



Three new species of *Neocamarosporium* isolated from saline environments: *N. aestuarinum* sp. nov., *N. endophyticum* sp. nov. and *N. halimiones* sp. nov.

Gonçalves MFM, Aleixo A, Vicente TFL, Esteves AC* and Alves A

Departamento de Biologia, CESAM, Universidade de Aveiro, 3810-193 Aveiro, Portugal

*current address: Universidade Católica Portuguesa, Institute of Health Sciences (ICS), Viseu, Portugal

Gonçalves MFM, Aleixo A, Vicente TFL, Esteves AC, Alves A 2019 – Three new species of *Neocamarosporium* isolated from saline environments: *N. aestuarinum* sp. nov., *N. endophyticum* sp. nov. and *N. halimiones* sp. nov. Mycosphe 10(1), 608–621, Doi 10.5943/mycosphe/10/1/11

Abstract

Neocamarosporium species are typically halotolerant, being commonly found in saline environments like saline water, hypersaline soils and especially in association with halophytes. Several isolates were obtained from saline water, dead leaves of the seaweed *Zostera noltii* and live tissues of the halophyte *Halimione portulacoides*. Phylogenetic analysis based on ITS sequence data placed these isolates into three clades within the genus *Neocamarosporium* distinct from the currently known species. Isolates from each clade showed clear differences in conidial morphology. Three new species *N. aestuarinum* sp. nov., *N. endophyticum* sp. nov. and *N. halimiones* sp. nov. are described and illustrated. Our results show that the salt marsh plant *H. portulacoides* harbours a high diversity of *Neocamarosporium* species.

Key words – *Neocamarosporiaceae* – Endophyte – Halophyte – Seagrass – Salt marsh

Introduction

Camarosporium-like taxa has been shown to be polyphyletic, thus resulting in the introduction of several novel genera, namely *Neocamarosporium* (Crous et al. 2014a), *Paracamarosporium* and *Pseudocamarosporium* (Wijayawardene et al. 2014), *Xenocamarosporium* (Crous et al. 2015), *Magnicamarosporium* (Tanaka et al. 2015), *Phragmocamarosporium* (Wijayawardene et al. 2015), *Didymellocomarosporium* and *Melanocamarosporium* (Wijayawardene et al. 2016).

Neocamarosporium was introduced by Crous et al. (2014a) with *N. goegapense* Crous & M.J. Wingf as the type species that was first isolated from dying leaves of *Mesembryanthemum* sp. in South Africa. Although morphologically very similar to *Camarosporium* (e.g. pycnidial conidiomata, conidiophores reduced to conidiogenous hyaline cells, proliferating percurrently and brown, smooth muriform conidia), it is phylogenetically distinguishable (Crous et al. 2014a, b, Wijayawardene et al. 2014, 2016, Wanasinghe et al. 2017, Ariyawansa et al. 2018). Currently, the genus comprises 15 species listed in the Index Fungorum (2019) and Mycobank databases. Although initially treated as a genus in the family *Pleosporaceae*, multi-gene phylogenies supported a new family, *Neocamarosporiaceae* Wanas., Wijayaw., Crous & K.D. Hyde in Pleosporales (Wanasinghe et al. 2017).

Most species of *Neocamarosporium* have been found in association with halophytes (salt-tolerant plants) in marine or estuarine habitats (Papizadeh et al. 2017). Some examples are *N. salicorniicola* Dayarathne, E.B.G. Jones & K.D. Hyde found on dead stems of *Salicornia* sp. in Thailand, *N. salsolae* Wanas., Gafforov & K.D. Hyde on dead stems of *Salsola* sp. in Uzbekistan, *N. obiones* Wanas. & K.D. Hyde on stems of *Halimione portulacoides* in Netherlands, and *N. calvescens* in *Atriplex hastata* in Germany (de Gruyter et al. 2013, Ariyawansa et al. 2015, Wijayawardene et al. 2017a). In addition, *N. chichastianum* was also found in saline soil of Lake Urima in Iran. Papizadeh et al. (2017) introduced three novel *Neocamarosporium* species: *N. jorjanensis* Papizadeh, Wijayaw, Amoozegar, Shahzadeh Fazeli, & K.D. Hyde, *N. persepolisi* Papizadeh, Wijayaw, Amoozegar, Shahzadeh Fazeli, & K.D. Hyde and *N. solicola* Papizadeh, Wijayaw, Amoozegar, Shahzadeh Fazeli & K.D. Hyde, all of them have also been isolated from saline environments in Iran.

Saline environments, including salt marshes, mangroves, saline soils and water cover a wide area of the planet and harbour a considerable fungal diversity with many new species described recently (Hyde & Jones 1998, Poon & Hyde 1998, Hyde & Pointing 2000, Sarma & Hyde 2001, Jones et al. 2015, Wijayawardene et al. 2017a, 2017b, Devadatha et al 2018a, 2018b). These environments also receive fungi from terrestrial sources that here find new niches for evolution (Azevedo et al. 2011). Despite this, the biology and ecology of fungi in these habitats are still poorly studied (Kis-Papo et al. 2001, 2003, Gunde-Cimerman et al. 2004, Butinar et al. 2005a, b).

During an extensive survey of the fungal diversity in marine and estuarine environments in Portugal, we have obtained a number of camarosporium-like isolates. The aim of this study was to characterise these isolates based on morphological, cultural and DNA sequence analyses.

Materials & Methods

Collection and Isolation

Water samples were collected from the sea at Vila Real de Santo António, near to the mouth of the estuary (37°09'54.2''N 7°24'04''W) and dead leaves of *Zostera noltii* in the salt marsh of Ria de Aveiro (40°39'33.3''N 8°43'27.4''W) in Portugal. Samples were placed in sterile plastic containers and maintained at 4 °C until fungal isolation. Water samples were vacuum filtered with sterile 0.22 µm cellulose membranes (Lifesciences). Then, the membranes were vigorously washed in 10 mL of autoclaved filtered saline water (AFSW). Aliquots of 100 µl from each water sample were spread onto Potato Dextrose Agar (PDA) containing 3 % sea salts (Sigma-Aldrich). *Z. noltii* samples were washed with AFSW, cut into small pieces and placed on PDA enriched with 3 % sea salts. Streptomycin and tetracycline, at final concentrations of 100 mg/L, were added to PDA medium to inhibit the growth of bacteria. Five replicates of agar plates were used for each sample. The plates were incubated at 25 °C and examined daily to observe the growth of fungal hyphae. Distinct fungal colonies were then transferred to new agar plates for further isolation and purification.

Endophytic isolates from the halophyte *Halimione portulacoides* were obtained in a previous study (Aleixo et al. unpublished). Briefly, *H. portulacoides* samples were collected from three distinct sites in Ria de Aveiro and the roots, stems and leaves tissues were used for isolation. After the surface sterilization, all tissues were cut into small pieces and placed on PDA and incubated at 25 °C for the growth of fungal colonies.

DNA isolation, amplification and analyses

Genomic DNA was extracted from fresh mycelium of cultures growing on PDA according to Möller et al. (1992). The primers ITS1 and ITS4 (White et al. 1990) were used for amplification and sequencing of the ITS region of the ribosomal DNA as described by Alves et al. (2004). The amplified PCR fragments were purified with the NZYGelpure kit (NZYTech, Portugal) before sequencing at GATC Biotech (Cologne, Germany). The nucleotide sequences were analysed with FinchTV v.1.4.0 (Geospiza Inc. www.geospiza.com/finchtv). A BLASTn search against GenBank

database using the ITS sequence was carried out to determine the closest matching sequences, which were added to the sequence alignment. Sequences were aligned with ClustalX v. 2.1 (Thompson et al. 1997), using the following parameters: pairwise alignment parameters (gap opening = 10, gap extension = 0.1) and multiple alignment parameters (gap opening = 10, gap extension = 0.2, transition weight = 0.5, delay divergent sequences = 25 %). Alignments were checked and edited with BioEdit Alignment Editor v.7.2.5 (Hall 1999). Phylogenetic analyses were done with MEGA7 v.7.0 (Kumar et al. 2016). All gaps were included in the analyses. MEGA7 v.7.0 was also used to determine the best substitution model to be used to build the Maximum Likelihood (ML) tree. ML analysis was performed on a Neighbour-Joining (NJ) starting tree automatically generated by the software. Nearest-Neighbour-Interchange (NNI) was used as the heuristic method for tree inference with 1,000 bootstrap replicates. The sequences generated in this study were deposited in GenBank and taxonomic novelties in Mycobank (www.mycobank.org) and Faces of Fungi (<http://www.facesoffungi.org>). Alignment and tree were deposited in TreeBase (S24085).

Morphology and growth studies

The morphological observations were performed using a SMZ1500 stereoscopic microscope and a Nikon Eclipse 80i microscope (Nikon, Japan). Measurements and photographs of the fungal structures, mounted in 100 % lactic acid, were taken with a Nikon DSR1 camera (Nikon, Japan) and the NIS-Elements D program (Nikon, Japan). Colony characters and pigment production were registered after 2 weeks of growth on PDA, Malt Extract Agar (MEA) and Oatmeal Agar (OA) incubated at 25 °C. Colony colours (surface and reverse) were assessed according to the colour charts of Rayner (1970). Morphological descriptions were based on cultures sporulating on PDA enriched with 3 % sea salts, after 2 weeks incubated at 25 °C.

The reaction of the isolates to a gradient of temperatures was observed. A 5-mm diameter plug was taken from the margin of an actively growing colony (14-day-old) and placed in the centre of PDA, MEA and OA plates. Three replicate plates per isolate were incubated at 10, 15, 20, 25, 30 and 35 °C in the darkness and the colony diameter was measured after 7 and 14 days.

To evaluate the requirement of sea salts for growth, each species was cultured on PDA with and without the addition of 3 % sea salts. Three replicate plates per isolate were incubated at 25 °C for 14 days in the dark. After incubation, the diameter of the colonies was measured and compared.

Results

Phenotypic characterisation

On the basis of conidial morphology, the isolates studied were divided into three groups: 1) isolates with aseptate golden-brown conidia; 2) isolates with 1-septate golden-brown conidia and 3) isolates with muriform septate golden-brown conidia.

For all isolates and all culture media tested, the minimum, maximum and optimal growth temperatures were 10, 30 and 25 °C, respectively, with the exception of OA where the maximum growth temperature was 35 °C. For all isolates tested no differences were observed in terms of colony diameter when grown in PDA with and without the addition of 3 % sea salts, thus showing that sea salts are not required for growth of the isolates.

Phylogeny

BLASTn searches against the NCBI GenBank nucleotide database using the ITS sequences of the isolates retrieved various hits, of which those with the highest sequence similarity belonged to members of the genus *Neocamarosporium*. Thus, the sequences generated in this study and sequences from *Neocamarosporium* species deposited in GenBank were included in a phylogenetic analysis (Table 1). The alignment of the ITS region contained 39 sequences (including the outgroup), and there was a total of 598 positions in the final dataset. The phylogenetic tree generated by ML analysis confirmed that the isolates studied fall into the genus *Neocamarosporium*

(*Neocamarosporiaceae*, *Pleosporales*). *Pyrenochaetopsis tabarestanensis* (IBRC-M 30051) was used as outgroup.

Table 1 List of isolates used in this study.

Species	Strain	Host/Substrate	Country	ITS
<i>Alternaria eureka</i>	CBS 193.86*	<i>Medicago rugosa</i>	Australia	MH861937
<i>Comoclathris spartii</i>	MFLUCC 13-0214*	<i>Spartium junceum</i>	Italy	KM577159
<i>Foliophoma fallens</i>	CBS 284.70	<i>Nerium oleander</i>	Italy	MH859609
	CBS 161.78	<i>Olea europaea</i>	New Zealand	KY929147
<i>Libertasomyces quercus</i>	CBS 134.97*	<i>Quercus ilex</i>	Spain	KY929152
<i>Neocamarosporium aestuarinum</i>	MUM 18.55/CMG 4*	Saline water	Portugal	MH397366
	CAA 803	<i>Halimione portulacoides</i>	Portugal	MK139931
<i>Neocamarosporium betae</i>	CBS 109410	<i>Beta vulgaris</i>	-	KY940790
	CBS 112.85	<i>Beta vulgaris</i>	Denmark	KY940782
	CBS 111.85	<i>Beta vulgaris</i>	Denmark	KY940781
	CBS 236.28	<i>Solanum tuberosum</i>	Netherlands	KY940746
	CBS 523.66	<i>Beta vulgaris</i>	Netherlands	FJ426981
<i>Neocamarosporium calvescens</i>	CBS 246.79	<i>Atriplex hastata</i>	Germany	KY940774
	CBS 343.78	<i>Atriplex hastata</i>	Netherlands	KY940773
	CBS 215.57	-	UK	KY940760
<i>Neocamarosporium chersinae</i>	CPC 27298*	Dead angulate tortoise shell	South Africa	NR_154261
<i>Neocamarosporium chichastianum</i>	CBS 137502/IBRC-M 30126*	Saline soil of Lake Urima	Iran	KM670458
<i>Neocamarosporium endophyticum</i>	MUM 18.56/CAA 808*	<i>Halimione portulacoides</i>	Portugal	MK139935
	CAA 809	<i>Halimione portulacoides</i>	Portugal	MK139936
	CAA 804	<i>Halimione portulacoides</i>	Portugal	MK139937
	CMG 10	Dead leaves of <i>Zostera noltii</i>	Portugal	MK492323
<i>Neocamarosporium goegapense</i>	CPC 23676/CBS 138008*	<i>Mesembryanthemum</i> sp.	South Africa	KJ869163
<i>Neocamarosporium halimiones</i>	MUM 18.54/CAA 807*	<i>Halimione portulacoides</i>	Portugal	MK139932
	CAA 805	<i>Halimione portulacoides</i>	Portugal	MK139933
	CAA 806	<i>Halimione portulacoides</i>	Portugal	MK139934
<i>Neocamarosporium jorjanensis</i>	IBRC-M 30243*	Hypersaline soil	Iran	KX817213
<i>Neocamarosporium korfii</i>	MFLUCC 17-0745*	-	Russia	NR_154268
<i>Neocamarosporium lamiacearum</i>	MFLU 15-2989*	<i>Lamiaceae</i> sp.	Russia	NR_154269
	MFLUCC 17-0750	-	Russia	MF434192
<i>Neocamarosporium obiones</i>	CBS 786.68	<i>Halimione portulacoides</i>	Netherlands	GU230753
	CBS 432.77	<i>Halimione portulacoides</i>	Netherlands	GU230752
<i>Neocamarosporium persepolisi</i>	IBRC-M 30134*	Hypersaline soil	Iran	KX817215
<i>Neocamarosporium phragmitis</i>	MFLUCC 17-0756*	<i>Phragmites australis</i>	UK	MG844345
<i>Neocamarosporium salicorniicola</i>	MFLU 15-0957*	<i>Salicornia</i> sp.	Thailand	NR_154270
<i>Neocamarosporium salsolae</i>	MFLU 17-0192*	<i>Salsola</i> sp.	Uzbekistan	NR_154271
<i>Neocamarosporium solicola</i>	IBRC-M 30257*	Hypersaline soil	Iran	KX817217
<i>Paradendryphiella salina</i>	CBS 142.60*	<i>Spartina</i> stem	UK	MH857928
<i>Pyrenochaetopsis tabarestanensis</i>	IBRC-M 30051*	Rice farm	Iran	NR_155636
<i>Stemphylium vesicarium</i>	CBS 191.86*	<i>Medicago sativa</i>	India	MH861935

CBS: Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; CAA: Culture collection of Artur Alves, housed at Department of Biology, University of Aveiro, Portugal; CMG: Culture collection of Micael Gonçalves, housed at Department of Biology, University of Aveiro, Portugal; CPC: Culture collection of Pedro Crous, housed at

CBS; MFLU: Herbarium of Mae Fah Luang University; IBRC: Iranian Biological Resource Center; MUM: Culture collection hosted at Center for Biological Engineering of University of Minho, Portugal. MFLUCC: Mae Fah Luang University Culture Collection, Chiang Rai, Thailand. Ex-type, ex-epitype, or isotype strains are marked with an asterisk. Sequences generated in this study are shown in **bold**.

In the ML ITS phylogenetic tree (Fig. 1) the isolates studied clustered into three distinct clades that received high bootstrap supports: clade 1 and 3 (99%) and clade 2 (91%). These three clades match exactly the same three groups previously defined on the basis of conidial morphology. Thus, these morphologically and phylogenetically distinct groups of isolates are deemed to represent three novel species of *Neocamarosporium*, which are described here in detail.

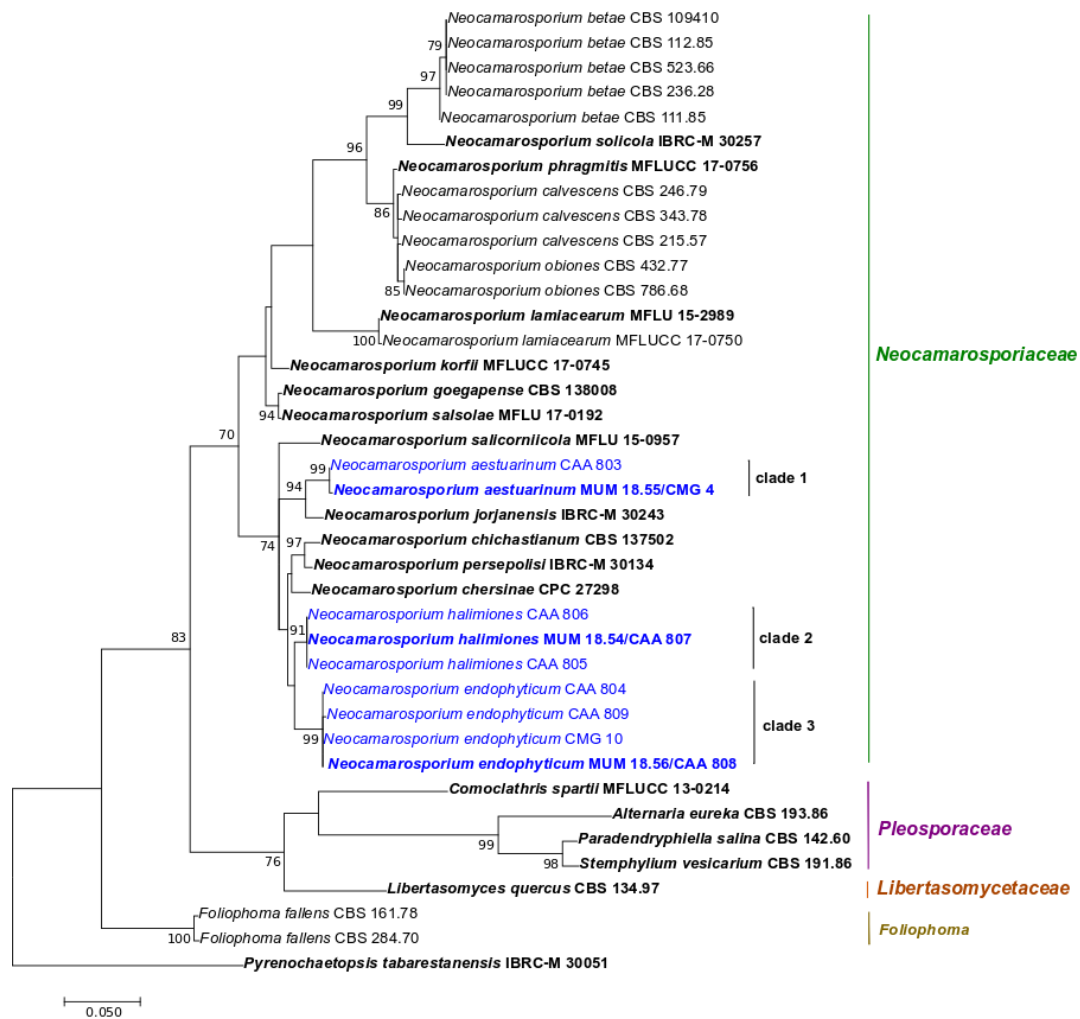


Figure 1 – Phylogenetic relationships of *Neocamarosporium* species based on ITS sequence data and inferred using the Maximum Likelihood method under the Kimura 2-parameter model. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site and rooted to *P. tabarestanensis* (IBRC-M 30051). Bootstrap values (> 70%) are shown at the nodes. Ex-holotype, ex-epitype, or ex-isotype strains are in bold and the isolates from the current study are in blue.

Taxonomy

Neocamarosporium aestuarinum M. Gonçalves & A. Alves, sp. nov.

Mycobank: MB829965; Facesoffungi number: FoF06133

Etymology – named for the environment it was first isolated from, namely an estuary.

Holotype – MUM-H 18.55

Fig. 2

Endophytic in marine habitats. Sexual morph: not observed. Asexual morph: coelomycetous. *Conidiomata* solitary, pycnidial, immersed to erumpent and globose. *Paraphyses* absent. *Conidia* (mean \pm S.D. = $10.7 \pm 2.5 \times 12.1 \pm 2.0 \mu\text{m}$, n = 50), solitary, initially hyaline, aseptate, thick-walled, developing a central septum and becoming muriform when mature with shape variable from globose to obovoid to ellipsoid or subcylindrical or irregular, golden-brown, finely roughened.

Culture characteristics – Colonies smooth, with fluffy aerial mycelium, lighter in the centre getting a colour towards the periphery. On PDA surface, margin semi regular, blood colour, smoke grey near the centre; reverse blood colour. On MEA surface, margins flesh with little tufts of aerial mycelium, dirty white near the centre; reverse light blood colour. On OA, olivaceous-black near the centre getting lighter towards the borders, with tufts of dirty white mycelium. Colonies growing slower on PDA and MEA, reaching 20 mm in 14 days at 25 °C, in darkness. On OA the colonies grew fast reaching 90 mm in the same time. At 35° C, there was only growth on OA with 50 mm in 14 days.

Known distribution – Portugal.

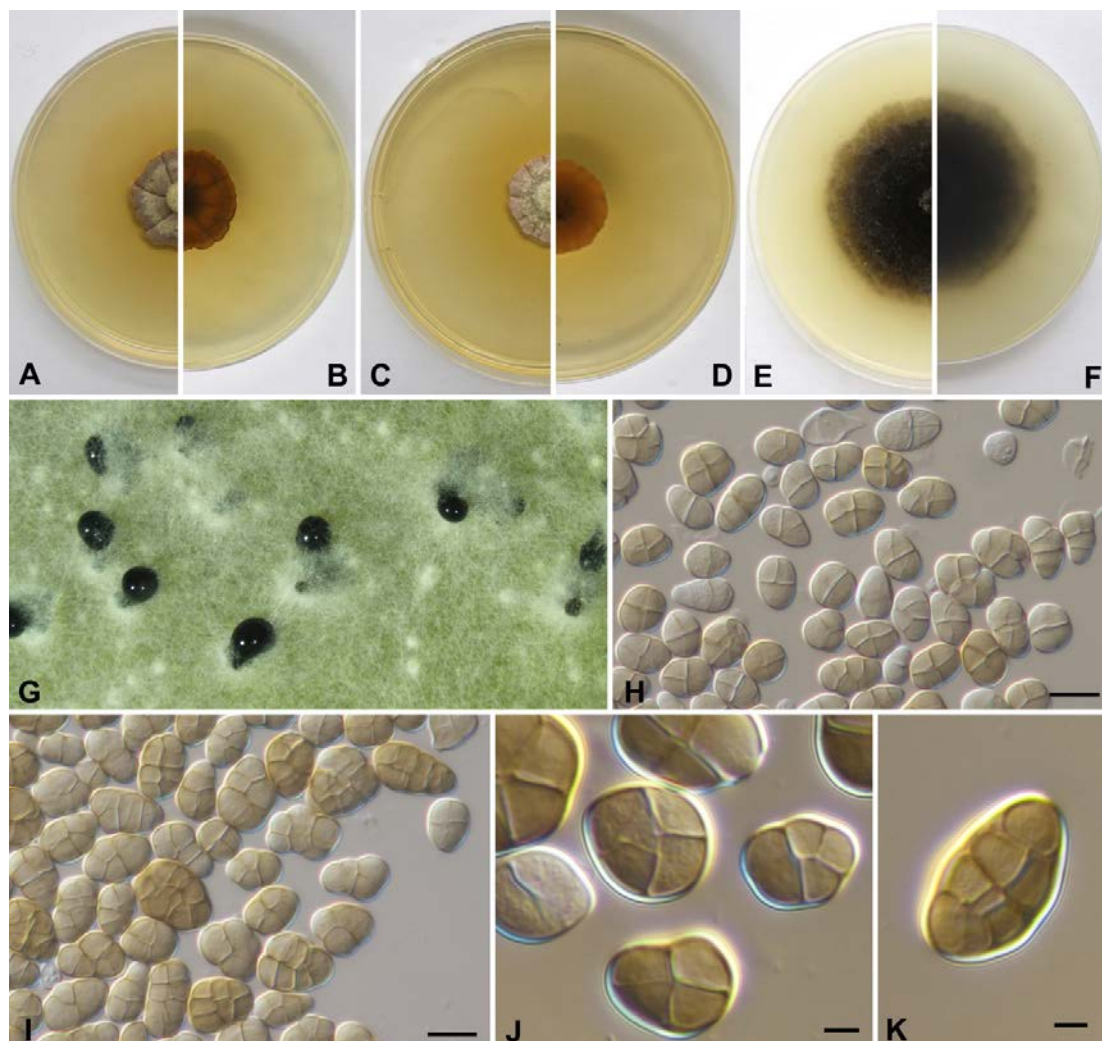


Figure 2 – *Neocamarosporium aestuarinum* (MUM 18.55). a-b Colony after 2 weeks at 25 °C on PDA (B reverse). c-d Colony after 2 weeks at 25 °C on MEA (D reverse). e-f Colony after 2 weeks at 25 °C on OA (F reverse). g Conidiomata developed on PDA. h-k Conidia. Scale bars: h–i = 10 μm , j–k = 2.5 μm .

Material examined – Portugal, Vila Real de Santo António, from sea water, October 2017, M. Gonçalves, (MUM-H 18.55 holotype), a dried culture sporulating, ex-holotype living culture, MUM 18.55 = CMG 4.

Note – *Neocamarosporium aestuarinum* is phylogenetically closely related but distinct from *N. jorjanensis* (IBRC-M 30243). Although conidial dimensions of both species are similar in length, conidia of *N. aestuarinum* are more globose with a wider width than *N. jorjanensis*. Also, they differ in 23 nucleotide positions in ITS (Tables 2, 3).

Neocamarosporium endophyticum M. Gonçalves & A. Alves, sp. nov. Fig. 3

Mycobank: MB829966; Facesoffungi number: FoF06135

Etymology – named for to the endophytic nature of the isolates obtained from plant tissues.

Holotype – MUM-H 18.56

Endophytic in marine habitats. Sexual morph: not observed. Asexual morph: coelomycetous. *Conidiomata* solitary, pycnidial, immersed to erumpent and globose. *Paraphyses* absent. *Conidia* (mean \pm S.D. = $7.8 \pm 1.2 \times 3.2 \pm 0.4 \mu\text{m}$, n = 50), solitary, initially hyaline, aseptate, thick-walled, developing a central septum (rarely developing additional septa) when mature with shape variable from obovoid to ellipsoid or subcylindrical, becoming golden-brown.

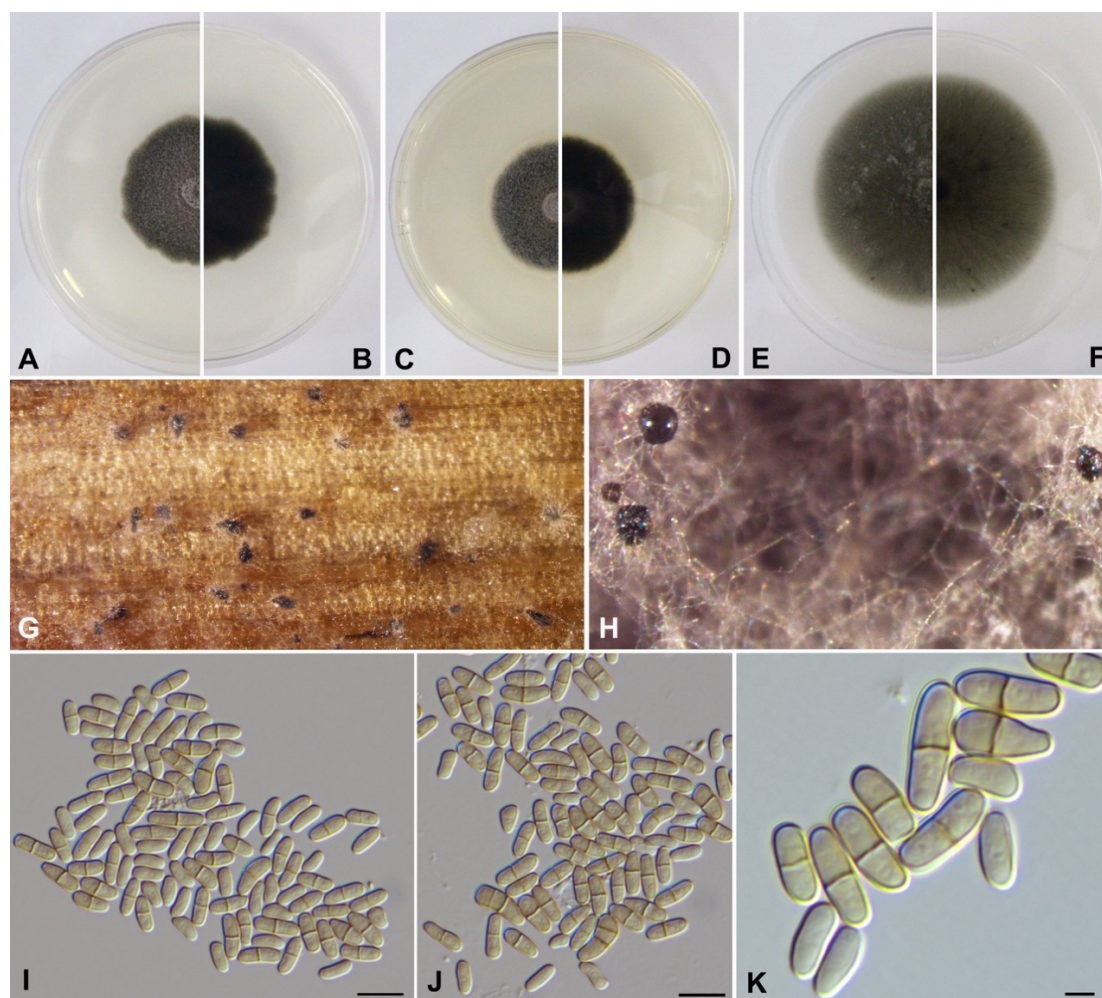


Figure 3 – *Neocamarosporium endophyticum* (MUM 18.56). a-b Colony after 2 weeks at 25 °C on PDA (B reverse). c-d Colony after 2 weeks at 25 °C on MEA (D reverse). e-f Colony after 2 weeks at 25 °C on OA (F reverse). g Conidiomata developed on fennel stems. h Conidiomata developed on PDA. i-k Conidia. Scale bars: i–j = 10 μm , k = 2.5 μm .

Culture characteristics – Colonies smooth, with fluffy aerial mycelium. On PDA, MEA and OA, olivaceous-grey to olivaceous-black. Colonies growing on PDA and MEA reaching 60 mm in 14 days at 25 °C, under dark. On OA the colonies grew fast reaching 70 mm in the same time. At 35° C, there was only growth in OA with 10 mm in 14 days.

Known distribution – Portugal.

Material examined – Portugal, Ria de Aveiro, endophytic isolate from *Halimione portulacoides*, October 2017, M. Gonçalves, (MUM-H 18.56 holotype), a dried culture sporulating, ex-holotype living culture MUM 18.56 = CAA808.

Note – *Neocamarosporium endophyticum* is phylogenetically closely related to *N. halimiones* (MUM 18.54). Although some culture characteristics of both species are similar, they differ in conidial morphology (septate vs. aseptate), dimensions and in 14 nucleotide positions in ITS (Table 2 and 3).

Neocamarosporium halimiones M. Gonçalves & A. Alves, sp. nov.

Fig. 4

Mycobank: MB829967; Facesoffungi number: FoF06132

Etymology – named for the host it was first isolated from, namely *Halimione portulacoides*.

Holotype – MUM-H 18.54

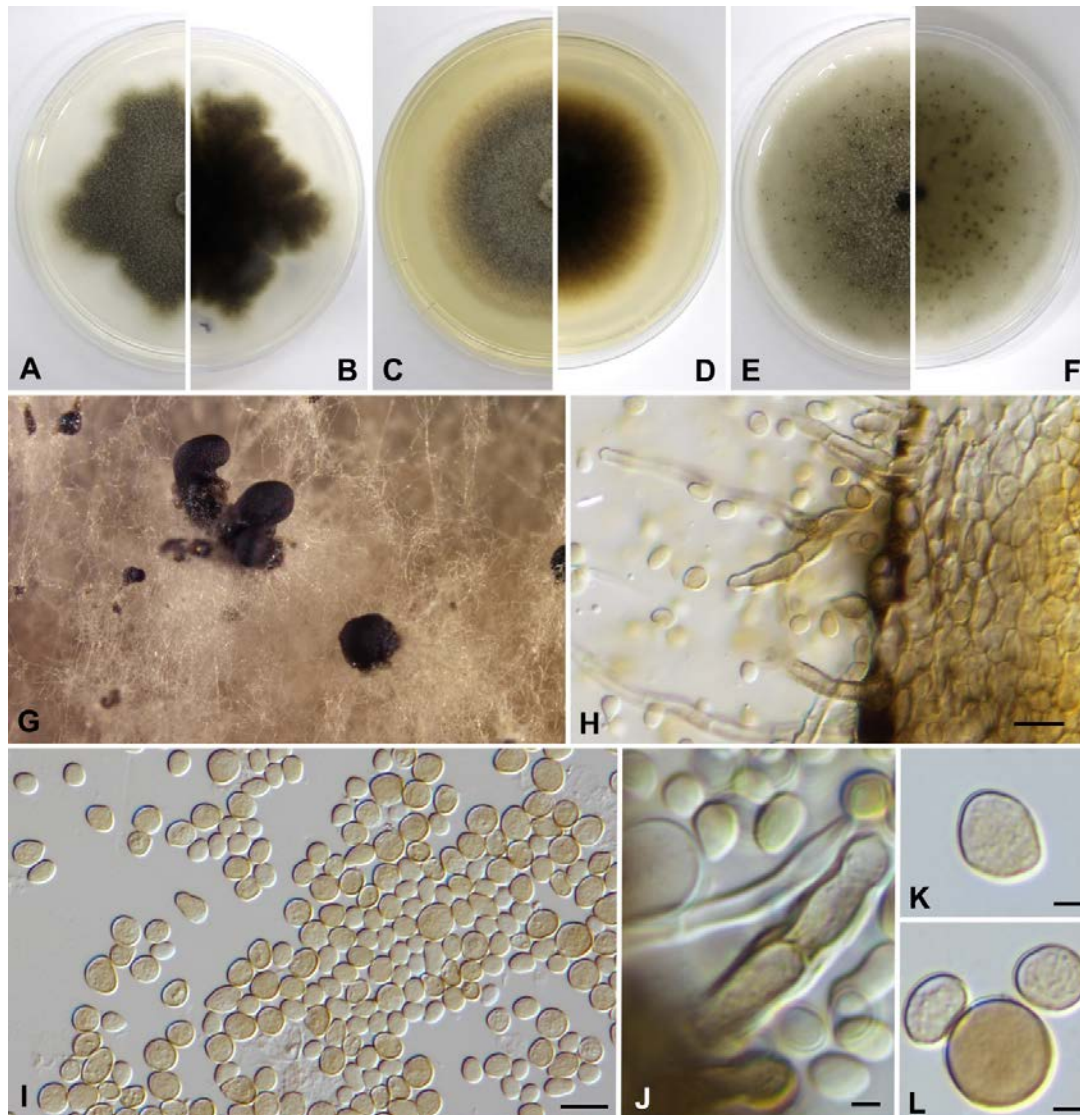


Figure 4 – *Neocamarosporium halimiones* (MUM 18.54). a-b Colony after 2 weeks at 25 °C on PDA (B reverse). c-d Colony after 2 weeks at 25 °C on MEA (D reverse). e-f Colony after 2 weeks at 25 °C on OA (F reverse). g Conidiomata developed on PDA. h Conidiogenous layer with conidiogenous cells and paraphyses. j Conidiogenous cells. i, k-l Conidia. Scale bars: h–i = 10 µm, j–l = 2.5 µm.

Endophytic in *Halimione portulacoides*. Sexual morph: not observed. Asexual morph: coelomycetous. *Conidiomata* solitary, pycnidial, immersed to erumpent and globose. *Paraphyses*

absent. *Conidiogenous cells* separate, hyaline, smooth, ampulliform. *Conidia* (mean \pm S.D. = $4.5 \pm 0.8 \times 4.0 \pm 0.7 \mu\text{m}$, n = 50), solitary, initially hyaline, aseptate, thick-walled, shape variable from globose, subglobose to obovoid or irregular, becoming golden-brown.

Culture characteristics – Colonies smooth, with fluffy aerial mycelium. On PDA surface, margin irregular, olivaceous-grey to olivaceous-black; reverse olivaceous-black. On MEA, olivaceous to olivaceous-grey, margins regular and dirty white. On OA, olivaceous-grey. Colonies growing on PDA and MEA, reaching 50 mm in 14 days at 25 °C, under dark. On OA the colonies grew faster reaching 65 mm in the same time. At 35° C, there was only growth on OA with 8 mm in 14 days.

Known distribution – Portugal.

Material examined – Portugal, Ria de Aveiro, endophytic isolate from *Halimione portulacoides*, October 2017, M. Gonçalves, (MUM-H 18.54 holotype), a dried culture sporulating, ex-holotype living culture MUM 18.54 = CAA 807.

Note – *Neocamarosporium halimiones* is phylogenetically closely related to *N. endophyticum* (MUM 18.56). They differ mainly in conidial morphology and in 14 nucleotide positions in ITS (Table 2 and 3).

Table 2 Synopsis of recorded *Neocamarosporium* species discussed in this study.

Species	Conidia Size (μm)	Septation	Colour	References
<i>N. aestuarinum</i>	$10.7 \pm 2.5 \times 12.1 \pm 2.0$	Muriform	Golden-brown	Present study
<i>N. betae</i>	$(2.5-4-6.5(-9.5) \times (1.5-2.5-4(-5.5))$	0	-	Boerema 1993
<i>N. calvescens</i>	-	1	Brown	Wijayawardene et al. 2016
<i>N. chersinae</i>	$(10-12-13(-15) \times (5-6(-7))$	Muriform	Golden-brown	Crous & Groenewald 2017
<i>N. chichastianum</i>	$(11-15-19(-22) \times (6-8-9(-11))$	Muriform	Brown	Crous et al. 2014b
<i>N. endophyticum</i>	$7.8 \pm 1.2 \times 3.2 \pm 0.4$	1 (-2)	Golden-brown	Present study
<i>N. goegapense</i>	$(15-20-22(-24) \times 15-17(-19))$	Muriform	Golden-brown	Crous et al. 2014a
<i>N. halimiones</i>	$4.5 \pm 0.8 \times 4.0 \pm 0.7$	0	Golden-brown	Present study
<i>N. jorjanensis</i>	$(9-11-12(-13) \times 4-7(-8))$	Muriform	Golden-brown	Papizadeh et al. 2017
<i>N. korfii</i>	$12-18 \times 8-10$	Muriform	Dark brown	Wanasinghe et al. 2017
<i>N. lamiacearum</i>	$14-20 \times 8-11$	Muriform	Pale brown	Wanasinghe et al. 2017
<i>N. obiones</i>	$8.5-9 \times 3-4$	1	Pale-yellow to brownish	Dickinson & Jones 1966
<i>N. persepolisi</i>	$(8-14-18(-19) \times 6-9$	Muriform	Golden-brown	Papizadeh et al. 2017
<i>N. phragmitis</i>	$12-16 \times 6-8$	Muriform	Pale brown	Hyde et al. 2018
<i>N. salicorniicola</i>	$8-12 \times 4-6$	Muriform	Dark brown	Wanasinghe et al. 2017
<i>N. salsolae</i>	$18-25 \times 12-20$	Muriform	Dark brown	Wanasinghe et al. 2017
<i>N. solicola</i>	$(8-12(-16) \times (2-2.5(-4))$	0	Hyaline	Papizadeh et al. 2017

Table 3 Major ITS base pair differences of *Neocamarosporium aestuarinum*, *N. chersinae*, *N. chichastianum*, *N. endophyticum*, *N. halimiones* and *N. jorjanensis*. n.b. nonexistent base.

Species	Base pair difference	Place of ITS nucleotides difference
<i>N. aestuarinum</i> and <i>N. jorjanensis</i>	G instead of A	36, 481
	A instead of n.b.	41, 180, 401
	C instead of T	42, 70,
	T instead of C	48, 395
	A instead of G	132, 402, 487
	A instead of C	382

Table 3 Continued.

Species	Base pair difference	Place of ITS nucleotides difference
	T instead of n.b.	96-400
	n.b. instead of A	422
	C instead of A	435
	n.b. instead of C	475, 496
	n.b. instead of T	482
<i>N. endophyticum</i> and <i>N. halimiones</i>	A instead of T	26
	T instead of C	33, 42, 44, 50, 378
	C instead of T	40, 347, 372,
	A instead of G	52
	G instead of A	113
	C instead of n.b.	474
	T instead of n.b.	475, 462
<i>N. endophyticum</i> and <i>N. chichastianum</i>	A instead of G	13, 19, 52
	A instead of T	26, 68
	C instead of T	32, 347, 372, 393, 447
	T instead of C	33, 50, 66, 378
	G instead of A	113
	C instead of n.b.	374
	T instead of n.b.	375, 462
	C instead of G	386
	G instead of C	389
T instead of G	448	
<i>N. endophyticum</i> and <i>N. chersinae</i>	G instead of A	7, 21, 113
	A instead of G	19, 25
	A instead of C	22
	A instead of T	26
	C instead of T	32, 347, 372
	T instead of C	33, 50, 140, 378
	G instead of T	39
	C instead of n.b.	374
	T instead of n.b.	375, 462
T instead of G	448	
<i>N. halimiones</i> and <i>N. chichastianum</i>	A instead of G	12, 18
	C instead of T	31
	T instead of C	39, 65, 390
	C instead of T	41, 43, 444
	A instead of T	77
	C instead of G	383
	G instead of C	386
T instead of G	445	
<i>N. halimiones</i> and <i>N. chersinae</i>	G instead of A	6, 20, 51
	A instead of G	8, 24
	A instead of C	21
	C instead of T	31
	G instead of T	38
	T instead of C	39, 139
	C instead of T	41, 43
T instead of G	445	

Discussion

The genus *Neocamarosporium* harbours 15 species described so far (Index Fungorum 2019). This study adds three novel species isolated from saline environments, namely *Neocamarosporium aestuarinum*, *N. endophyticum* and *N. halimiones*. The introduction of these species is well supported by morphological and phylogenetic data.

Crous et al. (2014a) introduced *Neocamarosporium* as a new genus distinct from *Camarosporium*. The presence of two distinct groups within *Neocamarosporium* with morphological and phylogenetic differences based on combined dataset of ITS and β -tubulin was reported by Papizadeh et al. (2017) suggesting the possibility of these representing two separate genera. One clade comprises the species *N. betae*, *N. calvescens* and *N. solicola* which have hyaline aseptate conidia and a second clade contains *N. goegapense*, *N. jorjanensis*, *N. chichastianum* and *N. persepolisi* with muriformly septate brown conidia. However, there was no β -tubulin sequence data available for most species and therefore the phylogenetic analysis presented did not encompass all known species in *Neocamarosporium*.

The three novel species described in this study are supported by morphological as well as DNA base pair differences as outlined by Jeewon & Hyde (2016). In our phylogenetic analysis, *N. aestuarinum* is more closely related to *N. jorjanensis* with high bootstrap support. Comparison of the ITS sequence from *N. aestuarinum* and *N. jorjanensis* revealed 23 base pair differences which support the establishment of *N. aestuarinum* as a distinct species. *Neocamarosporium aestuarinum* and *N. jorjanensis* have similar golden-brown and muriform conidia but can be differentiated by the size of conidia.

Neocamarosporium endophyticum and *N. halimiones* cluster together but their ITS sequences have 14 base pair differences. In addition, a comparison of the ITS sequences of *N. endophyticum* and other closely related *Neocamarosporium* species such as *N. chersinae* and *N. chichastianum* revealed 19 and 21 base pair differences, respectively. Same comparison with *N. halimiones* revealed 13 base pair differences with both *N. chersinae* and *N. chichastianum*. Morphologically, *N. endophyticum* and *N. halimiones* can be easily discriminated. The conidia of *N. halimiones* are aseptate, initially hyaline and soon become golden-brown while conidia of *N. endophyticum* are golden-brown and have a single central septum.

Two of the species described here, namely *N. halimiones* and *N. aestuarinum*, challenge the separation of the genus into two groups based on conidial characters as suggested by Papizadeh et al. (2017). Despite this, it is evident that the genus *Neocamarosporium* as currently circumscribed encompasses a wide diversity of conidial morphologies. It is possible that these different morphologies correspond to different genera. For example, Wijayawardene et al. (2014) established two genera, *Pseudocamarosporium* and *Paracamarosporium*. Although phylogenetically very close and have both pycnidial conidiomata, enteroblastic and phialidic conidiogenesis with percurrent proliferation and muriform conidia, they differ in microconidia morphology. However, multi-gene phylogenies including all *Neocamarosporium* species are needed to evaluate if these morphological groups represent distinct genera or not.

The majority of the *Neocamarosporium* species described so far appear to have some host and habitat preference, i.e. ecologically they can be found in estuarine or marine habitats in association with various genera of halophytes, such as, *Atriplex*, *Beta*, *Chenopodium*, *Halimione*, *Mesembryanthemum*, *Phragmites*, *Salicornia* and *Salsola* (Crous et al. 2014b, Ariyawansa et al. 2015, Wanasinghe et al. 2017). Papizadeh et al. (2017) reported the presence of *Neocamarosporium* species in hypersaline lakes soils in Iran, showing their halotolerance capacity by the ability to grow in media containing various concentrations of NaCl and MgCl₂. In fact, halotolerance seems to be a typical characteristic of the genus as suggested by Papizadeh et al. (2017). The species described here can be regarded as halotolerant as they grow equally well in the presence and absence of sea salts.

It is noteworthy that saline environments encompass such a high diversity of *Neocamarosporium* species and further studies will be necessary to understand the ecological role of these species in estuarine, marine and other saline habitats. Species of *Neocamarosporium* are regarded as pathogenic or saprobic on dying leaves and stems (Ariyawansa et al. 2015, Wanasinghe et al. 2017). However, their status as pathogens needs to be properly addressed as no clear evidence of their pathogenicity has been shown so far.

Here we describe three novel species that occur as endophytes on healthy tissues (leaves and stems) of the halophyte *H. portulacoides*, being *N. aestuarinum* also found in sea water and *N.*

endophyticum on dead leaves of *Z. noltii*. With four distinct species described so far (*N. aestuarinum*, *N. endophyticum*, *N. halimiones* and *N. obiones*), the salt-tolerant *H. portulacoides* seems to represent a diversity hotspot of the genus *Neocamarosporium* that deserves further investigation.

The relevance of the endophytic species reported for the host (*H. portulacoides*) harbouring them is unknown. However, as reported for other endophytes it is possible that these species have some plant-growth promoting effects thus contributing to the overall plant health and performance. Also, these species may have a significant contribution to the halophytes ability to tolerate salt stress as well as other environmental stresses (Sun et al. 2011, Ruppel et al. 2013). Future research should be conducted in order to better understand these *Neocamarosporium*-halophytes and their functional interactions.

Acknowledgements

The authors acknowledge the financial support from Fundação para a Ciência e a Tecnologia (FCT) to CESAM (UID/AMB/50017/2019) and the PhD grant of M. Gonçalves (SFRH/BD/129020/2017).

References

- Alves A, Correia A, Luque J, Phillips AJL. 2004 – *Botryosphaeria corticola* sp. nov. on *Quercus* species, with notes and description of *Botryosphaeria stevensii* and its anamorph *Diplodia mutila*. *Mycologia* 96, 598–613.
- Azevedo E, Caeiro MF, Rebelo R, Barata M. 2011 – Biodiversity and characterization of marine mycota from Portuguese waters. *Animal Biodiversity and Conservation* 34, 205–215.
- Ariyawansa HA, Thambugala KM, Manamgoda DS, Jayawardena R et al. 2015 – Towards a natural classification and backbone tree for *Pleosporaceae*. *Fungal Diversity* 71, 85–139.
- Ariyawansa HA, Phillips AJ, Chuang WY, Tsai I – 2018. *Tzeananiaceae*, a new pleosporalean family associated with *Ophiocordycepsmacroacicularis* fruiting bodies in Taiwan. *MycologyKeys* 37, 1–17.
- Boerema GH. 1993 – Contributions towards a monograph of *Phoma* (Coelomycetes) – II. Section peyronellaea. *Persoonia-Molecular Phylogeny and Evolution of Fungi* 15, 197–221.
- Butinar L, Santos S, Spencer-Martins I, Oren A et al. 2005a – Yeast diversity in hypersaline habitats. *FEMS Microbiology Letters* 244, 229–234.
- Butinar L, Sonjak S, Zalar P, Plemenitaš A et al. 2005b – Melanized halophilic fungi are eukaryotic members of microbial communities in hypersaline waters of solar salterns. *Botanica Marina* 48, 73–79.
- Crous PW, Shivas RG, Quaedvlieg W, van der Bank M et al. 2014a – Fungal Planet description sheets: 214–280. *Persoonia* 32, 184–306.
- Crous PW, Wingfield MJ, Schumacher RK, Summerell BA et al. 2014b – Fungal Planet description sheets: 281–319. *Persoonia* 33, 212–289.
- Crous PW, Wingfield MJ, Guarro J, Hernández-Restrepo M et al. 2015 – Fungal Planet description sheets: 320–370. *Persoonia* 34, 167–266.
- de Gruyter JC, Woudenberg JHC, Aveskamp MM, Verkley GJM et al. 2013 – Redisposition of phoma-like anamorphs in *Pleosporales*. *Studies in Mycology* 75, 1–36.
- Devadatha B, Sarma VV, Jeewon R, Wanasinghe DN et al. 2018a – *Thyridariella*, a novel marine fungal genus from India: morphological characterization and phylogeny inferred from multigene DNA sequence analyses. *Mycological progress* 17, 791–804.
- Devadatha B, Sarma VV, Jeewon R, Jones EG. 2018b – *Morosphaeria muthupetensis* sp. nov. (Morosphaeriaceae) from India: morphological characterization and multigene phylogenetic inference. *Botanica marina* 61, 395–405.

- Dickinson CH, Morgan-Jones G. 1966 – The mycoflora associated with *Halimione portulacoides*. IV. Observations on some species of Sphaeropsidales. Transactions of the British Mycological Society 49, 43–55.
- Gunde-Cimerman N, Zalar P, Petrovič U, Turk M et al. 2004 – Fungi in the salterns. In: Ventosa A (ed) Halophilic microorganisms. Springer, Berlin, Heidelberg.
- Hall TA. 1999 – BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series 41, 95–98.
- Hyde KD, Jones EBG. 1998 – Marine mangrove fungi. Marine Ecology 9, 15–33.
- Hyde KD, Pointing SB. 2000 – Marine mycology: a practical approach. Fungal Diversity Press, Hong Kong.
- Hyde KD, Chaiwan N, Norphanphoun C, Boonmee S et al. 2018 – Mycosphere notes 169–224. Mycosphere, 9, 271–430.
- Jeewon R, Hyde KD. 2016 – Establishing species boundaries and new taxa among fungi: recommendations to resolve taxonomic ambiguities. Mycosphere 7, 1669–1677.
- Jones EBG, Suetrong S, Sakayaroj J, Bahkali AH et al. 2015 – Classification of marine Ascomycota, Basidiomycota, Blastocladiomycota and Chytridiomycota. Fungal Diversity 73, 1–72.
- Kis-Papo T, Grishkan I, Oren A, Wasser SP et al. 2001 – Spatiotemporal diversity of filamentous fungi in the hypersaline Dead Sea. Mycology Research 105, 749–756.
- Kis-Papo T, Oren A, Wasser SP, Nevo E. 2003 – Survival of filamentous fungi in hypersaline Dead Sea water. Microbial Ecology 45, 183–190.
- Kumar S, Stecher G, Tamura K. 2016 – MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. Molecular Biology and Evolution 33, 1870–1874.
- Möller EM, Bahnweg G, Sandermann H, Geiger HH. 1992 – A simple and efficient protocol for isolation of high molecular weight DNA from filamentous fungi, fruit bodies, and infected plant tissues. Nucleic Acids Research 20, 6115–6116.
- Papizadeh M, Wijayawardene NN, Amoozegar MA, Saba F et al. 2017 – *Neocamarosporium jorjanensis*, *N. persepolisi* and *N. solicola* spp. nov. (*Neocamarosporiaceae*, *Pleosporales*) isolated from saline lakes of Iran indicate the possible halotolerant nature for the genus. Mycological Progress 17, 661–679.
- Poon MOK, Hyde KD. 1998 – Biodiversity of intertidal estuarine fungi on *Phragmites* at Mai Po marshes, Hong Kong. Botanica Marina 41, 141–155.
- Rayner RW. 1970 – A Mycological Colour Chart. Kew: Commonwealth Mycological Institute.
- Ruppel S, Franken P, Witzel K. 2013 – Properties of the halophyte microbiome and their implications for plant salt tolerance. Functional Plant Biology 40, 940–951.
- Sarma VV, Hyde KD. 2001 – A review on frequently occurring fungi in mangroves. Fungal Diversity 8, 1–34.
- Sun Y, Wang Q, Lu XD, Okane I et al. 2011 – Endophytic fungi associated with two *Suaeda* species growing in alkaline soil in China. Mycosphere 2, 239–248.
- Tanaka K, Hirayama K, Yonezawa H, Sato G et al. 2015 – Revision of the *Massarineae* (Pleosporales, Dothideomycetes). Studies in Mycology 82, 75–136.
- Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F et al. 1997 – The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research 25, 4876–4882.
- Wanasinghe DN, Hyde KD, Jeewon R, Crous PW et al. 2017 – Phylogenetic revision of *Camarosporium* (*Pleosporineae*, *Dothideomycetes*) and allied genera. Studies in Mycology 87, 207–256.
- White TJ, Bruns T, Lee S, Taylor J. 1990 – Amplification and direct sequencing of fungal genes for phylogenies. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds), PCR Protocols: A guide to methods and applications. Academic Press, California, pp. 315–322.

- Wijayawardene NN, Crous PW, Kirk PM, Hawksworth DL et al. 2014 – Naming and outline of dothideomycetes – 2014 including proposals for the protection or suppression of generic names. *Fungal Diversity* 69, 1–55.
- Wijayawardene NN, Hyde KD, Bhat DJ, Goonasekara ID et al. 2015 – Additions to brown spored coelomycetous taxa in Massarinae, Pleosporales: introducing *Phragmocamarosporium* gen. nov. and *Suttonomyces* gen. nov. *Cryptogamie Mycologie* 36, 213–225.
- Wijayawardene NN, Hyde KD, Wanasinghe DN, Papizadeh M et al. 2016 – Taxonomy and phylogeny of dematiaceous coelomycetes. *Fungal Diversity* 77, 1–316.
- Wijayawardene NN, Papizadeh M, Phillips AJL, Wanasinghe DN et al. 2017a – Mycosphere Essays 19: recent advances and future challenges in taxonomy of coelomycetous fungi. *Mycosphere* 8, 934–950.
- Wijayawardene NN, Hyde KD, Rajeshkumar KC, Hawksworth DL et al. 2017b – Notes for genera: Ascomycota. *Fungal Diversity* 86, 1–594.