in *Hypocrea/Trichoderma*. Mycologia. 2005; 97:1365–1378. doi:10.3852/mycologia.97.6.1365. [PubMed: 16722227]

Lima CE, Forchiassin F, Ranalli ME. Systematic and biological study of Hypocreales of Argentina IV. *Nectria catalinensis* sp. nov. Nova Hedwig. 1988; 46:149–156.

Liu YL, Whelen S, Hall BD. Phylogenetic relationships among ascomycetes: evidence from an RNA polymerase II subunit. Mol Biol Evol. 1999; 16:1799–1808. [PubMed: 10605121]

Posada D, Crandall KA. Modeltest: testing the model of DNA substitution. Bioinformatics. 1998; 14:817–818. doi:10.1093/bioinformatics/14.9.817. [PubMed: 9918953]

Riethmüller A, Voglmayr H, Göker M, Weiß M, Oberwinkler F. Phylogenetic relationships of the downy mildews (Peronosporales) and related groups based on nuclear large subunit ribosomal DNA sequences. Mycologia. 2002; 94:834–849. doi:10.2307/3761698. [PubMed: 21156557]

Rossman AY. The genus *Ophionectria* (Euascomycetes, Hypocreales). Mycologia. 1977; 69:355–391. doi:10.2307/3758661.

Rossman AY. The phragmosporous species of *Nectria* and related genera. Mycol Pap. 1983; 150:1–164.

Rossman AY. A synopsis of the *Nectria cinnabarina*-group. Mem New York Bot Garden. 1989; 49:253–265.

Rossman AY, Samuels GJ, Rogerson CT, Lowen R. Genera of Bionectriaceae, Hypocreaceae and Nectriaceae (Hypocreales, Ascomycetes). Stud Mycol. 1999; 42:1–248.

Samuels GJ, Brayford D. Species of *Nectria* (sensu lato) with red perithecia and striate ascospores. Sydowia. 1994; 46:75–161.

Samuels GJ, Petrini O, Kuhls K, Lieckfeldt E, Kubicek CP. The *Hypocrea schweinitzii* complex and *Trichoderma* sect. *Longibrachiatum*. Stud Mycol. 1998; 41:1–54.

Samuels, GJ.; Rossman, AY. Conidia and classification of the nectrioid fungi. In: Kendrick, WB., editor. The Whole Fungus. Vol. 1. 1979. p. 167-179.

Seeler EV. A monographic study of the genus *Thyronectria*. J Arnold Arboretum Harvard Univ. 1940; 21:429–460.

Seifert KA. A monograph of *Stilbella* and some allied Hyphomycetes. Stud Mycol. 1985; 27:1–235.

Stamatakis E. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics. 2006; 22:2688–2690. doi:10.1093/bioinformatics/btl446. [PubMed: 16928733]

Swofford, DL. PAUP*: phylogenetic analysis using parsimony (*and other methods). Version 4.0b10. Sinauer Associates; Sunderland, Massachusetts: 2002.

Tamura K, Nei M. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol Biol Evol. 1993; 10:512–526. [PubMed: 8336541]

Vilgalys R, Hester M. Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. J Bacteriol. 1990; 172:238–4246.

Voglmayr H, Jaklitsch WM. *Prosthecium* species with *Stegonsporium* anamorphs on *Acer*. Mycol Res. 2008; 112:885–905. doi:10.1016/j.mycres.2008.01.020. [PubMed: 18554889]

Werle E, Schneider C, Renner M, Völker M, Fiehn W. Convenient single-step, one tube purification of PCR products for direct sequencing. Nucleic Acids Res. 1994; 22:4354–4355. doi:10.1093/nar/22.20.4354. [PubMed: 7937169]

White, TJ.; Bruns, T.; Lee, S.; Taylor, J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, MA.; Gelfand, DH.; Sninsky, JJ.; White, TJ., editors. PCR protocols: a guide to methods and applications. Academic Press; Dan Diego: 1990. p. 315-322.

Europe PMC Funders Author Manuscripts

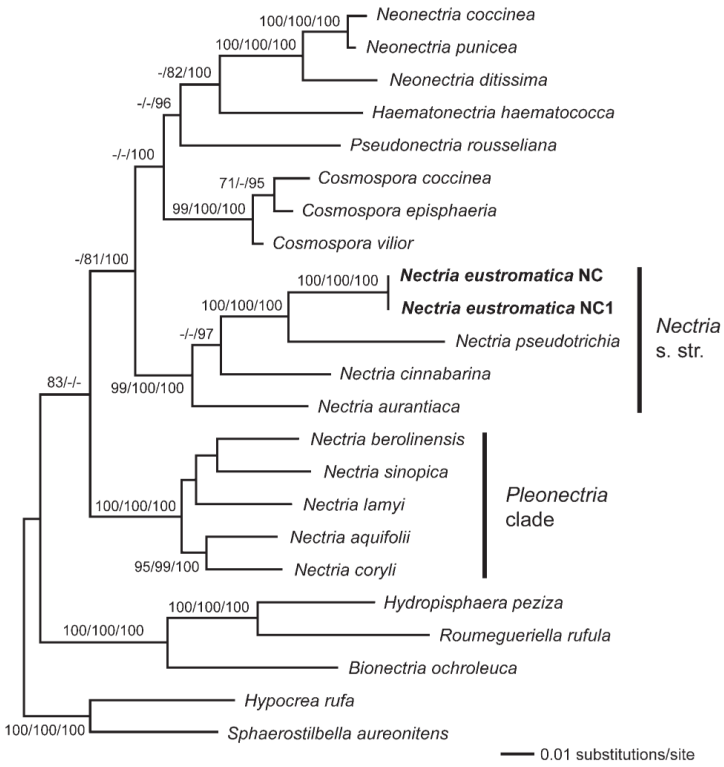

Fig. 1.

Phylogram of the best ML tree (lnL = −21091.24) revealed by RAxML from an analysis of the combined LSU-ITS-*rpb2*-*tef1* matrix of selected Hypocreales, showing the phylogenetic position of *Nectria eustromatica* within *Nectria* sensu stricto. MP bootstrap support above 70%, ML bootstrap support above 70% and Bayesian posterior probabilities above 90% are given at first, second and third position above or below the branches.

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

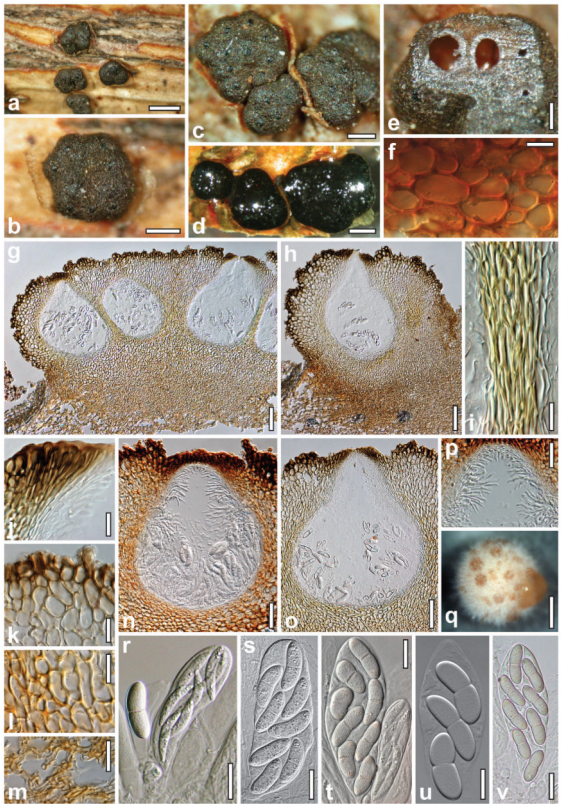

Fig. 2.
Teleomorph and sporodochia of *Nectria eustromatica*. a–c. Dry stromata (a. habit). d. Rehydrated stromata. e. Stroma cut horizontally showing orange-brown interior. f. Subcortical cells in cross-section in 3% KOH. g, h. Stromata in vertical section (h. uniperitheciate stroma; in lactic acid). i. Walls of two adjacent perithecia in section. j. Ostiolar apex cells. k. Cortical and subcortical tissue in section. l. Subperithecial tissue in section. m. Stroma base in section. n, o. Perithecia in section (n. in 50% glycerol; o. in lactic acid). p. Apical paraphyses. q. Sporodochium (CBS 125578, OA, 42 d). r–v. Asci and ascospores (r. immature ascus; t. mature and immature asci). Sources: a–c, e,f, r, t–v. WU 30194. d, g–p, s. WU 30195. Bars: a, q = 0.8 mm. b–d = 0.4 mm. e = 0.2 mm. f = 10 μm. g, h = 0.1 mm. i, j, l, u = 15 μm. k, r–t, v = 20 μm. m, p = 30 μm. n = 50 μm. o = 70 μm.

Europe PMC Funders Author Manuscripts

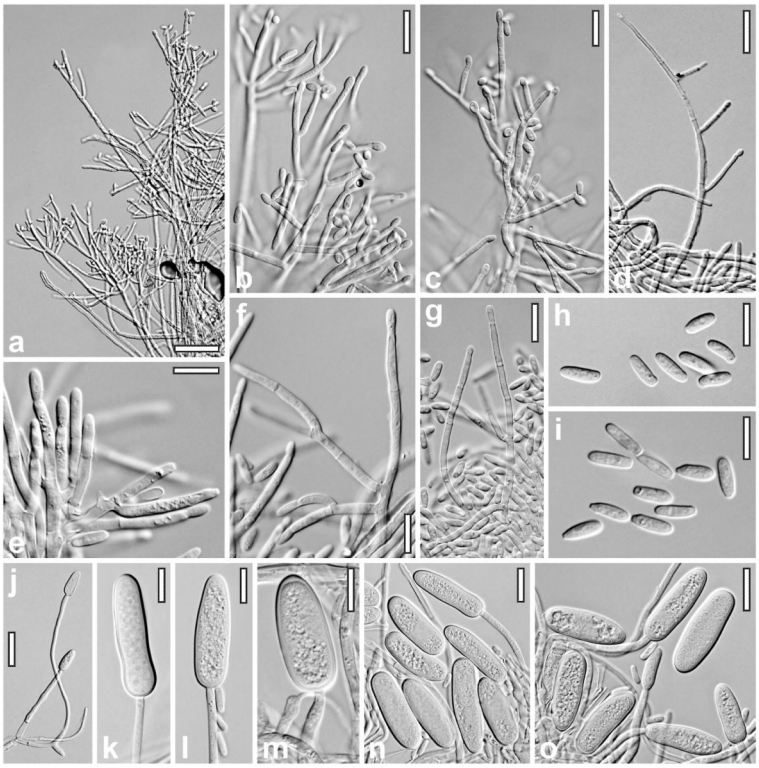

Fig. 3.

Anamorph of *Nectria eustromatica*. a–d. Conidiophores (a–c. MEA, 79 d; d. OA, 42 d). e–g. Phialides and conidia (e, f. PDA, 34 d; g. SNA, 26 d). h, i. Conidia (h. SNA, 26 d; i. PDA, 34 d). j. Macroconidia-forming conidiophore (OA, 42 d). k–o. Macroconidia (OA, 42 d). a–d, g, h, j–o. CBS 125578. e, f, i. CBS 121896. a–o. All at 20 ± 5 C. Bars: a = 30 μm. b, c, g, n, o = 15 μm. d, j = 20 μm. e, f, h, i, k–m = 10 μm.

Europe PMC Funders Author Manuscripts

Table I

GenBank accession numbers of the sequences used for multigene phylogenetic analyses. Sequences starting with HM were generated in the present study

Taxon	Strain	LSU	ITS	*rpb2*	*tef1*
Bionectria ochroleuca (Schwein.) Schroers & Samuels	CCFC 226708 CBS 376.55 AFTOL-ID 187	AY283558 — —	— AF358239 —	— — DQ862013	— — DQ862029
Cosmospora coccinea Rabenh.	A.R. 2741 CBS 114050	AY489734 —	— FJ474072	— DQ522438	AY489629 —
Cosmospora episphaeria (Tode) Rossman & Samuels	G.J.S. 98-160 G.J.S. 88-29	— AY015625	FJ474073 —	— —	— —
Cosmospora vilior (Starbäck) Rossman & Samuels	Guardbridge 20 G.J.S. 96-186	— AY015626	GU726755 —	— —	— —
Haematonectria haematococca (Berk. & Broome) Samuels & Rossman	voucher 83364 not specified ATCC MYA-4622	DQ119558 —	GU327638	Genome[a] —	Genome[a] —
Hydropisphaera peziza (Tode) Dumort.	G.J.S. 92-101 CBS 102038	AY489730 —	— —	— DQ522444	AY489625 —
Hypocrea rufa (Pers.) Fr.	CBS 114374 (G.J.S. 89-127) C.P.K. 1998	AY489726 —	— DQ677656	EF692510 —	— DQ672616
Nectria aquifolii (Fr.) Berk.	CBS 127381	HM534891	HM534891	HM534881	HM534870
Nectria aurantiaca (Tul. & C. Tul.) Jacz.	CBS 236.29	HM534892	HM534892	HM534882	HM534871
Nectria berolinensis (Sacc.) Cooke	CBS 127382	HM534893	HM534893	HM534883	HM534872
Nectria cinnabarina (Tode) Fr.	CBS 127383	HM534894	HM534894	HM534884	HM534873
Nectria coryli Fuckel	CBS 127384	HM534895	HM534895	HM534885	HM534874
Nectria eustromatica Jaklitsch & Voglmayr	CBS 121896 (NC)	HM534896	HM534896	HM534886	HM534875
Nectria eustromatica	CBS 125578 (NC1)	HM534897	HM534897	HM534887	HM534876
Nectria lamyi (Desm.) De Not.	CBS 127385	HM534898	HM534898	HM534888	HM534877
Nectria pseudotrichia (Schwein.) Berk. & M.A. Curtis	CBS 641.83	HM534899	HM534899	HM534889	HM534878
Nectria sinopica (Fr.) Fr.	CBS 127386	HM534900	HM534900	HM534890	HM534879
Neonectria coccinea (Pers.) Rossman & Samuels	CBS 237.29 CBS 29181 CBS 119159 CBS 118914	AY677327 — — —	— FJ474075 — —	— — DQ789819 —	— — — DQ789688

Europe PMC Funders Author Manuscripts

Taxon	Strain	LSU	ITS	*rpb2*	*tef1*
Neonectria ditissima (Tul. & C. Tul.) Samuels & Rossman	CBS 226.31	AY677330	—	—	—
	CBS 117752	—	DQ178168	—	—
	G.J.S. 94-12	—	—	DQ789823	—
	CBS 118927	—	—	—	DQ789743
Neonectria punicea (J.C. Schmidt) Castl. & Rossman	CBS 124262	HM534901	HM534901	—	HM534880
	CBS 119724	—	—	DQ789753	—
Pseudonectria rousseliana (Mont.) Wollenw.	A.R. 2716	U17416	—	—	AF543780
	not specified	—	FJ555527	—	—
	CBS 114049	—	—	DQ522459	—
Roumegueriella rufula (Berk. & Broome) Malloch & Cain	G.J.S. 91-164	EF469082	—	EF469116	EF469070
Sphaerostilbella aureonitens (Tul. & C. Tul.) Seifert, Samuels & W. Gams	TFC 96-77	AF160246	FJ442633	FJ442763	DQ834452
	G.J.S. 74-87				

[a]Retrieved from the JGI database (http://genome.jgi-psf.org/).

Europe PMC Funders Author Manuscripts

Table II

Source data, CBS culture numbers and herbarium vouchers of the specimens sequenced in the present study

Taxon	Geographic origin, year, collector	Host	Strain	Herbarium voucher
Nectria aquifolii	UK, Surrey, Royal Botanic Gardens Kew, 11 Nov 2008, H. Voglmayr	*Ilex aquifolium*	CBS 127381	WU 30360
Nectria aurantiaca	UK, Bristol, Oct 1929, E.W. Mason	*Ulmus campestris*	CBS 236.29	—
Nectria berolinensis	Austria, Wien, Floridsdorf, 13 Apr 2009, W. Jaklitsch	*Ribes sanguinea*	CBS 127382	WU 30361
Nectria cinnabarina	Austria, Niederösterreich, Litschau, 14 Sep 2009, W. Jaklitsch	*Frangula alnus*	CBS 127383	—
Nectria coryli	Austria, Oberösterreich, St. Willibald, 22 May 2009, H. Voglmayr	*Pyrus communis*	CBS 127384	WU 30362
Nectria eustromatica	Croatia, Opatija, Mošcenička Draga, 29 Mar 2007, W. Jaklitsch & H. Voglmayr	*Hippocrepis emerus*	CBS 121896 (NC)	WU 30194
Nectria eustromatica	Italy, Lazio, Bagnaia, Prov. Viterbo, 28 Jul 2009, W. Jaklitsch & H. Voglmayr	*Hippocrepis emerus*	CBS 125578 (NC1)	WU 30195
Nectria lamyi	Austria, Wien, Floridsdorf, 31 May 2009, W. Jaklitsch	*Berberis thunbergii*	CBS 127385	WU 30363
Nectria pseudotrichia	Venezuela; Edo Tachira, near La Fria, 21 Jul 1971, K.P. Dumont & al.	unidentified wood	CBS 641.83	—
Nectria sinopica	Austria, Niederösterreich, Maierhö fen, 20 Jun 2009, W. Jaklitsch & H. Voglmayr	*Hedera helix*	CBS 127386	WU 30364
Neonectria punicea	Austria, Kärnten, St. Margareten i. Rosental, 2 Nov 2008, W. Jaklitsch	*Frangula alnus*	CBS 124262	WU 30365