Stilbocrea walteri sp. nov., an unusual species of Bionectriaceae

Hermann Voglmayr 1, Walter M. Jaklitsch 1,2

Abstract
The new species Stilbocrea walteri is described and illustrated from Quercus ilex collected in Portugal. Phylogenetic analyses of LSU rDNA, rpb1, rpb2 and tef1 sequence matrices place S. walteri in the Bionectriaceae, Hypocreales, within a clade of specimens morphologically identified as Stilbocrea macrostoma, the generic type of Stilbocrea. Stilbocrea walteri differs from S. macrostoma in dark olive green to blackish ascomata basally immersed in a stroma, KOH+ and LA+ ascomata and the lack of a stilbella-like asexual morph on natural substrate and pure culture. A simple phialidic asexual morph is formed in pure culture. To enable a morphological comparison, Stilbocrea macrostoma is illustrated.

Keywords Ascomycota · Hypocreales · Nectria · Phylogenetic analysis · Sordariomycetes · Taxonomy

Introduction
During a collecting trip to Portugal, a black stromatic pyrenomycete was collected on dead corticated branches of Quercus ilex. Microscopic analyses revealed a nectriaceous fungus, which could not be identified to genus or species, and also the familial affiliation remained unclear. The partial immersion of ascomata in a well-developed stroma and reddening of the ascomatal walls in KOH pointed towards Nectriaceae, but molecular phylogenetic analysis based on LSU rDNA, rpb1, rpb2 and tef1 sequences revealed a placement within Bionectriaceae. Based on this evidence, a new species of Stilbocrea is described.

Materials and methods
Culture preparation, isolates and specimens
Cultures were prepared from ascospores and maintained as described previously (Jaklitsch 2009). Germinating ascospores were placed on CMD (CMA: Sigma, St Louis, Missouri; supplemented with 2% (w/v) D(+)-glucose-monohydrate) or 2% malt extract agar (MEA; 2% w/v malt extract, 2% w/v agar-agar; Merck, Darmstadt, Germany). The plates were sealed with laboratory film and incubated at room temperature. Cultures used for the study of the asexual morph were grown on 2% MEA or CMD at room temperature (22 ± 3 °C) under alternating 12 h daylight and 12 h darkness. The ex-type culture was deposited at the Westerdijk Fungal Biodiversity Centre (CBS-KNAW), Utrecht, The Netherlands, and specimens in the Fungarium of the Institute of Botany, University of Vienna (WU). The following specimens of Stilbocrea macrostoma were sequenced for the phylogenetic analyses and/or used for morphological illustration and comparison but are not described in detail here: Panama, Parque Nacional Altos de Campana, on dead branch of an unidentified tree, S.
Table 1  List of taxa and GenBank accessions used in the current phylogenetic study. The references are according to the NCBI Nucleotide database. Sequences in bold were generated during the present study.

| Taxon                        | LSU       | rpb1     | rpb2     | tef1     | References                                           |
|------------------------------|-----------|----------|----------|----------|-----------------------------------------------------|
| “Acremonium” acutatum        | NG_056976|          |          |          | Summerbell et al. (2011)                             |
| “Acremonium” alternatum      | NG_056977|          |          |          | Summerbell et al. (2011)                             |
| “Acremonium” fusidioides     | NG_056984|          |          |          | Summerbell et al. (2011)                             |
| “Acremonium” hennebertii     | NG_056987|          |          |          | Summerbell et al. (2011)                             |
| “Acremonium” sclerotigenum   | NG_057139| KC998999 | KC998988 |          | Hijikawa et al. (2017), Grum-Grzhimaylo et al. (2013b) |
| “Acremonium” zeylanicum      | HQ232154  |          |          |          | Summerbell et al. (2011)                             |
| Bryocentria brongniartii     | EU940125  |          |          |          | Stenroos et al. (2010)                               |
| Bryocentria metzgeriae       | EU940106  |          |          |          | Stenroos et al. (2010)                               |
| Bulbithecium hyalosporum     | AF096187  |          |          |          | Suh and Blackwell (1999)                             |
| Bullanockia australis        | KY173506  |          |          |          | Crous et al. (2016a)                                 |
| Calonectria cylindrospora    | U17409    |          |          |          | Rehner and Samuels (1995)                            |
| Chaetopsina fulva            | DQ119554  |          |          |          | Zhang and Zhuang (unpubl.)                           |
| Clonostachys buxi            |           | KM232416 |          |          | Lombard et al. (2015)                                |
| Clonostachys byssicola       | GQ506040  |          |          | LT220768 | Hirooka et al. (2010), Sharma and Marques (unpubl.) |
| Clonostachys compactuscula    | GQ506036  |          |          |          | Hirooka et al. (2010)                                |
| Clonostachys epichloe         | DQ363259  |          |          |          | Kirschner (unpubl.)                                 |
| Clonostachys grammicospora   | AF193238  |          |          |          | Rossman et al. (2001)                                |
| Clonostachys pityrodes        | AY489728  | AY489658 |          |          | Castlebury et al. (2004)                             |
| Clonostachys rosea           | AY283558  | GQ506038 | DQ522415 | AY489611 | Seifert et al. (2003), Hirooka et al. (2010), Spatafora et al. (2007), Castlebury et al. (2004) |
| Clonostachys setosa          | AF210670  |          |          |          | Schroers (2001)                                      |
| Cosmospora coccinea          | AY489734  |          |          |          | Castlebury et al. (2004)                             |
| Cyanonectria cyanostoma      | FJ474081  |          |          |          | Samuels et al. (unpubl.)                             |
| Cylindrocladiella microcylindrica | AY793432      |          |          |          | Crous et al. (2005)                                  |
| Dialonectria epispheeria     | AY015625  |          |          |          | Zhang and Blackwell (2002)                           |
| Emericellopsis alkalina      |           |          |          | KC999029 | KC998993 | Grum-Grzhimaylo et al. (2013) |
| Emericellopsis glabra        | GQ505993  | GQ506023 |          |          | Hirooka et al. (2010)                                |
| Emericellopsis maritima      | FJ176861  |          |          | KC999033 | KC998997 | Grum-Grzhimaylo et al. (2013b) |
| Emericellopsis minima        |           |          |          | KC999031 | KC998996 | Grum-Grzhimaylo et al. (2013b) |
| Emericellopsis pallida       |           |          |          | KC999034 |          | Grum-Grzhimaylo et al. (2013b) |
| Emericellopsis terricola     | U57082    |          |          |          | Glenn and Bacon (unpubl.)                            |
| Eucapheria capensis          | EF110619  |          |          |          | Crous et al. (2007)                                  |
| Eucapheria rustica           | KY173501  |          |          |          | Crous et al. (2016a)                                 |
| Flammocladiella decorata     | NG_058175|          |          |          | Crous et al. (2015a)                                 |
| Geonectria subalpina         | MH155487  |          |          | KY872747 |          | Lechat et al. (2018)                                 |
| Geosmithia brunnnea          |          |          |          |          |          | Huang et al. (unpubl.)                               |
| Geosmithia langdonii         |           |          |          | HG799928 |          | Kolarik et al. (unpubl.)                             |
| Geosmithia lavendula         |          |          |          | HG799879 |          | Stielow et al. (unpubl.)                             |
| Geosmithia microcortyli      |          |          |          | FM986794 |          | Kolarik and Kirkendall (2010)                        |
| Geosmithia pallida           |           |          |          | HG799930 |          | Kolarik et al. (unpubl.)                             |
| Geosmithia proliferans       |           |          |          | KY872749 |          | Huang et al. (unpubl.)                               |
| Taxon                           | LSU         | rpb1        | rpb2        | tef1        | References                                                                 |
|--------------------------------|-------------|-------------|-------------|-------------|-----------------------------------------------------------------------------|
| Geosmithia putterillii         | KT155185    | HG799907    | HG799853    |             | Stielow et al. (unpubl.), Kolarik et al. (unpubl.)                         |
| Gliomastix massaei             | HQ232060    |             |             |             | Summerbell et al. (2011)                                                   |
| Gliomastix murorum             |             |             | FJ238363    |             | Schoch et al. (unpubl.)                                                   |
| Gliomastix roseogrisea         | HQ232122    |             |             |             | Summerbell et al. (2011)                                                   |
| Heleococcum aurantiacum        | JX158463    | JX158463    | JX158397    |             | Grum-Grzhimaylo et al. (2013a)                                             |
| Heleococcum japonense          | JX158442    | JX158464    | JX158398    |             | Grum-Grzhimaylo et al. (2013a)                                             |
| Heleococcum japonicum          | U17429      |             |             |             | Rehner and Samuels (1995)                                                  |
| Hydropisphaera erubescens      |             | DQ518182    |             | DQ522344    | James et al. (unpubl.), AFTOL (unpubl.), Spatafora et al. (2007)           |
| Hydropisphaera fungicola       |             | GQ506025    |             |             | Hirooka et al. (2010)                                                      |
| Hydropisphaera peciza          |             |             |             | DQ522444    | Castlebury et al. (2004), Spatafora et al. (2007)                          |
| Hydropisphaera suffulta        | GU017530    |             |             |             | Lechat (unpubl.)                                                           |
| Ijuhya chilensis               | KY607553    | KY607579    |             |             | Ashrafi et al. (2017)                                                      |
| Ijuhya corynospora             | KY607580    |             |             |             | Ashrafi et al. (2017)                                                      |
| Ijuhya faveliana               | KY607582    |             |             |             | Ashrafi et al. (2017)                                                      |
| Ijuhya fournieri               | KP899118    |             |             |             | Lechat et al. (2015)                                                       |
| Ijuhya paraparilis             | GQ506041    |             |             |             | Hirooka et al. (2010)                                                      |
| Ijuhya parilis                 | KY607584    |             |             |             | Ashrafi et al. (2017)                                                      |
| Ijuhya peristomialis           | KY607559    | KY607585    |             |             | Ashrafi et al. (2017)                                                      |
| Ijuhya vitellina               | KY607577    |             |             |             | Ashrafi et al. (2017)                                                      |
| Kallichroma glabrum            | AF193233    |             |             |             | Rossman et al. (2001)                                                      |
| Kallichroma tethys             | AF193234    |             |             |             | Rossman et al. (2001)                                                      |
| Lasionectria mantuana          |             |             |             | GQ506024    | Rossman et al. (2001)                                                      |
| Lasionectriella rubioi         | KU593581    |             |             |             | Lechat and Fournier (2016)                                                 |
| Leuconectria clusiae           | U17412      |             |             |             | Rehner and Samuels (1995)                                                  |
| Leucosphaerina arxii           | NG_057892   |             |             |             | Summerbell et al. (2011)                                                   |
| Mycoarachis inversa            | NG_059437   | GQ506021    |             | HM484840    | Hirooka et al. (2010), Chaverri et al. (2011)                              |
| Myrothecium inundatum          | KU846474    |             |             |             | Lombard et al. (2016)                                                      |
| Nectria aurantiaca             | HM534892    |             |             | HM484577    | Jaklitsch and Voglmayr (2011b)                                             |
| Nectria cinnabarina            | HM534894    |             | JQ014125    | AF543785    | Jaklitsch and Voglmayr (2011b), Hirooka et al. (2011), Schoch et al. (2012), Currie et al. (2003) |
| Nectria pseudotrichia          | HM534899    |             |             |             | Jaklitsch and Voglmayr (2011b)                                             |
| Nectriopsis epimyocota         | GQ506037    |             |             |             | Hirooka et al. (2010)                                                      |
| Nectriopsis exigua             | GQ506014    |             |             | HM484852    | Hirooka et al. (2010), Chaverri et al. (2011)                              |
| Nectriopsis violacea           | AF193242    |             |             |             | Rossman et al. (2001), Castlebury et al. (2004)                            |
| Neocosmospora haematococca     | DQ119558    |             |             |             | Zhang and Zhuang (unpubl.), Castlebury et al. (2004)                        |
| Neocosmospora vasinfecta       | U17406      |             |             |             | Rehner and Samuels (1995)                                                  |
| Neonecristia coccinea          | AY677327    |             |             |             | Halleen et al. (2004)                                                      |
Table 1 (continued)

| Taxon                   | LSU   | rpb1            | rpb2            | tef1            | References                                      |
|-------------------------|-------|-----------------|-----------------|-----------------|------------------------------------------------|
| Neonectria ditissima     | AY677330 |                 |                 |                 | Halleen et al. (2004)                           |
| Neonectria punicea       | HM534901 |                 |                 |                 | Jaklitsch and Voglmayr (2011b)                  |
| Niesslia exilis          | AY489720 |                 |                 |                 | Castlebury et al. (2004)                        |
| Nigrosabulum globosum    | AF096195 |                 |                 |                 | Suh and Blackwell (1999)                        |
| Ochronectria calami      | AF193243 | AY489644 | EF692515 | AY489612 | Rossman et al. (2001), Castlebury et al. (2004), Sung et al. (2008) |
| Ovicillium attenuatum    | KU382232 |                 |                 |                 | Zare and Gams (2016)                            |
| Paracylindrocarpon aloicola | KX228328 |                 |                 |                 | Crous et al. (2016b)                            |
| Peethambara spirostriata | AY489724 |                 |                 |                 | Castlebury et al. (2004)                        |
| Peethambara sundara      | AY193245 |                 |                 |                 | Rossman et al. (2001)                           |
| Penicillifer diparietispora | AY489735 |                 |                 |                 | Castlebury et al. (2004)                        |
| Persiciospora africana   | AY015631 |                 |                 |                 | Zhang and Blackwell (2002)                      |
| Protocreopsis korfii     | KT852955 |                 |                 |                 | Lechat and Fournier (2015)                      |
| Protocreopsis pertusa    | GQ506002 |                 |                 |                 | Hirooka et al. (2010)                           |
| Pseudocosmospora vilior  | AY015626 |                 |                 |                 | Zhang and Blackwell (2002)                      |
| Rosasphaeria moravica    | JF440985 |                 |                 |                 | Jaklitsch and Voglmayr (2012)                    |
| Roumegueriella rafula    | EF469082 | GQ506029 | EF469116 | EF469070 | Sang et al. (2007a), Hirooka et al. (2010)      |
| Sarcopodium macalpinei   | DQ195666 |                 |                 |                 | Zhang and Zhuang (unpubl.)                      |
| Selinia pulchra          | AY489264 | GQ506022 |                 | HM484841 | Rossman et al. (2001), Hirooka et al. (2010), Chaverri et al. (2011) |
| Stachybotrys chartarum   | KU846792 |                 |                 |                 | Lombard et al. (2016)                           |
| Stephanonectria keithii  | AY489727 |                 |                 | AY489622 | Castlebury et al. (2004)                        |
| Stilbocrea macrostoma    | AY489725, GQ506033, AY489655, MH562716 | GO506004, | EF692520, MH577043 | MH562716, MH562716 | Hirooka et al. (2010), Castlebury et al. (2004), Sung et al. (2008), this study |
| Stilbocrea sp.           | JQ733407 |                 |                 |                 | Supaphon et al. (2017)                          |
| “Stilbocrea” sp.         | KX578037 |                 |                 |                 | Lechat (unpubl.)                                |
| Stilbocrea walteri       | MH562717 | MH562715 | MH577042 | MH562714 | this study                                      |
| Stromatonecrtia caraganae | HQ112287 | HQ112290 | HQ112286 |         | Jaklitsch and Voglmayr (2011a)                  |
| Synnemellisia aurantia   | KX866396 |                 |                 |                 | Lisboa et al. (unpubl.)                         |
| Thyronectria aquifoliol | HM534891 |                 |                 |                 | Jaklitsch and Voglmayr (2011b)                  |
| Thyronectria berolinensis | HM534893 |                 |                 |                 | Jaklitsch and Voglmayr (2011b)                  |
| Thyronectria coryli      | HM534895 |                 |                 |                 | Jaklitsch and Voglmayr (2011b)                  |
| Thyronectria lamyi       | HM534898 |                 |                 |                 | Jaklitsch and Voglmayr (2011b)                  |
| Thyronectria rhodochlora | HM534900 |                 |                 | KJ570728 | KJ570751 | Jaklitsch and Voglmayr (2014)                  |
| Thyronectria sinopica    | GQ506013 | GQ506018 |                 |                 | Jaklitsch and Voglmayr (2011b)                  |
| Verrucostoma freycinetiae | GQ506013 |                 |                 |                 | Hirooka et al. (2010)                           |
| Verrucostoma martinicensis | KP192672 |                 |                 |                 | Crous et al. (2015b)                            |
| Volutella buxi           | U17416 |                 |                 |                 | Rehner and Samuels (1995)                       |
| Xanthonecrtia pseudopezica | KU946964 |                 |                 |                 | Lechat et al. (2016)                            |
Morphological observations

Microscopic preparations were mounted in water, 3% potassium hydroxide (KOH) or lactic acid (LA). Stereomicroscopy illustrations and measurements were done with a Keyence VHX-6000 system. Light microscopy was performed with Nomarski differential interference contrast (DIC) using the Zeiss Axio Imager.A1 compound microscope, and images and data were gathered using the Zeiss AxioCam 506 colour digital camera and measured by using the Zeiss ZEN Blue Edition software. 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.

DNA extraction, PCR and sequencing

Growth of liquid culture and extraction of genomic DNA was done according to Voglmayr and Jaklitsch (2011), using the DNeasy Plant Mini Kit (QIAgen GmbH, Hilden, Germany). To confirm the identity of the culture, DNA was also extracted from stromata following the protocol of Voglmayr and Jaklitsch (2011) for herbarium specimens, but using the DNeasy Plant Mini Kit. The complete ITS region and D1 and D2 domains of 28S rDNA region (ITS-LSU) were amplified with primers V9G (de Hoog and Gerrits van den Ende 1998) and LR5 (Vilgalys and Hester 1990), a ca. 750 bp fragment of the RNA polymerase II subunit 1 (rpb1) gene with primers RPBI-Ac (Schoch et al. 2012) and RPBI1Cr (Sung et al. 2007b), a ca. 1.1 kb fragment of the RNA polymerase II subunit 2 (rpb2) gene with primers fRPB2-5F and fRPB2-7cR (Liu et al. 1999) or dRPB2-5f and dRPB2-7r (Voglmayr et al. 2016) and a ca. 1.4 kb fragment of the translation elongation factor 1-α (tefl1) gene with primers EF1-728F (Carbone and Kohn 1999) and EF1-2218R (Rehner and Buckley 2005). From stromatal DNA, only the ITS-LSU was amplified and sequenced. PCR was performed with a Taq polymerase, with annealing temperatures of 55 °C for ITS-LSU, tefl1 and rpb2 (primer pair fRPB2-5F, rPB2-7cR) and 51 °C for rpb1 and rpb2 (primer pair dRPB2-5f, dRPB2-7r). PCR products were purified using an enzymatic PCR cleanup (Werle et al. 1994) as described in Voglmayr and Jaklitsch (2008). DNA was cycle-sequenced using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit v. 3.1 (Applied Biosystems, Warrington) and the PCR primers; in addition, primers ITS4 (White et al. 1990), LR3 (Vilgalys and Hester 1990) and LR2R-A (Voglmayr et al. 2012) were used for the ITS-LSU region. Sequencing was performed on an automated DNA sequencer (ABI 3730x1 Genetic Analyser, Applied Biosystems).

Phylogenetic analyses

As the LSU rDNA is the most representative marker available for many genera of Bionectriaceae, an extended LSU matrix was produced for phylogenetic analyses. For this, the sequence matrix of Jaklitsch and Voglmayr (2011a) was supplemented with selected sequences from Summerbell et al. (2011) and a few additional GenBank sequences. Only few rpb1, rpb2 and tefl1 sequences of Bionectriaceae were available from GenBank to phylogenetically place Stilbocrea. For the same reason, ITS rDNA was not phylogenetically analysed. The GenBank accession numbers of sequences downloaded for phylogenetic analyses are given in Table 1 and in the phylogenetic trees (Figs. 1 and 2), following the taxon names. Generic classification of the Nectriaceae follows Lombard et al. (2015), of Stachybotryaceae Lombard et al. (2016) and of Bionectriaceae the taxonomy implemented in NCBI GenBank, with a few additions of recently published new genera.

The downloaded GenBank sequences were aligned with the sequences generated in our study with the server version of MAFFT (www.ebi.ac.uk/Tools/mafft) using the default settings and checked and refined with BioEdit v. 7.0.9.0 (Hall 1999). The four matrices were analysed separately. The final matrices used for phylogenetic analyses contained 863, 750, 1072 and 951 alignment characters for the LSU, rpb1, rpb2 and tefl1, respectively.

Maximum parsimony (MP) analyses were performed with PAUP v. 4.0a161 (Swofford 2002), using 1000 replicates of heuristic search with random addition of sequences and subsequent TBR branch swapping.
Sequencing and molecular phylogeny

The ITS-LSU sequences obtained from the culture and the stromata of the newly described fungus were identical. Sequence similarity of the ITS of the newly described fungus and the newly sequenced *Stilbocrea macrostoma* accession from Panama (SM) was 83.5% (71 nucleotide substitutions and 14 gaps).

Of the 866 nucleotide characters included in the LSU analyses, 163 were parsimony informative. Maximum parsimony analyses revealed 24 MP trees 1202 steps long, one of which is shown as Fig. 1. The MP trees differed mainly in the deeper nodes of Nectriaceae (Fig. 1); in some of the MP trees, Stachybotryaceae were embedded within the Nectriaceae (not shown). In the phylogenetic analyses, the Stachybotryaceae were moderately supported, while the clade comprising Bionectriaceae plus Flammocadiellaceae received high support. The Flammocadiellaceae were revealed as sister group to Bionectriaceae in the MP analyses; however, the latter did not receive significant bootstrap support (Fig. 1). Within Bionectriaceae, backbone support of deeper nodes was mostly low or absent. The GenBank accessions of *Stilbocrea* included in our LSU analyses did not form a monophyle in this

unpublished accession KX578037 from Spain labelled *Stilbocrea* sp. was placed outside the *Stilbocrea* clade. The three accessions of *Stilbocrea macrostoma*, the fungus from Portugal and two GenBank accessions of endophyte isolates from tropical marine seagrasses (JQ733407; GU017530) formed a monophylum with low support (Fig. 1). However, the various accessions of *Stilbocrea macrostoma* did not form a monophylum, as the newly sequenced *S. macrostoma* specimen from Panama was in a basal position to a highly supported subclade containing the new *Stilbocrea* species from Portugal, the GenBank accessions of *S. macrostoma* from New Zealand and Sri Lanka and the two endophyte isolates.

Of the 750 nucleotide characters included in the *rpb1* analyses, 367 were parsimony informative. Maximum parsimony analyses revealed two MP trees 2320 steps long, one of which is shown as Fig. 2a. The two MP trees were identical except for an interchanged position of *Ijuhya peristomialis* and *Ijuhya parilis* (not shown). Of the 1072 nucleotide characters included in the *rpb2* analyses, 533 were parsimony informative. Maximum parsimony analyses revealed a single MP tree 2597 steps long which is shown as Fig. 2b. Of the 951 nucleotide characters included in the *tef1* analyses, 231 were parsimony informative. Maximum parsimony analyses revealed a single MP tree 957 steps long which is shown as Fig. 2c.

In the analyses of the protein-coding genes (*rpb1, rpb2, tef1*), many of the deeper nodes within Bionectriaceae received no or low support (Fig. 2a–c), and only limited comparisons are possible between these trees due to a different taxon selection. However, the new fungus from Portugal and the GenBank accessions of *Stilbocrea macrostoma* from New Zealand (all three markers available) and Sri Lanka (only *rpb1* available) consistently formed a clade with maximum support (Fig. 2a–c), while the newly sequenced Panamese accession of *Stilbocrea macrostoma* was not contained in this

RESULTS

**Sequencing and Molecular Phylogeny**

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Of the 866 nucleotide characters included in the LSU analyses, 163 were parsimony informative. Maximum parsimony analyses revealed 24 MP trees 1202 steps long, one of which is shown as Fig. 1. The MP trees differed mainly in the deeper nodes of Nectriaceae (Fig. 1); in some of the MP trees, Stachybotryaceae were embedded within the Nectriaceae (not shown). In the phylogenetic analyses, the Stachybotryaceae were moderately supported, while the clade comprising Bionectriaceae plus Flammocadiellaceae received high support. The Flammocadiellaceae were revealed as sister group to Bionectriaceae in the MP analyses; however, the latter did not receive significant bootstrap support (Fig. 1). Within Bionectriaceae, backbone support of deeper nodes was mostly low or absent. The GenBank accessions of *Stilbocrea* included in our LSU analyses did not form a monophylum as the

unpublished accession KX578037 from Spain labelled *Stilbocrea* sp. was placed outside the *Stilbocrea* clade. The three accessions of *Stilbocrea macrostoma*, the fungus from Portugal and two GenBank accessions of endophyte isolates from tropical marine seagrasses (JQ733407; GU017530) formed a monophylum with low support (Fig. 1). However, the various accessions of *Stilbocrea macrostoma* did not form a monophylum, as the newly sequenced *S. macrostoma* specimen from Panama was in a basal position to a highly supported subclade containing the new *Stilbocrea* species from Portugal, the GenBank accessions of *S. macrostoma* from New Zealand and Sri Lanka and the two endophyte isolates.

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clade (Fig. 2a, b). Remarkably, in the rpb1 tree (Fig. 2a), the fungus from Portugal was placed in a sister group position to the GenBank accessions of Stilbocrea macrostoma from New Zealand and Sri Lanka with medium (84%; MP) to high (95%; ML) support.

Considering morphological and molecular data, the specimen from Portugal is described as a new species.

**Taxonomy**

**Stilbocrea walteri** Voglmayr & Jaklitsch, sp. nov. Figs. 3 and 4.

MycoBank MB 826919.

Etymology: in honour of Walter Gams.

Stromata when dry (460–)680–1100(–1600) µm diam (n = 50), (260–)300–430(–520) µm high (n = 30), scattered, less commonly in groups of 2–3, erumpent from bark, pulvinate; round, elliptical or irregular in outline. Stromata at the base compact, white in section. Perithecia (2–)5–15(–20) per stroma, basally immersed in the uppermost layer of the stroma, dark olive green to black when dry, black in water; in 3% KOH with a reddish tinge, reversible after addition of LA, no pigment dissolved. Ostiolar dots (24–)31–42(–47) µm diam (n = 33), umbilicate, black.

Subperithecial and basial tissue of the stroma mostly consisting of a t. angularis of thin-walled, hyaline cells (6–)7.5–15(–18.5) × (3.5–)5–8.5(–11) µm (n = 30), becoming hyphal adjacent to the host tissue; stroma tissue without colour change in KOH or LA. Perithecia (205–)216–271(–277) µm high, (153–)171–234(–250) µm wide (n = 12), globose to subglobose, partially immersed in stroma, apical parts exposed. Ostioles periphysate, periphyses 12–34 µm long, 1.2–2 µm wide (n = 10). Peridium 35–90 µm thick, consisting of three layers: a 6–24-µm thick inner layer of hyaline to subhyaline, thick-walled (outermost) to thin-walled (innermost) elongate cells (6–)8–15(–19) × (1–)2–4(–5) µm (n = 50); a 13–24-µm-thick medium layer of dark olive green, thick-walled, elongate cells (6–)8–15(–18.5) × (4–)5–8(–11) µm (n = 30) turning red brown in KOH and olivaceous toumber brown in LA; and a 16–49-µm-thick outer layer of subhyaline to light olive green, thick-walled, elongate to isodiamic cells (5.5–)6.5–10.5(–12.5) × (3.5–)5–5.5(–7) µm (n = 30); surface sometimes covered by a thin outer layer of collapsed cells and amorphous material. Ascii oblong, narrowly clavate or fusoid, lacking a differentiated apical apparatus, upper part with eight uniseriate ascospores (45–)53–66(–72) × (8–)9–10.5(–11) µm (n = 27), lower part stipe-like, ca. 8–20 µm long. Ascospores (8.5–)9.5–11(–12.5) × (4.0–)4.5–5(–5.5) µm, l/w (1.6–)1.9–2.4(–2.9) (n = 130), ellipsoid, oblong or fusoid, hyaline, with a median or slightly eccentric septum, straight, symmetric or slightly curved, slightly constricted at the septum, with broadly rounded ends, distinctly verrucose in water and LA, smooth in 3% KOH, with one large guttule per cell. Asexual morph on natural substrate not seen.

Cultures and asexual morph: colonies slow-growing, reaching 29 mm diam in 10 days on CMD; on MEA compact, flat, with white surface and yellowish reverse, after 1 month irregularly lobate, greyish brown in the centre, ochraceous with whitish patches at the margin; on CMD cottony, white, with abundant surface mycelium of hyphae commonly aggregated to hyphal strands, reverse yellowish. Conidiophores consisting of intercalary phialides with short lateral conidiiferous pegs (0.7–)0.8–3.0(–4.3) × (0.9–)1.1–1.6(–1.8) µm (n = 22), and terminally and laterally formed phialides. Phialides abundant on aerial mycelium, lageniform to cylindrical, (3–)7–15.5(–22) × (1.2–)1.6–2.3(–2.5) µm (n = 40). Conidia (3.5–)4.5–5.5(–6.5) × (1.3–)1.5–2.0(–2.5) µm, l/w (2.0–)2.6–3.3(–3.7) (n = 100), unicellular, allantoid, hyaline, smooth, commonly with a guttule at or towards one or both ends; after few days swelling to irregular shapes and up to ca. 9 × 3.5 µm. Blastoconidia formed on CMD in masses in the colony centre a few days after inoculation, hyaline, first ellipsoid to subglobose, globose and thick-walled with age, (2.5–)3–4.5(–5.5) µm diam (n = 120).

Distribution: Only known from a single collection in Portugal

Host: On dead corticated branches of *Quercus ilex*; probably saprobic

Holotype: Portugal, Parque Natural de Sintra-Cascais, S Monserrate, on *Quercus ilex*, 18 Feb. 2017, H. Voglmayr (WU 39972); ex-holotype culture NQI = CBS 144627; ex-holotype sequences MH562717 (ITS-LSU rDNA), MH562715 (rpb1), MH577042 (rpb2), MH562714 (tefl)
In the phylogenetic analyses (Figs. 1 and 2), the fungus described here was unexpectedly placed in Bionectriaceae. Dark stromata and/or ascomata are not typically seen in Hypocreales, although they are formed by numerous nectriaceous species such as Nectria eustromatica (Jaklitsch and Voglmayr 2011b) or Thyronectria obscura (Jaklitsch and Voglmayr 2014). The species also shows a KOH-positive reaction of the ascomatal wall which is commonly seen in Nectriaceae (Rossman et al. 1999), but phylogenetic analyses of LSU sequences clearly placed the new fungus within Bionectriaceae, in a clade containing three accessions identified as StilbocREA macrostoma (Fig. 1). Based on morphological distinctness, we consider the specimen from Portugal to represent a new species, described here as S. walteri. It differs substantially from S. macrostoma, and all putative synonyms listed in Seifert (1985) and Rossman et al. (1999), in its dark olive green to black perithecia, KOH and LA-positive reactions, compact stromata and a lack of a stilbella-like asexual morph. Stilbocrea walteri also contains much fewer perithecia which are apically free and only basally immersed in the stroma, whereas S. macrostoma contains numerous, up to several hundred ascomata almost entirely immersed in the stroma, resulting in a hypocrea-like appearance (Seifert 1985). Also, the stroma texture differs between the two species (a textura angularis-globulosa of thick-walled cells in S. walteri; a hyphal textura intricata with a surface layer of irregularly branched hyphae (cf. Figs. 2q and 4F-i; Seifert 1985, Rossman et al. 1999) in S. macrostoma). In addition, S. macrostoma is primarily a tropical to subtropical species, which to our knowledge has not been recorded from Europe. Notably, there are also a few characters of Stilbocrea walteri shared with S. macrostoma, like ascospores of similar size with a verruculose ornamentation disappearing in KOH (see Figs. 4e–v and 5j–q). Due to these marked discrepancies which could cast doubts on the reliability of the DNA sequences, DNA extraction was repeated directly from stromata, which revealed identical ITS-LSU sequences from stromata and culture, confirming that the sequences originate from the target fungus.

Our analyses (Figs. 1 and 2) may suggest that morphology of the sexual morph is not a good character for classification within Bionectriaceae and Nectriaceae. Asexual morphs, however, are not superior in this regard, as e.g. synnematous, stilbella-like asexual morphs also occur in the Nectriaceae, e.g. in Nectria pseudotrichia (Hirooka et al. 2012), and acremonium-like forms also in several other unrelated families of the Sordariomycetes (see, e.g. Summerbell et al. 2011). Also, the simple phialidic asexual morph of S. walteri observed in pure culture does not provide much phylogenetic information, as similar asexual morphs occur in various hypocrealean lineages.

Except for the commonly sequenced LSU, very few additional sequence data are available for most genera of Bionectriaceae. Apart from the well-studied genera Geosmithia and Clonostachys, even the ITS rDNA is lacking for many taxa. From the four species currently accepted in Stilbocrea (Rossman et al. 1999, de Beer et al. 2013), sequence data are available only for the generic type, Stilbocrea macrostoma. However, all three LSU sequences labelled as S. macrostoma differ substantially, and the two accessions from Sri Lanka and New Zealand form a highly supported subclade with the morphologically deviating S. walteri (Fig. 1), which is also seen in the analyses of the protein-coding genes (Fig. 2). Remarkably, this clade also contains two LSU sequences of endophyte isolates from the tropical marine seagrasses Enhalus acoroides (Sakayaraj et al. 2010) and Thalassia hemprichii (Supaphon et al. 2017), but unfortunately, no morphological data are available for them. In the LSU tree, the newly sequenced Panamese accession of S. macrostoma occupies a basal position in the poorly supported Stilbocrea clade (Fig. 1), but it is placed outside the Stilbocrea clade in the rpb1 and rpb2 trees (Fig. 2a, b), indicating that these accessions represent distinct species which may even not be congeneric. These results, together with the poor backbone support in the phylogenetic analyses (Figs. 1 and 2), suggest that a single gene alone is insufficient to provide a sound basis for defining phylogenetic generic concepts within the Bionectriaceae. A
wide pantropical to warm-temperate distribution of *S. macrostoma* has been derived in the premolecular era (Seifert 1985), but if all sequences are correct in terms of generation from morphologically identical fungal material, then *S. macrostoma* will most probably be split into several species in future. Several taxa described from the Old and New World and synonymised with *S. macrostoma* based on morphology (Seifert 1985, Rossman et al. 1999) will then need to be reconsidered and re-examined in detail. Remarkably, in their description of *S. macrostoma*, Seifert (1985) and Rossman et al. (1999) mentioned ascomata occasionally becoming red-brown to dark olive green with age; however, we have not seen any dark green colour in our material investigated. Much more sampling and generation of molecular data including protein-coding phylogenetic markers of Bionectriaceae are necessary to reveal a clearer picture of phylogenetic relationships within the family and to achieve a robust species classification and delimitation.
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References

Ashrafi S, Helaly S, Schroers HJ, Stadler M, Richert-Poeggeler KR et al (2017) *Ibuha vietlina* sp. nov., a novel source for chaetoglobosin A, is a destructive parasite of the cereal cyst nematode *Heterodera filipjevi*. PLoS One 12:e0180032

Carbone I, Kohn LM (1999) A method for designing primer sets for speciation studies in filamentous ascomycetes. Mycologia 91:553–556

Castlebury LA, Rossman AY, Sung GH, Hyten AS, Spatafora JW (2004) Multigene phylogeny reveals new lineage for *Stachybotrys chartarum*, the indoor air fungus. Mycol Res 108:864–872

Chaverri P, Salgado C, Hirooka Y, Rossman AY, Samuels GJ (2011) Delimitation of *Neonectria* and *Cylindrocarpon* (Nectriaceae, Hypocreales, Ascomycota) and related genera with *Cylindrocarpon-*like anamorphs. Stud Mycol 68:57–78

Crous PW, Allegrucci N, Arambari AM, Cazau MC, Groenewald JZ, Wingfield MJ (2005) *Deamatiocladium cervidis* gen. sp. nov. (Nectriaceae, Hypocreales), a new genus from *Cells* leaf litter in Argentina. Mycol Res 109:833–840

Crous PW, Mohammed C, Glen M, Verkley GJM, Groenewald JZ (2007) *Eucalyptus* microfungi known from culture. 3. *Eucasphearia* and *Sympoventuria* genera nova, and new species of *Furcaspora*. *Harknessia*, *Heterocumba* and *Phacidieilla*. Fungal Divers 25:19–36

Crous PW, Schumacher RK, Wingfield MJ, Lombard L, Giraldo A et al (2015a) Fungal systematics and evolution: FUSE 1. Sydowia 67:81–118

Crous PW, Wingfield MJ, Guaro J, Hernández-Restrepo M, Sutton DA et al (2015b) Fungal planet description sheets. 320–370. Persoonia 36:245–310

Crous PW, Wingfield MJ, Burgess TI, Hardy GE, Crane C et al (2016a) Fungal planet description sheets. 469–556. Persoonia 37:218–266

Crous PW, Wingfield MJ, Richardson DM, Le Roux JJ, Strasberg D et al (2016b) Fungal planet description sheets. 400–468. Persoonia 37:316–458

Currie CR, Wong B, Stuart AE, Schultz TR, Rehner SA et al (2003) Ancient tripartite coevolution in the attine ant-microbe symbiosis. Science 299:386–388

de BeerZW, Seifert KA, Wingfield MJ (2013) A nomenclator for ophiostomatoid genera and species in the Ophiostomatales and Microascales. CBS Biodiv Ser 12:245–322

de Hoog GS, Gerrits van den Ende AHG (1998) Molecular diagnostics of clinical strains of filamentous basidiomycetes. *Myco keys* 41:183–189

Grum-Grzhimaylo AA, Debets AJ, van Diepeningen AD, Georgieva ML, Bilanenko EN (2013a) *Sodiumyces alkalinus*, a new holomorph algalpohilic ascomycete within the Plecostosphaerellaceae. Persoonia 31:147–158

Grum-Grzhimaylo AA, Georgieva ML, Debets AJ, Bilanenko EN (2013b) Are alkali tolerant fungi of the *Emericellopsis* lineage (Bionectriaceae) of marine origin? IMA Fungus 4:213–228

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis, program for Windows 95/98/NT. Nucleic Acids Symp Ser 41:95–98

Halleen F, Schroers H-J, Groenewald JZ, Crous PW (2004) Novel species of *Cylindrocarpon* (*Neonectria*) and *Campylocarpon* gen. nov. associated with black foot disease of grapevines (*Vitis* spp.). Stud Mycol 50:431–455

Hijikawa Y, Matsuzaki M, Suzuki S, Inaoka DK, Tatsumi R et al (2017) Re-identification of the ascofun-runone-producing fungus *Ascochyta viciae* as *Acremonium sclerotigenum*. *J Antibiot* 70:304–307

Hirooka Y, Kobayashi T, Ono T, Rossman AY, Chaverri P (2010) *Verrucostoma*, a new genus in the Bionectriaceae from the Bonin Islands, Japan. Mycologia 102:418–429

Hirooka Y, Rossman AY, Chaverri P (2011) A morphological and phylogenetic revision of the *Nectria cinnabarina* species complex. Stud Mycol 68:35–56

Hirooka Y, Rossman AY, Samuels GJ, Lechat C, Chaverri P (2012) A monograph of *Allantonectria*, *Nectria*, and *Pleonectria* (Nectriaceae, Hypocreales, Ascomycota) and their pycnidial, sporodochial, and synnematous anamorphs. Stud Mycol 71:1–210

Jaklitsch WM (2009) European species of *Hypocreapart I*. The green-sored species. Stud Mycol 63:1–91

Jaklitsch WM, Voglmayr H (2011a) *Stromatonecota* gen. nov. and notes on *Myrmecia*. Mycologia 103:431–440

Jaklitsch WM, Voglmayr H (2011b) *Nectria eustromatica* sp. nov., an exceptional species with a hypocreaceous stroma. Mycologia 103:209–218

Jaklitsch WM, Voglmayr H (2012) Phylogenetic relationships of five genera of Xylariales and *Rosasphaeria* gen. nov. (Hypocreales). Fungal Divers 52:75–98

Jaklitsch WM, Voglmayr H (2014) Persistent hamathelial threads in the Nectriaceae, Hypocreales: *Thryonecota* revisited and re-instated. Persoonia 33:182–211

Kolarik M, Kirkendall LR (2010) Evidence for a new lineage of primary ambrosia fungi in *Geosmithia* Pitt (Ascomycota: Hypocreales). Fungal Biol 114:676–689

Lechat C, Fournier J (2015) *Prototheca korfii*, a new species from Martinique (French West Indies). Ascomycete.org 7:307–310

Lechat C, Fournier J (2016) *Lasionectriella*, a new genus in the Bionectriaceae, with two new species from France and Spain, *L. herbicola* and *L. rubioi*. Ascomycete.org 8:59–65

Lechat C, Lesage-Meesen L, Favel A (2015) A new species of *Ibuha*, *I. fournieri*, from French Guiana. Ascomycete.org 7:101–104

Lechat C, Fournier J, Moreau-P A (2016) *Xanthonecota*, a new genus for the nectrioid fungus *Nectria pseudoepeza*. Ascomycete.org 8:172–178

Lechat C, Fournier J, Vega M, Priou J-P (2018) *Geonectria*, a new genus in the Bionectriaceae from France. Ascomycete.org 10:81–85

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

Lombard L, van der Merwe NA, Groenewald JZ, Crous PW (2015) Generic concepts in Nectriaceae. Stud Mycol 80:189–245

Lombard L, Houbraken J, Decock C, Samson RA, Meijer M et al (2016) Generic hyper-diversity in Stachybotriaceae. Persoonia 36:156–246

Rehner SA, Buckley E (2005) A *Beauveria* phylogeny inferred from nuclear ITS and EF-1-α sequences: evidence for cryptic diversification and links to *Cordyceps* teleomorphs. Mycologia 97:84–98

Rehner SA, Samuels GJ (1995) Molecular systematics of the Hypocreales: a teleomorph gene phylogeny and the status of their anamorphs. Can J Bot 73(Suppl 1):S816–S823
