Westerdykella reniformis sp. nov., producing the antibiotic metabolites melinacidin IV and chetracin B

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Abstract: *Westerdykella reniformis* Ebead & Overy sp. nov. is described based on morphology and phylogenetic analyses using ITS, nLSU rDNA, and β-tubulin gene sequences. *Westerdykella reniformis* is characterized by the production of cleistothecioid ascomata, containing small globose to subglobose asci with 32, aseptate, dark colored, pronouncedly reniform ascospores having a concave central groove. The isolate was obtained from a red alga (*Polysiphonia* sp.) collected from the tidal zone in Canada at low tide. Organic extracts enriched in extrolites, obtained from fermentation on a rice-based media, inhibited the growth of methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus faecium* (VRE), *S. warneri*, and *Proteus vulgaris*. Presented here is the identification of the compounds responsible for the observed antimicrobial activity, the taxonomic description of *W. reniformis*, and a dichotomous key to the known species of *Westerdykella* based on macro- and micromorphological characters.

INTRODUCTION

While screening organic solvent extracts of isolates of algicolous fungi obtained from Prince Edward Island (Canada) for antimicrobial activity, we found several strains with unique ITS rDNA gene sequences and associated extracts having antibiotic activity. Of particular interest was an isolate that was phylogenetically related to the genus *Westerdykella* within the family *Sporormiaceae*. Taxa in *Sporormiaceae* occur worldwide, especially on dung, but also as endophytes and as soil saprobes. The family currently comprises seven genera representing around 100 species: *Chaetopreussia*, *Pleophragmia*, *Preussia*, *Sporormia*, *Sporormiella*, *Spororminula,* and *Westerdykella* (Kruys *et al.* 2006, Lumbsch & Huhndorf 2007, Kruys & Wedin 2009).

The genus *Westerdykella*, first described by Stolk in 1955, was named after Johanna Westerdijk, the founding director of what is now the KNAW-CBS Fungal Biodiversity Centre in Utrecht, The Netherlands (Stolk 1955). *Westerdykella* species occur worldwide on a variety of substrates including soil, mud, dung, and plant material (Clum 1955, Ito & Nakagiri 1995, Stolk 1955, Cain 1961, Rai & Tewari 1962, Malloch & Cain 1972). Kruys & Wedin (2009) retypified the genus, and distinguished it from other genera in the family by the production of cleistothecioid ascomata containing small asci

(< 50 μm tall) with a short or almost absent stipe, encasing one-celled ascospores without germ slits.

Species delineation within the genus historically has been based primarily on asci and ascospore shape. Originating with the description of the ex-type strain, *W. ornata* (Stolk 1955), to date nine species have been described within *Westerdykella*: *W. ornata*, *W. angulata*, *W. aurantiaca*, *W. cylindrica*, *W. dispersa*, *W. globosa*, *W. multispora*, *W. nigra*, and *W. purpurea*. Over time and through various taxonomic revisions, several species of the genera *Preussia*, *Pycnidiophora,* and *Eremodothis* have been reclassified in *Westerdykella*. *Pycnidiophora multispora* was the first taxon to be transferred into the genus by Cejp & Milko (1964). Subsequently, Arx (1973) reclassified *Preussia cylindrica* in the genus due to the production of cylindrical, larger ascospores and the presentation of an asexual *Phoma*-like state, and also *P. nigra* due to the production of short cylindrical asci ellipsoidal ascospores, and the absence of a conidial state. Subsequently, *Preussia purpurea* was also transferred to the genus by Arx (1975) due to the production of an orange pigment in culture, non-ostiolate ascomata with often a central columnar body and ascospores without germ pores. Ito & Nakagiri (1995) added *P. globosa* on the basis of the production of asci containing 32 ascospores, each having a single semicircular spiral ridge on the spore surface, and so conforming to the generic concept

of *Westerdykella* as described by Stolk (1955). From a multilocus phylogenetic study based of ITS and nLSU rDNA, mtSSU and β-tubilin gene sequences, Kruys & Wedin (2009) reclassified *Pycnidiophora dispersa* and *P. aurantiaca* in the genus. Furthermore, *Eremodothis angulata* was found to be phylogenetically related to *Westerdykella*, despite producing eight pyramidal star-shaped ascospores per ascus compared to the typical 32 ascospores of other *Westerdykella* species, and was therefore also reclassified within it.

In this study, a unique *Westerdykella* isolate from algae collected in the littoral zone was evaluated for morphological similarity to other taxa of the genus and for phylogenetic relatedness within *Sporormiaceae*. Multi-gene phylogenies were constructed using data sets of ITS and nLSU rDNA and β-tubulin gene sequences. Two extrolites were responsible for the observed antibiotic activity and these metabolites were isolated and identified from organic extracts of rice-based medium fermentations of the isolate. Presented here is the taxonomic description of the novel *Westerdykella* species, *W. reniformis* sp. nov., a dichotomous key to the known species of the genus based on macro- and micromorphological characteristics, and the isolation and identification of the antibiotic secondary metabolites melinacidin IV and chetracin B, production which is particular feature of *W. reniformis*.

MATERIALS AND METHODS

Sample collection
Algal material (*Polysiphonia* sp.) was collected by G.A.E. from the shoreline at Point Prim, Prince Edward Island (46'04"N, 62'59"W) at low tide on 4 June 2009. Immediately after removal from the sea, the algal sample was deposited in a sterile plastic bag and seawater was added. The sample was kept cold until arrival at the laboratory where it was maintained at 4 °C until the following day. Before being processed, the sample was shaken three times with sterile seawater in order to wash the surface free of any adhering particulate material.

Sample plating and fungal isolation
The sample was homogenized in sterile seawater and the resulting homogenate was plated on a 9 cm Petri dish containing YM media (Yeast extract Malt agar; 2 g yeast extract, 10 g malt extract, 10 g glucose, 20 g agar, 50 mg chloramphenicol, 18 g Instant Ocean in 1 L Millipore H_2O) and inspected daily for fungal growth. The plates were incubated at 22 °C for 5 d and then examined under the dissecting microscope. Emerging fungal colonies were transferred via a flame-sterilized needle to another Petri dish containing YM. After obtaining a pure isolate, seed inoculum was prepared by excising cubes (1–3 mm^3) from an actively growing culture into 15 mL of yeast extract-maltose medium (10 g peptone, 40 g maltose, 10 g yeast extract, 18 g Instant Ocean and 1 g agar in 1 L Millipore H_2O) in a 50 mL test tube and incubated at 22 °C, 200 rpm for 5 d, after which 500 μL of mycelial suspension was removed for DNA extraction and the remainder reserved to inoculate fermentations.

Culture characteristics and morphology
For morphological and molecular comparisons, six *Westerdykella* isolates were obtained from CBS: *W. cylindrica* CBS 454.72, *W. dispersa* CBS 297.56, *W. multispora* CBS 391.51, *W. nigra* CBS 416.72, *W. ornata* CBS 379.55, and *W. rapa-nuiensis* ined. CBS 604.97. For macro-morphological comparisons, fungi were grown on OA (Oatmeal Agar; 30 g oatmeal, 15 g agar in 1 L Millipore H_2O), Mannitol Soya agar (20 g mannitol, 20 g soya flour, 20 g agar in 1L Millipore H_2O) and Rice agar (75 g brown rice, 20 g agar in 1 L Millipore H_2O) at 22 °C and their growth rates were measured and colonies were evaluated after 7 and 14 d of incubation. Colour descriptions were qualified using Kornerup & Wanscher (1978). Measurements were repeated twice. For micro-morphological measurements and photographs, fungal structures from 26 d-old cultures were mounted on glass slides in lactic acid; photographs were taken while viewing using either bright field or phase contrast microscopy. For measurements, a Leica DME light microscope with phase contrast optics accompanied by a Leica EC3 camera (Leica Microsystems, Switzerland), was used at 100× magnification and a total of 25 ascospores and 25 asci were measured from crushed mounts and the dimension range (minimum and maximum) and average were determined (measurements were adjusted to the nearest 0.5 microns to avoid false impression of accuracy). Bright-field photomicrographs were obtained with a Carl Zeiss microscope, Axio Imager A1m model with a HRc Axiocam digital camera and AxioVision v. 3.1 software (Carl Zeiss, Heerbrugg, Switzerland).

Salt tolerance testing
Isolate RKGE 35 was point-inoculated onto OA media and OA media with artificial seawater (+ASW; 18 g L^{-1} Instant Ocean) and plates were incubated at 22 °C. Radial growth rates and colony features were noted after 7 d and 14 d of incubation.

Fermentation and extraction
Strains were fermented on a rice-based medium (10 g brown rice; 50 mL YNB (6.7 g YNB + 5 g sucrose in 1 L Millipore H_2O)) in 250 ml Erlenmeyer flasks. The brown rice medium was autoclaved twice for 20 min at 121 °C, first with only brown rice, which was allowed to cool before YNB was added and the mixture was autoclaved again. Flasks were inoculated with 1.5 mL of seed inoculum. An uninoculated control flask was used to inspect medium purity and to be used as a negative control for antimicrobial screening. All experiments were incubated under stationary conditions at 22 °C for 21 d.

After 21 d of incubation, fermentations were extracted by first disrupting the fungal colony using a sterile spatula and adding 30 mL EtOAc:MeOH (1:1), followed by shaking at 50 rpm for 1 h at room temperature. Organic extracts were then vacuum-filtered through Whatman #3 filter paper and dried using a GeneVac vacuum evaporating system (model: EZ-2 MK2) prior to fractionation. Extracts were fractionated on Thermo HyperSep C-18 Sep Pack columns (500 mg C-18, 6 mL column volume) using a vacuum manifold by eluting with 14 mL of each of the following solvent combinations: 8:2 H_2O:MeOH (fraction 1), 1:1 H_2O:MeOH (fraction 2), 2:8 H_2O:MeOH (fraction 3), EtOH (fraction 4), and 1:1

MeOH:DCM (fraction 5). The eluent representing fractions 2–5 were retained and using a GeneVac (model: EZ-2 MK2) evaporating system, weighed and submitted for antimicrobial testing, and analyzed by LC/HRMS using a Kinetex 1.7 µm C18 UPLC column (Phenomenex, 50 × 2.1 mm) and Accela Thermo equipment coupled with MS-ELSD-UV detection (Orbitrap Exactive mass spectrometer fitted with an ESI source, PDA, and LT-ELSD Sedex 80 (Sedere)).

Additional fermentation of strain RKGE 35 was carried out in 10 Erlenmeyer flasks and following the same growth conditions and extraction protocol as described above in order to obtain sufficient material to determine the structural identity of the secondary metabolites responsible for the observed antimicrobial activity. The resulting EtOAc:MeOH extract was partitioned between EtOAc:H_2O (1:1) and the organic layer (EtOAc) was dried under vacuum. The resulting gum was resuspended in a biphasic solvent mixture of Hexane:MeOH:H_2O (6:7:2) and the H_2O:MeOH layer after evaporation was subjected to a flash chromatography using bulk C-18 to yield to five fractions: 9:1 H_2O:MeOH, 1:1 H_2O:MeOH, 2:8 H_2O:MeOH, EtOH, acetone, and 1:1 MeOH:DCM. The 2:8 H_2O:MeOH fraction was further fractionated on normal phase silica by using automated medium pressure chromatography system (Combiflash Rf200 (Teledyne Isco)) to yield to 15 fractions. Fractions 3–5 were purified by semi-preparative normal phase HPLC (Phenomenex Luna Silica, 250 × 10 mm, 5 µm) with isocratic conditions using 15% CHCl$_3$:MeOH (9:1) in 85 % CHCl$_3$ and a flow rate of 2.5 ml min^{-1} to afford melanicidin IV and chetracin B.

Antimicrobial bioassay

All microbroth antibiotic susceptibility testing was carried out in 96-well plates in accordance with Clinical Laboratory Standards Institute testing standards (Ferraro, 2003) using the following pathogens: methicillin-resistant *Staphylococcus aureus* ATCC 33591 (MRSA), *S. warneri* ATCC 17917, vancomycin-resistant *Enterococcus faecium* EF379 (VRE), *Pseudomonas aeruginosa* ATCC 14210, *Proteus vulgaris* ATCC 12454, and *Candida albicans* ATCC 14035. Extract fractions and pure compounds were tested in triplicate against each organism. Extract fractions were resuspended in sterile 20 % DMSO and assayed at 250 µg/mL with a final well volume concentration of 2 % DMSO while pure compounds were serially diluted to generate a range of twelve concentrations (128 µg mL^{-1} to 0.0625 µg mL^{-1}) in a final well volume concentration of 2% DMSO. Each plate contained eight uninoculated positive controls (media + 20 % DMSO), eight untreated negative controls (Media + 20 % DMSO + organism), and one column containing a concentration range of a control antibiotic (vancomycin for MRSA, and *S. warneri*, rifampicin for VRE, gentamycin for *P. aeruginosa*, ciprofloxacin for *P. vulgaris*, or nystatin for *C. albicans*). The optical density of the plate was recorded using a BioTek Synergy HT plate reader at 600 nm at time zero and then again after incubation of the plates for 22 h at 37 °C. After subtracting the time zero OD600 from the final reading, the percentages of microorganism survival relative to vehicle control wells were calculated.

DNA extraction and PCR amplification

Genomic DNA was obtained from all strains using the fast DNA extraction kit (FASTDNA SPIN KIT FOR SOIL®, MP Biomedicals) according to the manufacturer's protocols. Double-stranded copies of the ITS and nLSU rRNA gene and the β-tubulin gene were obtained by polymerase chain reaction (PCR) amplifications using 50 µL of reaction mixture consisting of 25 µL of Econo Taq® PLUS GREEN 2× Master Mix (Lucigen), 17 µL of sterile ddH$_2$O, 2 µL of each primer and 4 µL of genomic DNA. Reactions were run in a Biometra thermocycler using the following settings for ITS amplicon generation: an initial denaturation step at 96 °C for 3 min, 35 cycles consisting of denaturation at 96 °C for 45 s, primer annealing at 54.5 °C for 45 s and extension at 72 °C for 1 min. The PCR was completed with a final extension step of 10 min at 72 °C. Amplification protocols were similar for both β-tubulin and nLSU rDNA genes with the exception of the employed annealing temperatures: 58 °C for β-tubulin and for 50 °C nLSU. Primers used for the ITS rDNA gene were ITS-1 and ITS-4 (White *et al.* 1990), for the β-tubulin gene were BT1819R and BT2916 (Miller & Hundorf 2005); for the nLSU rDNA gene were LROR and LR7 (Vilglys & Hester 1990, Rehner & Samuels 1994). PCR amplicons were checked for correct length and concentration by electrophoresis in 1 % agarose gel in 1× TAE buffer (Tris Base 2.42 g, glacial acetic acid 0.572 IL, 0.5 M EDTA 1 mL; add ddH$_2$O to 500 mL.

DNA sequencing and sequence alignment

The ITS, nLSU, and β-tubulin amplicons were sent to a commercial sequencing facility (Eurofins MWG Biotech) and sequenced on a 3730xl DNA Analyzer coupled with BigDye Terminator v. 3.1 Cycle Sequencing reagents, Applied Biosystems (ABI). The generated sequences were compared with other fungal DNA sequences from NCBI's GenBank sequence database using a Blastn search algorithm. Phylogenetic analysis of the ITS rDNA gene were performed using the software Molecular Evolutionary Genetics Analysis v. 5 (MEGA5) (Tamura *et al.* 2011). Sequence data generated in this study were aligned with additional sequences of representative *Westerdykella* spp. as well as several isolates belonging to *Sporormiaceae* and other *Pleosporales* available in GenBank (Table 1). In total, 34 sequences were aligned using the ClustalW algorithm, with a DNA Gap Open Penalty = 15.0, DNA Gap Extension Penalty = 6.66 and a delay divergent cutoff of 30 %. For result optimization, the alignments were refined by manual correction when needed. The evolutionary history was inferred using the neighbor-joining method employing the maximum composite likelihood model using pairwise deletion and the clade stability was evaluated using the bootstrap method (*n* = 2000 bootstrap replications). Novel sequences were accessioned in GenBank under accession numbers JX235699–JX235707.

A multigene phylogeny was constructed using 23 isolates (Table 1). Relevant sequence data were downloaded from GenBank and used to construct aligned and trimmed ITS, nLSU and β-tubulin data matrices in MEGA5. A Bayesian analysis was performed using MrBayes 3.2 (Ronquist *et al.* 2012) with the following settings: nst = 6, therefore using GTR (General Time Reversible) model; rates = invgama, setting across-site rate variation for gamma distribution with a proportion of

Table 1. Sequences included in this study, newly generated sequences are highlighted in bold.

Species	Isolate	Origin	GenBank accession no.		
			ITS	28S	β-tubulin
Herpotrichia juniper	CBS 468.64	Switzerland, *Pinus mugo*	GQ203759	DQ384093	GQ203681
Pleospora herbarum	ATCC 11681	USA, onion leaf	AF229479	AF382386	AY749032
Preussia australis	Lundqvist 20884-a	France, rabbit dung	GQ203773	GQ203732	GQ203695
P. funiculata	Huhndorf 2577	USA, porcupine dung	GQ203762	GQ203722	GQ203685
P. isomera	CBS 388.78	Venezuela, cow dung	GQ203763	GQ203723	GQ203686
P. lignicola	CBS 363.69	Netherlands, rabbit dung	GQ203783	DQ384098	GQ203703
P. tenerifae	CBS 354.86	Tenerife, rabbit dung	GQ203794	GQ203752	GQ203713
P. terricola	CBS 317.65	Honduras, *Musa sapientum*	GQ203765	GQ203725	GQ203688
P. terricola	CBS 527.84	Tanzania, elephant dung	GQ203764	GQ203724	GQ203687
P. typharum	CBS 107.69	Japan, deer dung	GQ203766	GQ203726	GQ203689
P. vulgaris	Strid 18884	Sweden, hare dung	GQ203767	GQ203727	GQ203690
Sporormia fimetaria	Lundqvist 2302-c	Sweden, cow dung	GQ203768	GQ203728	GQ203691
Sporomiella affinis	Lundqvist 17739-j	Denmark, rabbit dung	GQ203770	–	–
S. dakotensis	Thulin 2570-g	Ethiopia, cow dung	GQ203776	–	–
S. heptamera	Lundqvist 3090-b	Sweden, horse dung	GQ203778	–	–
S. irregularis	Lundqvist 16568-f	Hungary, cow dung	GQ203780	GQ203739	GQ203700
S. leporina	Lundqvist 19873-a	Sweden, hare dung	GQ203781	–	–
S. pulchella	Richardson MJR67/01	USA, dung	GQ203789	–	–
S. vexans	UME23	Sweden, moose dung	GQ203793	–	–
Trematosphaeria heterospora	CBS 644.86	Switzerland, *Iris sp.*	GQ203795	AY016369	GQ203714
Westerdykella angulata	IMI 090323	India, rice-field soil	GQ203758	GQ203720	GQ203680
W. angulata	CBS 610.74	India, rice-field soil	GQ203757	–	–
W. aurantiaca	IMI 086825	India, mud	AY943057	–	–
W. aurantiaca	FNBR-03	India, soil	JN118571	–	–
W. cylindrica	ATCC 24077 = CBS 454.72	Kenya, cow dung	AY943056	AY004343	**JX235707**
W. dispersa	CBS 297.56	Virginia, damp seedlings	GQ203797.1	GQ203753.1	GQ203716.1
W. dispersa	CBS 156.67	Nigeria, soil	DQ468016	–	–
W. dispersa	CBS 508.75	Armenia, salt-marsh soil	GQ203798	–	–
W. dispersa	CBS 712.71	The Netherlands, greenhouse soil	DQ468031	–	–
W. globosa	IFO 32588	India, soil	AY943046	–	–
W. multispora	CBS 383.69	France, saline soil	GQ203799	GQ203754	GQ203717
W. multispora	CBS 391.51	Japan	AY943048	–	–
W. nigra	CBS 416.72	Pakistan, soil	GQ203800	GQ203755	GQ203718
W. nigra	ATCC 12756		AY943049	–	–
W. ornata	CBS 379.55	Mozambique, mangrove mud	GQ203801	AY853401	GQ203719
W. purpurea	CBS 297.75	Togo, sandy soil	AY943050	–	–
W. purpurea	HN6-5B	China, mangrove	FJ624258	–	–
W. rapa-nuiensis ined.	CBS 604.97	Chile, soil	**JX235699**	**JX235703**	**JX235705**
W. reniformis	RKGE35 = DAOM 242243	Canada, red algae	**JX235700**	**JX235704**	**JX235706**
Verruculina enalia	CBS 304.66	Liberia, drift wood	GQ203796	AY016363	GQ203715

invariant sites; MCMC heated chain set with nchains = 4 and temp = 0.2, ngen = 500 000, samplefreq = 100, sumt burnin = 1250; the analysis was continued for 500 000 generations in order obtain an average standard deviation of split frequencies below 0.01. The first 25 % of sampled trees were discarded as burn-in. Resulting trees were viewed in FigTree v. 1.3.1. Sequence alignments and trees presented were deposited in TreeBASE (accession number 13676).

RESULTS

Sequencing analysis

In order to verify the taxonomic placement of isolate RKGE 35, gDNA was extracted and amplified by PCR for different genes resulting in sequence lengths of 472, 1295 and 935 nucleotides for the ITS rDNA, nLSU rDNA, and β-tubulin genes respectively. The Blastn search for
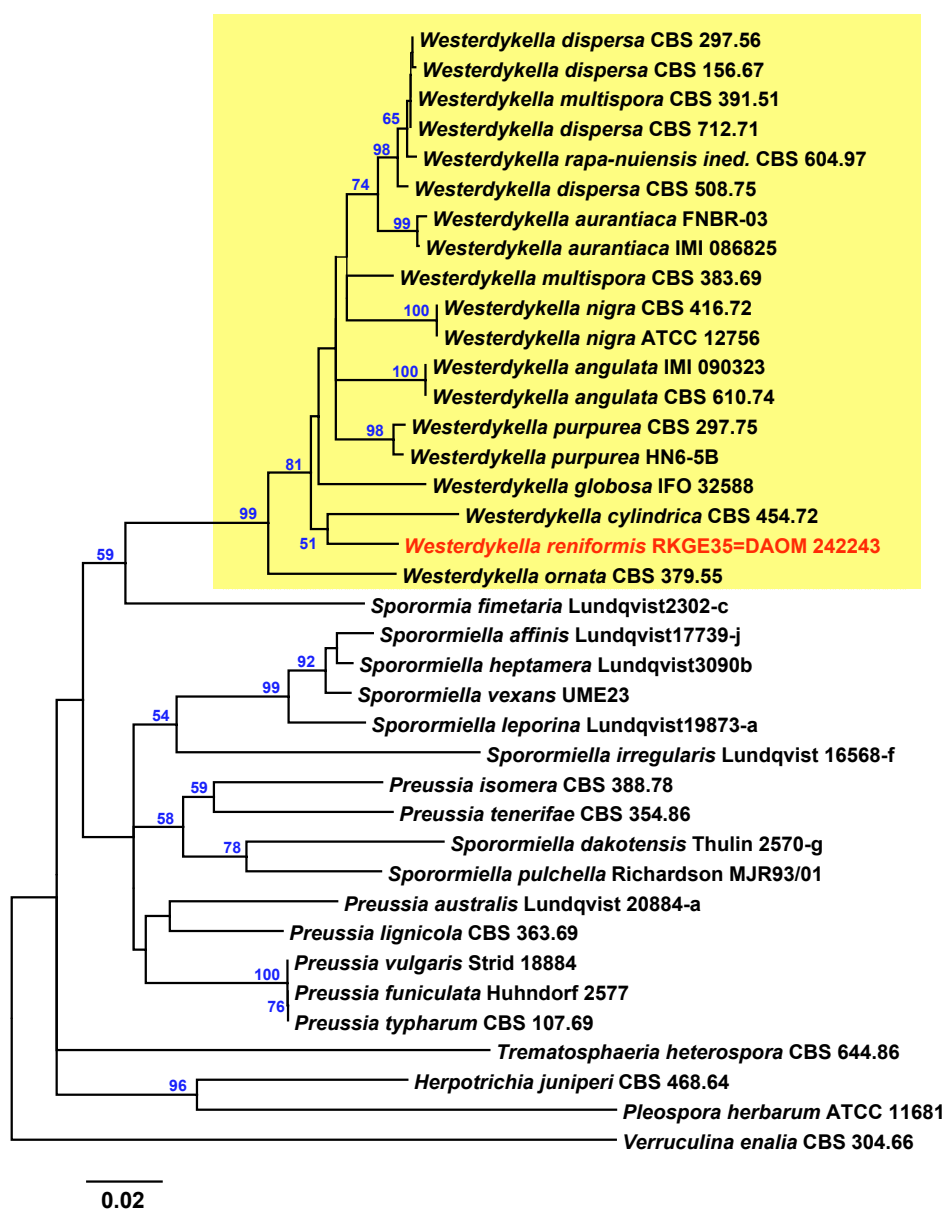
Fig. 1. Bootstrap consensus tree inferred from 2000 replicates using the neighbor-joining method based on ITS rDNA sequences. The percentage of replicate trees (> 50 %) in which the associated taxa clustered together in the bootstrap tests of 2000 replicates are shown next to the branches. Evolutionary distances were computed using the maximum composite likelihood method and are in the units of the number of base substitutions per site. The tree was rooted with *Verruculina enalia* (CBS 304.66).

the sequences showed that isolate RKGE 35 is classified within the genus *Westerdykella.* For the ITS region, the closest sequence matches with 95 % maximum identity and complete coverage were to those of *W. ornata* CBS 379.55 (AY943045.1; matching 455/477 bases with 7 gaps), *W. dispersa* CBS 297.56 (AY943055.1; matching 454/477 bases with 6 gaps), and *W. aurantiaca* IMI 08625 (AY943048.1; matching 452/477 bases with 6 gaps). For the amplified nLSU region, the closest sequence matches with complete coverage were to *W. angulata* IMI 090323 (GQ203720.1; matching 1281/1296 bases with 1 gap) with 99 % maximum identity followed by *W. cylindrica* ATCC 24077 (NG027595.1; matching 1266/1269 bases with 1 gap) with 98 % maximum identity. For the β-tubulin gene, the closest sequence matches with complete coverage were to

W. dispersa CBS 297.56 (GQ203716.1; matching 876/941 bases with 6 gaps) with 93 % maximum identity and *W. ornata* CBS 379.55 (GQ203719.1; matching 869/945 bases with 10 gaps) with 92 % maximum identity as well as with *W. angulata* IMI090323 (GQ203680.1; matching 368/926 bases with 6 gaps) with 94 % maximum identity and only 98 % coverage.

Phylogenetic analyses

The ITS rDNA gene was analysed to determine the relative evolutionary history of isolate RKGE 35 with multiple isolates of other representative *Westerdykella* spp. The evolutionary history was inferred by the bootstrap consensus tree (Fig. 1) constructed using the neighbour-joining method and 2000 bootstrap replicates. The analysis involved 39 sequences
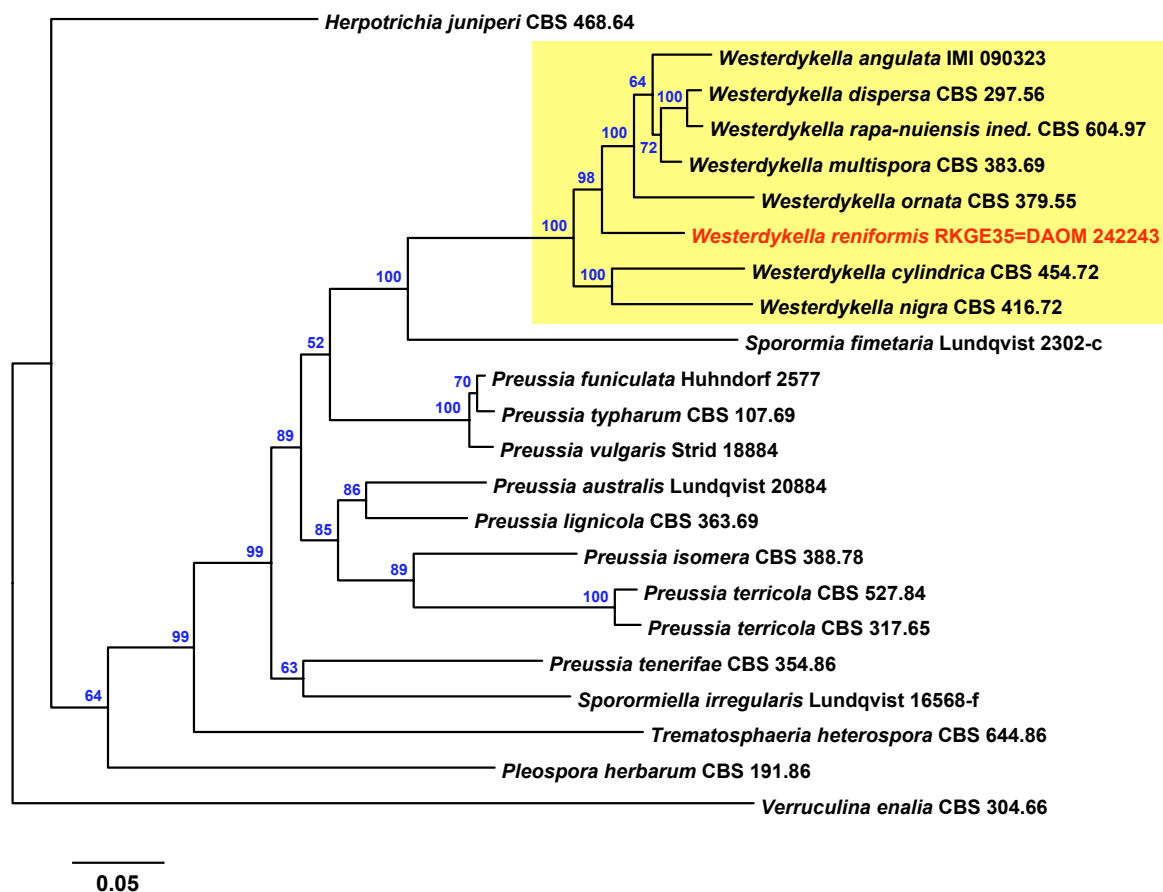
ARTICLE

Fig. 2. Consensus tree inferred from a Bayesian analysis of ITS and nLSU rDNA and β-tubulin gene sequences. Bayesian posterior probabilities are given as % values at the nodes. The tree was rooted with *Verruculina enalia* (CBS 304.66).

and included 409 nucleotide positions in the final dataset with an overall mean distance calculated as 0.137 with a standard error of 0.013. The genus *Westerdykella* formed a well-supported monophyletic clade distinct from other members of *Sporormiaceae*. Within the *Westerdykella* clade, sequences from individual isolates formed distinct species groups with high bootstrap support; however, evolutionary relatedness of species within the genus was difficult to infer due to separation with low associated bootstrap values. Isolate RKGE 35 formed a sister clade to that of *W. cylindrica,* represented by isolate CBS 454.72 (= ATCC 24077). Isolate CBS 604.97, representing the unpublished species *W. rapa-nuiensis* ined., clustered together within the *W. dispersa* clade along with the *W. multispora* isolate CBS 391.51.

Evolutionary history within the genus *Westerdykella* was also inferred by a multigene Bayesian analysis involving sequences of the ITS and nLSU rDNA and β-tubulin genes from 23 strains. The aligned dataset consisted of 417 nucleotides from the ITS rDNA, 872 nucleotides from the nLSU rDNA and 511 nucleotides from the β-tubulin gene sequences. Convergence was assumed as an average standard deviation of split frequencies of 0.007432 was achieved following 500 000 generations. From the generated phylogenetic tree (Fig. 2), representative isolate sequences of *Westerdykella* species once again clustered together forming a distinct clade with 100 % Bayesian posterior probability support. Isolate RKGE 35 clustered within the *Westerdykella*

clade, forming its own discrete lineage with 100 % posterior probability support. Isolate CBS 604.97 representing the not yet formally named *W. rapa-nuiensis* clustered together and most proximal to *W. dispera* (CBS 297.56).

Antimicrobial metabolite identification

Two successive orthogonal fractionations (reverse phase then normal phase) of the MeOH:H_2O extract obtained after liquid-liquid partitions yielded three fractions (3–5) exhibiting strong antimicrobial activities. Chemical profiling by LC-HRMS coupled to a universal detector (ELSD) suggested that two major compounds were responsible for the observed biological activities (Fig. 3). The interpretation of the HRMS data indicated the molecular formulae $C_{30}H_{28}N_6O_8S_4$ (m/z 729.09076 [M+H]$^+$, Δ -2.2 ppm) and $C_{30}H_{28}N_6O_8S_5$ (m/z 761.06274 [M+H]$^+$, Δ -2.3 ppm) respectively and was in agreement with the observed isotopic pattern and the presence of sulfur atoms. After searches in databases Antibases and SciFinder, the two prominent components with antimicrobial properties were identified as the known metabolites melinacidin IV and chetracin B (Fig. 4) which belong to the important class of biologically active metabolites: epipolythiodioxopiperazines (ETPs) (Argoudelis & Mizsak 1977, Li *et al*. 2012). This conclusion was further confirmed by ^{1}H NMR analysis after the purification of both metabolites by normal phase HPLC.
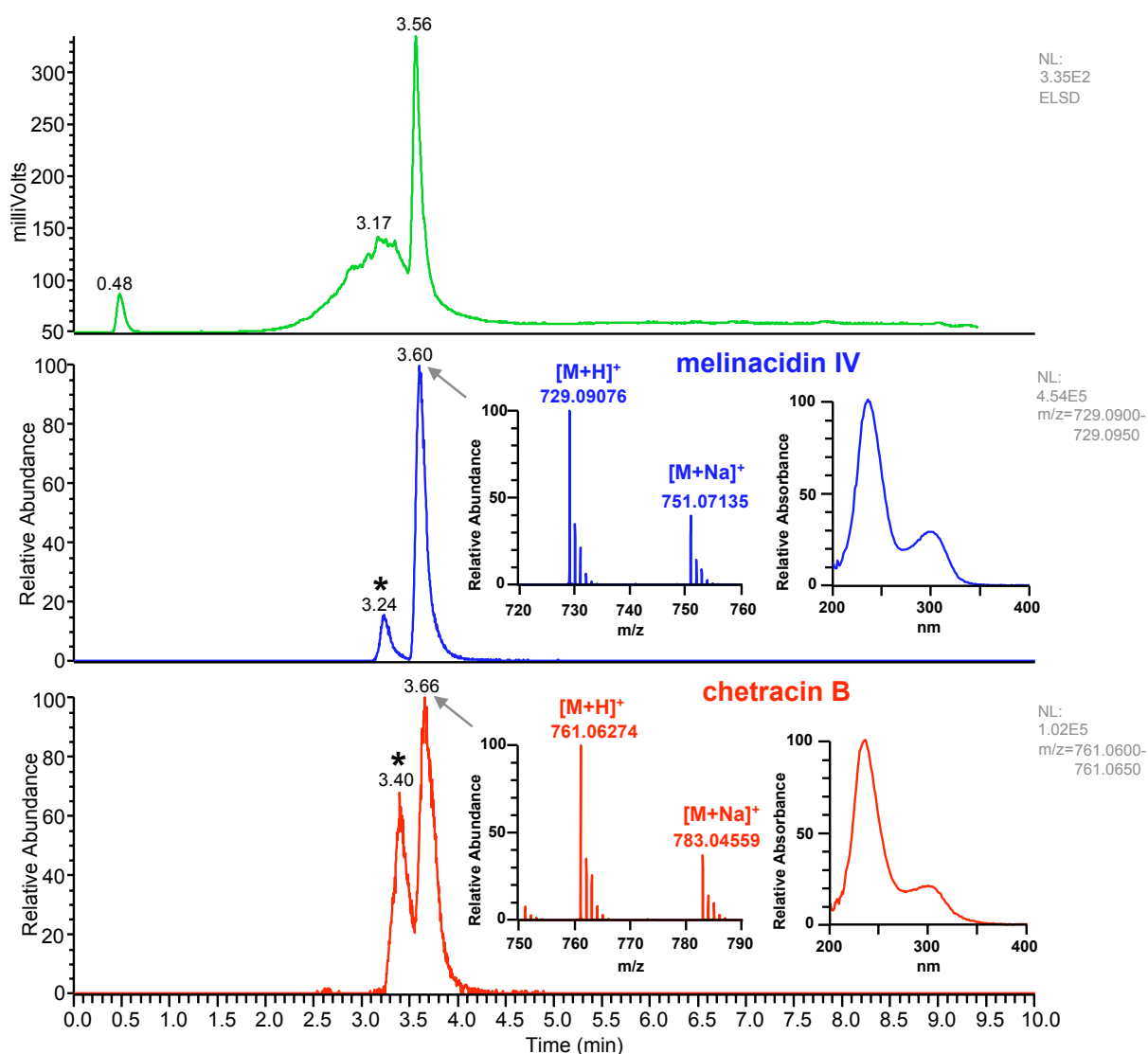
Fig. 3. ELSD (top) and single ion monitoring LCMS traces (middle, bottom) of fraction 3 (2:8 H_2O:MeOH) generated from rice fermentations of *W. reniformis*. Due to differences in tubing length, there is a 4 sec delay between the ELSD and MS detectors. High resolution mass spectra and UV absorbance traces (200–400 nm) are provided confirming the production of melinacidin IV and chetracin B. *Denotes possible artifacts due to reverse phase chromatography or presence of analogs.

Bioactivity

Microbroth dilution antibiotic susceptibility was determined at a concentration of 250 μg mL^{-1} for all of the fractions generated from rice fermentation extracts of the representative *Westerdykella* strains. Antibiotic activity was observed for all of the strains tested and the more potent antimicrobial response was observed in fraction 3 (2:8 H_2O:MeOH) (summarized in Table 2). It is notable that none of these fractions inhibited the growth of *Pseudomonas aeruginosa* and the yeast *Candida albicans* at a concentration of 250 μg mL^{-1}. Gram positive antibiotic activity against methicillin-resistant *Staphylococcus aureus* (MRSA) and *S. warneri* was observed for all of the strains tested whereas activity against vancomycin-resistant *Enterococcus faecium* (VRE) and the Gram negative bacterium *Proteus vulgaris* was exhibited only for the strain RKGE 35. Strain RKGE 35 exhibited a distinct antibiotic phenotype relative to the other strains tested. This observation was further confirmed by the comparison

of the LC-HRMS data. Indeed, melinacidin derivatives were exclusively detected for the isolate RKGE 35 and constituted the main components of fraction 3 (2:8 H_2O:MeOH). LC-HRMS analysis of the extract fractions of the remaining *Westerdykella* strains examined confirmed the absence of melinacidin IV and chetracin B from both fraction 3 and fraction 4. Rather, the metabolite profiles of these other strains for fraction 3 and fraction 4 were dominated by the presence of fatty acids characterized by the molecular formulae $C_{16}H_{32}O_2$, $C_{18}H_{34}O_2$, and $C_{18}H_{32}O_2$ and oxidized fatty acids characterized by the molecular formulae $C_{18}H_{34}O_3$ and $C_{18}H_{32}O_3$. Further purification and identification of the metabolites responsible for the antibacterial effect observed from the remaining *Westerdykella* strains has not been followed up here as it beyond the intended scope of this manuscript. Additional antimicrobial testing was carried out on purified melinacidin A and chetracin B to determine minimal inhibitory concentration (MIC) and half maximal inhibitory concentration (IC$_{50}$) values
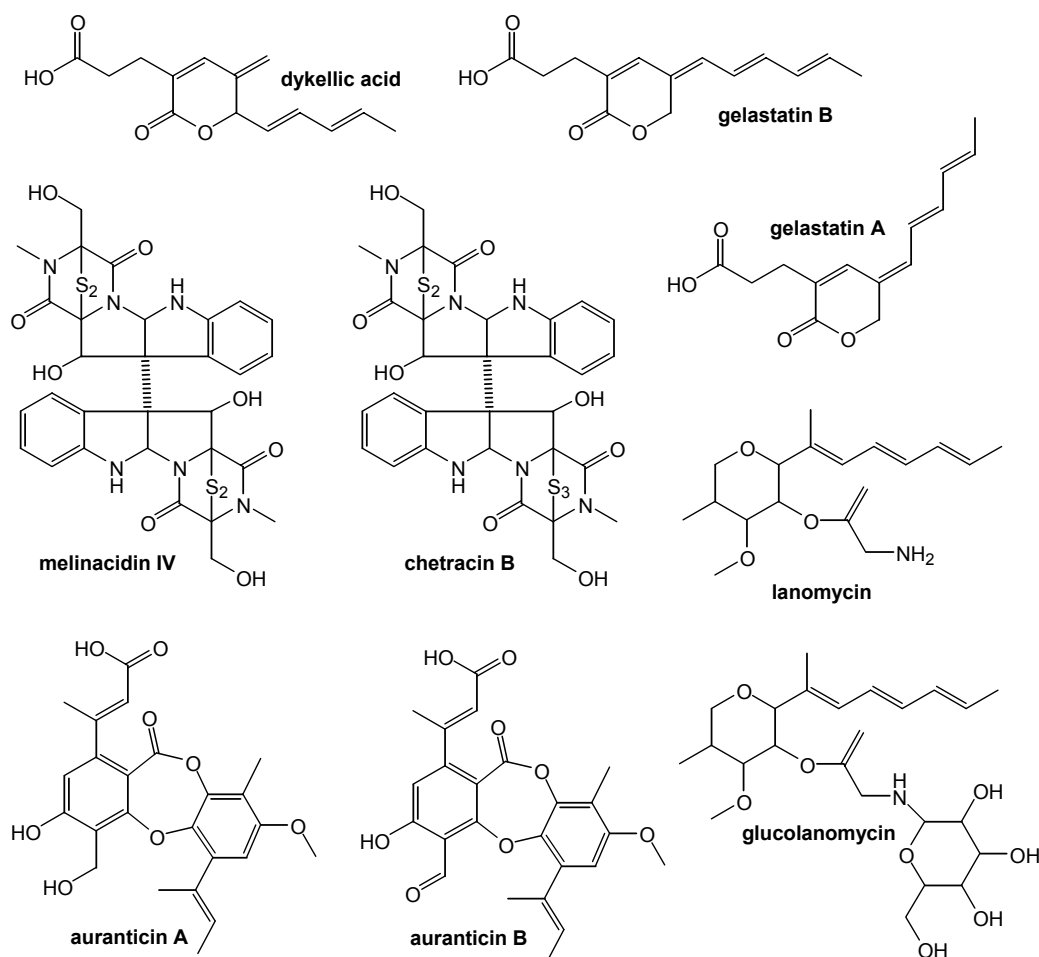
Fig. 4. Chemical structures of biologically active secondary metabolites produced by *Westerdykella* species.

Table 2. Observed biological activity, presented as a percentage of inhibition, of fraction 3 (2:8 H$_2$O:MeOH) generated from organic extracts of rice fermentations of various *Westerdykella* spp. against various pathogens tested at 250 µg mL^{-1} in a microbroth dilution assay (results of values less than 50 % were not included).

Species	Strain	MRSA	VRE	*P. aeruginosa*	*P. vulgaris*	*S. warneri*	*C. albicans*
W. reniformis	RKGE35 = DAOM 242243	100	100	–	99	100	–
W. ornata	CBS 379.55	87	–	–	–	74	–
W. nigra	CBS 416.72	93	–	–	–	84	–
W. multispora	CBS 383.69	87	–	–	–	73	–
W. cylindrica	CBS 454.72	74	–	–	–	–	–
W. dispersa	CBS 297.56	97	–	–	–	86	–
W. rapa-nueinsis ined.	CBS 604.97	93	–	–	–	82	–

Table 3. Biological activity of melinacidin IV and chetracin B against the drug resistant Gram-positive bacteria, methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus faecium* (VRE) and the Gram-negative bacterium *Proteus vulgaris* along with antibiotics tested as a positive control. All assays were run in triplicate, averaged and activity values are expressed in µM. If an assay was not performed, table entry was left blank. MIC: minimal inhibitory concentration. IC$_{50}$: half maximal inhibitory concentration.

	MRSA		VRE		*P. vulgaris*	
Compound	MIC	IC$_{50}$	MIC	IC$_{50}$	MIC	IC$_{50}$
melinacidin IV	0.7	0.1	22	6	175.8	71.4
chetracin B	0.7	0.2	10.5	1.1	168.4	76.3
vancomycin	1.4	0.6	–	–	–	–
rifampicin	–	–	2.4	1	–	–
ciprofloxacin	–	–	–	–	0.024	0.012

of the compounds against MRSA, VRE and *P. vulgaris* (summarized in Table 3). Both melinacidin IV and chetracin B were slightly more efficacious than vancomycin against MRSA and were considerably less efficacious than rifampicin and ciprofloxacin against VRE and *P. vulgaris* respectively.

TAXONOMY

The *Westerdykella* isolate RKGE 35 was clearly distinguished from other *Westerdykella* species studied based on DNA sequence comparisons of three gene regions, and growth inhibition to both Gram positive and Gram negative bacteria due to the production of melinacidin IV and chetracin B. Additional differences in both macro- and micro-morphological characters were also observed from those of the closest phylogenetically related species, confirming this isolate as representing a new taxon:

Westerdykella reniformis G.A. Ebead & D.P. Overy, **sp. nov.**
MycoBank MB800917
(Fig. 5)

Etymology: The species name reflects the pronounced reniform (kidney shape) of the ascospores.

Diagnosis: Colonies appressed, velvety, faint brown on oatmeal agar; attaining 16 mm diam after 7 d at 22 °C; producing distinct, glabrous, brownish black cleistothecia after 26 d; containing globose to subglobose, rarely ovoid asci (12–18 × 11–17 µm); each bearing 32, black, glabrous, reniform ascospores with a distinct central grove (2–4 × 4–6 µm); pycnidial stage unknown.

Type: **Canada**: *Prince Edward Island*: Point Prim (46'04"N, 62'59"W), from *Polysiphonia* sp. collected from littoral tidal zone at low tide, 4 June 2009, *G.A. Ebead*, (DAOM 242243 – holotype; culture ex-type RKGE35).

Description: *Cleistothecia* discrete, occurring in the upper layer of the culture medium, normally forming underneath a dense mat of hyphae exuding clear exudates, globose, glabrous and brownish black to black. *Ascus* initials somewhat clavate, asci later becoming globose to subglobose, occasionally ovoid when mature, measuring 12–18 (av. 14.7) × 10.5 – 16.5 (av. 12.8) µm, containing 32 ascospores. *Ascospores* 2.5–4 (av. 2.9) × 4 – 6 (av. 4.9) µm, black, glabrous, pronouncedly reniform in shape, having a central groove on the concave side, no oil droplets or germ-slits observed, germinating readily in 24 h at 22 °C. No conidial stage observed.

Colony morphology: Colonies on oatmeal agar slow growing, attaining 16 mm diam in 7 d, and 40 mm diam in 14 d at 22 °C. Mycelial development appressed, velvety, with no or little aerial hyphae. The colonies are faint brown in colour (6E4), the reverse brown (6E4) to dark grey-black in parts according to age of the colony. In older cultures (26 d), forming white mycelial aggregates covering brownish black cleistothecia, overall texture of the culture varies with density of the cleistothecia and the extent of hyphal overgrowth. Colonies on oatmeal agar (with artificial seawater) attaining 20 mm diam in 7 d and 40 mm diam in 14 d at 22 °C, colony and reverse faint brown (6E4) and mycelia appressed to the agar surface, cleistothecia absent at 26 d, appearing later after 32 d. Colonies on mannitol soya agar slow growing, attaining 12 mm diam in 7 d and 36 mm diam in 14 d at 22 °C, mycelia appressed and velvety, greyish brown (6D4), later becoming darker (6E4) with age, aerial mycelia present. Concentric circles of black cleistothecia apparent upon review of the colony reverse after 26 d. Colonies on rice agar, reaching 15 mm diam after 7 d and 44 mm diam after 14 d at 22 °C, dense, floccose aerial mycelia apparent, reverse progressing from a lighter to darker brown with age (6E4–6F4), cleistothecia absent after 26 d.

Key to the known species of *Westerdykella*

As previously evaluated by Kruys & Wedin (2009), the morphological characteristics of ascus shape and dimensions, along with ascospore shape, dimensions and ornamentation were found to be diagnostic in distinguishing species within the genus. The following dichotomous key was produced to facilitate the morphological identification of *Westerdykella* species.

1	Ascospores ornamented; asci 32-spored	2
	Ascospores not ornamented; asci 8- or 32-spored.	3
2 (1)	Ascospores globose with semicircular ridge; asci subglobose-ovoid	**globosa**
	Ascospores globose with 4–5 spiral bands, asci subglobose-elliptical	**ornata**
3(1)	Ascospores reniform, cylindrical, or subglobose; asci 32-spored	4
	Ascospores angular with rounded ends, asci globose, 8-spored	**angulata**
4(3)	Ascospores reniform	5
	Ascospores subglobose or cylindrical	6
5 (4)	Asci globose; pycnidial state present; conidia globose to pyriform	**dispersa**
	Ascospores with a pronounced central groove; asci globose to subglobose, sometimes ovoid; pycnidial state absent	**reniformis**

DISCUSSION

The new taxon, *Westerdykella reniformis*, conforms to the classical morphological characterization of the genus *Westerdykella,* including the production of cleistothecioid ascomata containing small asci with an almost absent ascus stipe, and each ascus containing 32, 1-celled, dark-coloured ascospores lacking germ slits. Phylogenetic analyses using ITS and combined ITS and nLSU rDNA and β-tublin genes confirmed the placement in *Westerdykella*.

Morphologically, *W. reniformis* is differentiated from both *W. ornata* and *W. globosa* on ascospore characters, as both those species produce globose, ornate ascospores (Stolk 1955, Ito & Nakagiri 1995) while *W. reniformis* produces reniform ascospores lacking ornamentation. *Westerdykella reniformis* is also easily distinguished from *W. cylindrica* and *W. nigra* as both species produce clavate asci (Cain 1961, Malloch & Cain 1972), while the asci of *W. reniformis* are globose to subglobose. Both the asci and ascospores of *W. reniformis* are morphologically most similar to those of *W. dispersa*, *W. multispora,* and *W. purpurea* in both shape and dimension ranges (Cain 1961); however, phylogenetically *W. reniformis* is distinct from *W. dispersa*, *W. multispora,* and *W. purpurea* in both the ITS and the multigene analyses. Additionally, *W. dispersa* produces a pycnidial asexual morph (Clum 1955), which is absent in *W. reniformis*. CBS 604.97, previously classified and deposited but yet not validly published under the name *W. rapa-nueinsis,* also has reniform ascospores; however based on phylogenetic analyses of all three genes sequenced and compared, *W. rapa-nueinsis* was phylogenetically distinct from *W. reniformis* and most similar to *W. dispersa*. Moreover, CBS 604.97 presented a pycnidial asexual morph comparable to *W. dispersa.* Based on micromorphological observations and the ITS and multigene phylogenetic comparisons, CBS 604.97 should be considered as a strain of *W. dispersa.* CBS 391.51, identified as *W. multispora,* was also found to cluster with *W. dispersa* in both our ITS phlyogenetic analysis as well as a previous ITS-nLSU phylogeny (Kruys & Wedin, 2009), suggesting that the strain has been misidentified. In order to confirm this synonmy, a morphological comparison of this strain to that of the ex-type strain of *W. dispersa* is warranted.

Westerdykella species have been isolated from a wide variety of environmental substrates, including soil/sediment, and dung and plant debris. Our isolate was obtained from algal debris collected from the littoral zone at low tide. Growth measurement with *W. reniformis* on OA media varying in salt concentrations demonstrated that this fungus is capable of growing and sporulating in both a saline and non-saline environment. The ability of this fungus to grow and sporulate in the absence of salt suggests that this fungus is not obligate marine (as defined by Kohlmeyer & Kohlmeyer 1979); an obligate marine fungus must be able to grow and sporulate exclusively in a marine or estuarine habitat. Although *Westerdykella* species are commonly isolated from terrestrial environments, they have also been isolated from both aquatic, estuarine and marine environments. In particular, *W. aurantiaca* and *W. multispora* have been isolated from mangrove sediments (Lee & Baker 1973, Poch & Gloer 1991) while *W. dispersa* has been isolated from a saline lake in Egypt (El-Sharouny *et al*. 2009). *Westerdykella dispersa* and *W. multispora* have also been isolated from low salinity and fresh water environments, both from sediment samples from lakes (Mishra 1995), and river delta flood plains (Bettucci *et al*. 2002) and estuaries (da Silva *et al*. 2003). Furthermore, they occurred as endophytes within the leaves of the freshwater lake reed *Phragmites australis* (Angelini *et al*. 2012). Therefore species of the genus *Westerdykella* appear to be widespread and most likely play a saprobic role in the decomposition of plant organic material within these ecosystems.

Several research groups have previously examined *Westerdykella* isolates for the production of bioactive compounds (Fig. 4). Dykellic acid is an apoptosis inhibitor, isolated from *W. multispora* with indications as a therapeutic in a range of apotopsis-mediated diseases, such as hepatitis, neurodegeneration, and stroke (Lee *et al*. 1999a, 2003). Dykellic acid inhibited Ca^{2+} influx, Ca^{2+}-activated DNA endonuclease activity and suppressed caspase-3 protease activation preventing the cell from entering the execution of apoptosis (Lee *et al*. 2003). The gelastatins (A and B) are stereoisomers structurally related to dykellic acid that were isolated from the same strain of *W. multispora* (Lee *et al*. 1997). A mixture of the isomers was found to selectively inhibit
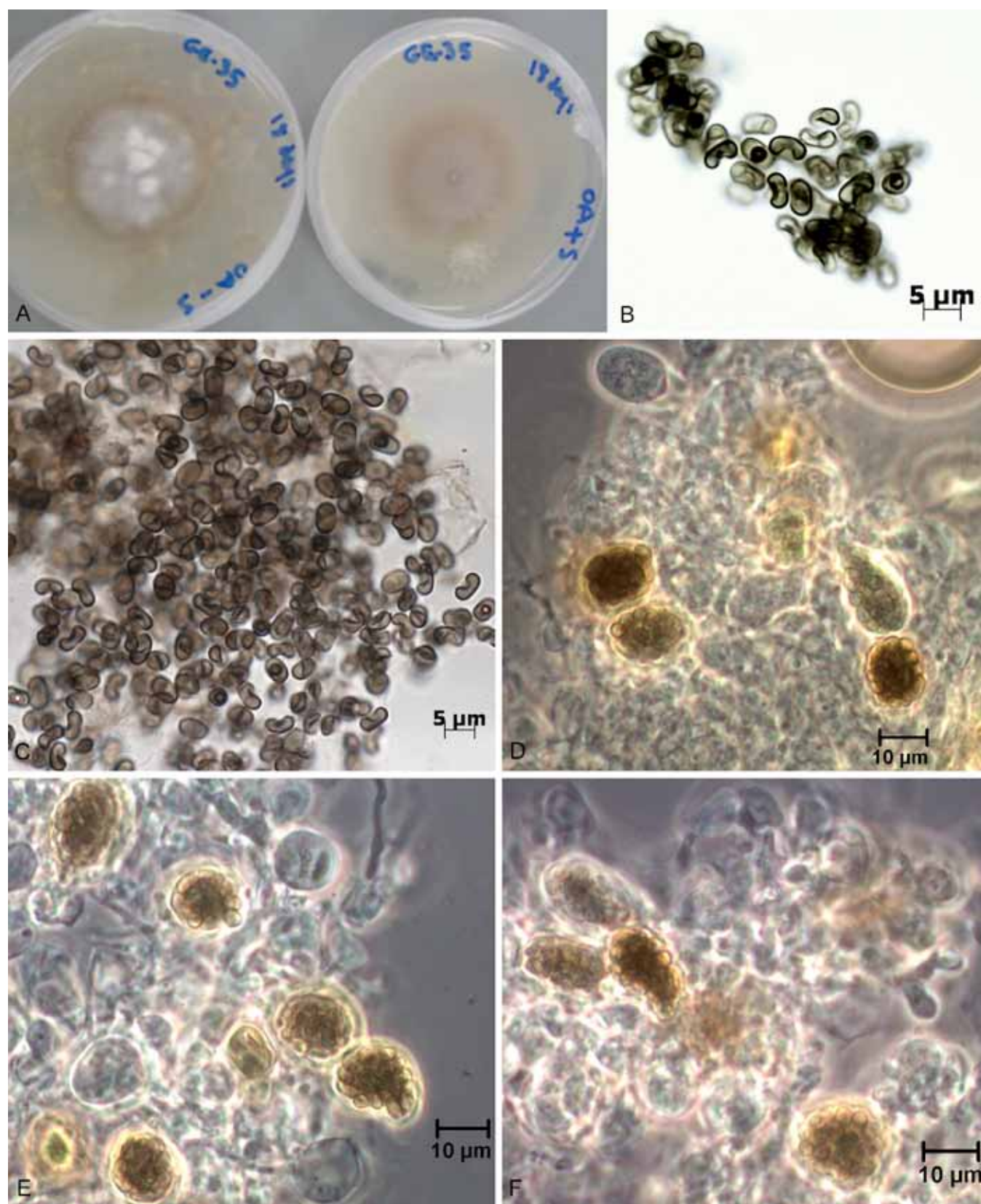
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Fig. 5. Macro and micro-morphology of *Westerdykella reniformis* (RKGE 35 = DAOM 242243). **A.** Colony grown on oatmeal agar (left) and oatmeal agar with sea salts (right) at 14d. **B–C.** Ascospores. **D–F.** Asci.

the metalloproteinase gelatinase MMP-2 (involved in the cleavage of type IV collagen), demonstrating reversible and competitive inhibition of the enzyme; the mixture has therefore been proposed as lead compounds for the development as an antimetastatic agent (Lee *et al*. 1999b). Lanomycin and glucolanomycin, were two antifungal metabolites isolated from liquid fermentations of *W. dispersa* inhibiting growth of various dermatophytes and some species of *Candida,* but

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were inactive against *C. albicans*, *Aspergillus flavus* and Gram-positve and Gram-negative bacteria (O'Sullivan *et al.* 1992). Antifungal activity of lanomycin and glucolanomycin was attributed to the inhibition of lanosterol 14α-demethylase, suggesting a similar mode of action to the azole and bis-triazole class of antifungal agents (O'Sullivan *et al.* 1992).

From this survey, culture extracts generated from each of the *Westerdykella* species strains tested, demonstrated an antibiotic effect. Antibiotic activity was first associated with the genus *Westerdykella* from a mangrove isolate of *W. aurantiaca*. The depsidones, auranticins A and B, were isolated and demonstrated to have activity against both the Gram-positive bacteria *Staphylococcus aureus* and *Bacillus subtilis*, with auranticin A being more potent in antibiotic activity compared to auranticin B using a disc diffusion assay (Poch & Gloer 1991). In our survey, antibiosis, measured as growth inhibition, was assayed against several Gram-positive and Gram-negative bacteria, including the drug resistant pathogens MRSA and VRE, and the pathogenic yeast, *C. albicans*. All of the *Westerdykella* isolates tested inhibited growth of the Gram-positive bacteria MRSA and *S. warneri*; however activity against VRE and *Proteus vulgaris* was unique to *W. reniformis*. LC-HRMS analysis confirmed the absence of auranticin A and B in each of the fraction 3's obtained from *W. cylindrica*, *W. dispersa*, *W. multispora*, *W. nigra*, *W. ornata*, and *W. reniformis*. The observed biological activity of fraction 3 derived from culture extracts from *W. reniformis* was attributed to the production of melinacidin IV and chetracin B, which was found to be exclusive within the genus to *W. reniformis.* Melinacidin derivatives have been reported previously from a variety of different fungi: *Acrostalagmus luteoalbus* (syn. *A. cinnabarinus*; Argoudelis & Mizsak 1977), *Chaetomium nigricolor* (syn. *C. abuense*; Saito *et al.* 1985), *Cladobotryum* sp. (Feng *et al.* 2003), and *Oidiodendron truncatum* (Li *et al.* 2012); indicating that melinacidin production is not uncommon, nor limited to a particular taxonomic order. Both melinacidin IV and chetracin B are epipolythiodioxopiperazines, an important class of biologically active metabolites which possess a wide variety of biological activities, including antiproliferative, cytotoxic, immunomodulatory, antiviral, and antimicrobial activities (Li *et al.* 2012). We have reported potent antibiotic activity of melinacidin IV against the drug resistant bacteria methicillin-resistant *S. aureus* and vancomycin-resistant *Enterococcus faecium* for the first time.

ACKNOWLEDGEMENTS

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