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# 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*

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## 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), *Didymellocamarosporium* and *Melanocamarosporium* (Wijayawardene et al. 2016).

*Neocamarasporium* 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).

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Corresponding Author: Artur Alves – e-mail – artur.alves@ua.pt

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 (37º09’54.2’’N 7º24’04’’W) and dead leaves of *Zostera noltii* in the salt marsh of Ria de Aveiro (40º39’33.3’’N 8º43’27.4’’W) in Portugal. Samples were placed in sterile plastic containers and maintained at 4 ºC 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 ºC 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 DSRi1 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 ºC. 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 ºC.

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 ºC 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 ºC, respectively, with the exception of OA where the maximum growth temperature was 35 ºC. 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 |
|---|---|---|---|---|
| *Alternaria eureka* | CBS 193.86* | *Medicago rugosa* | Australia | MH861937 |
| *Comoclathris spartii* | MFLUCC 13-0214* | *Spartium junceum* | Italy | KM577159 |
| *Foliophoma fallens* | CBS 284.70 | *Nerium oleander* | Italy | MH859609 |
| | CBS 161.78 | *Olea europaea* | New Zealand | KY929147 |
| *Libertasomyces quercus* | CBS 134.97* | *Quercus ilex* | Spain | KY929152 |
| ***Neocamarosporium aestuarinum*** | **MUM 18.55/CMG 4*** | **Saline water** | **Portugal** | **MH397366** |
| | **CAA 803** | ***Halimione portulacoides*** | **Portugal** | **MK139931** |
| *Neocamarosporium betae* | CBS 109410 | *Beta vulgaris* | - | KY940790 |
| | CBS 112.85 | *Beta vulgaris* | Denmark | KY940782 |
| | CBS 111.85 | *Beta vulgaris* | Denmark | KY940781 |
| | CBS 236.28 | *Solanum tuberosum* | Netherlands | KY940746 |
| | CBS 523.66 | *Beta vulgaris* | Netherlands | FJ426981 |
| *Neocamarosporium calvescens* | CBS 246.79 | *Atriplex hastata* | Germany | KY940774 |
| | CBS 343.78 | *Atriplex hastata* | Netherlands | KY940773 |
| | CBS 215.57 | - | UK | KY940760 |
| *Neocamarosporium chersinae* | CPC 27298* | Dead angulate tortoise shell | South Africa | NR_154261 |
| *Neocamarosporium chichastianum* | CBS 137502/IBRC-M 30126* | Saline soil of Lake Urima | Iran | KM670458 |
| ***Neocamarosporium endophyticum*** | **MUM 18.56/CAA 808*** | ***Halimione portulacoides*** | **Portugal** | **MK139935** |
| | **CAA 809** | ***Halimione portulacoides*** | **Portugal** | **MK139936** |
| | **CAA 804** | ***Halimione portulacoides*** | **Portugal** | **MK139937** |
| | **CMG 10** | **Dead leaves of *Zostera noltii*** | **Portugal** | **MK492323** |
| *Neocamarosporium goegapense* | CPC 23676/CBS 138008* | *Mesembryanthemum* sp. | South Africa | KJ869163 |
| ***Neocamarosporium halimiones*** | **MUM 18.54/CAA 807*** | ***Halimione portulacoides*** | **Portugal** | **MK139932** |
| | **CAA 805** | ***Halimione portulacoides*** | **Portugal** | **MK139933** |
| | **CAA 806** | ***Halimione portulacoides*** | **Portugal** | **MK139934** |
| *Neocamarosporium jorjanensis* | IBRC-M 30243* | Hypersaline soil | Iran | KX817213 |
| *Neocamarosporium korfii* | MFLUCC 17-0745* | - | Russia | NR_154268 |
| *Neocamarosporium lamiacearum* | MFLU 15-2989* | *Lamiaceae* sp. | Russia | NR_154269 |
| | MFLUCC 17-0750 | - | Russia | MF434192 |
| *Neocamarosporium obiones* | CBS 786.68 | *Halimione portulacoides* | Netherlands | GU230753 |
| | CBS 432.77 | *Halimione portulacoides* | Netherlands | GU230752 |
| *Neocamarosporium persepolisi* | IBRC-M 30134* | Hypersaline soil | Iran | KX817215 |
| *Neocamarosporium phragmitis* | MFLUCC 17-0756* | *Phragmites australis* | UK | MG844345 |
| *Neocamarosporium salicorniicola* | MFLU 15-0957* | *Salicornia* sp. | Thailand | NR_154270 |
| *Neocamarosporium salsolae* | MFLU 17-0192* | *Salsola* sp. | Uzbekistan | NR_154271 |
| *Neocamarosporium solicola* | IBRC-M 30257* | Hypersaline soil | Iran | KX817217 |
| *Paradendryphiella salina* | CBS 142.60* | *Spartina* stem | UK | MH857928 |
| *Pyrenochaetopsis tabarestanensis* | IBRC-M 30051* | Rice farm | Iran | NR_155636 |
| *Stemphylium vesicarium* | CBS 191.86* | *Medicago sativa* | 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.

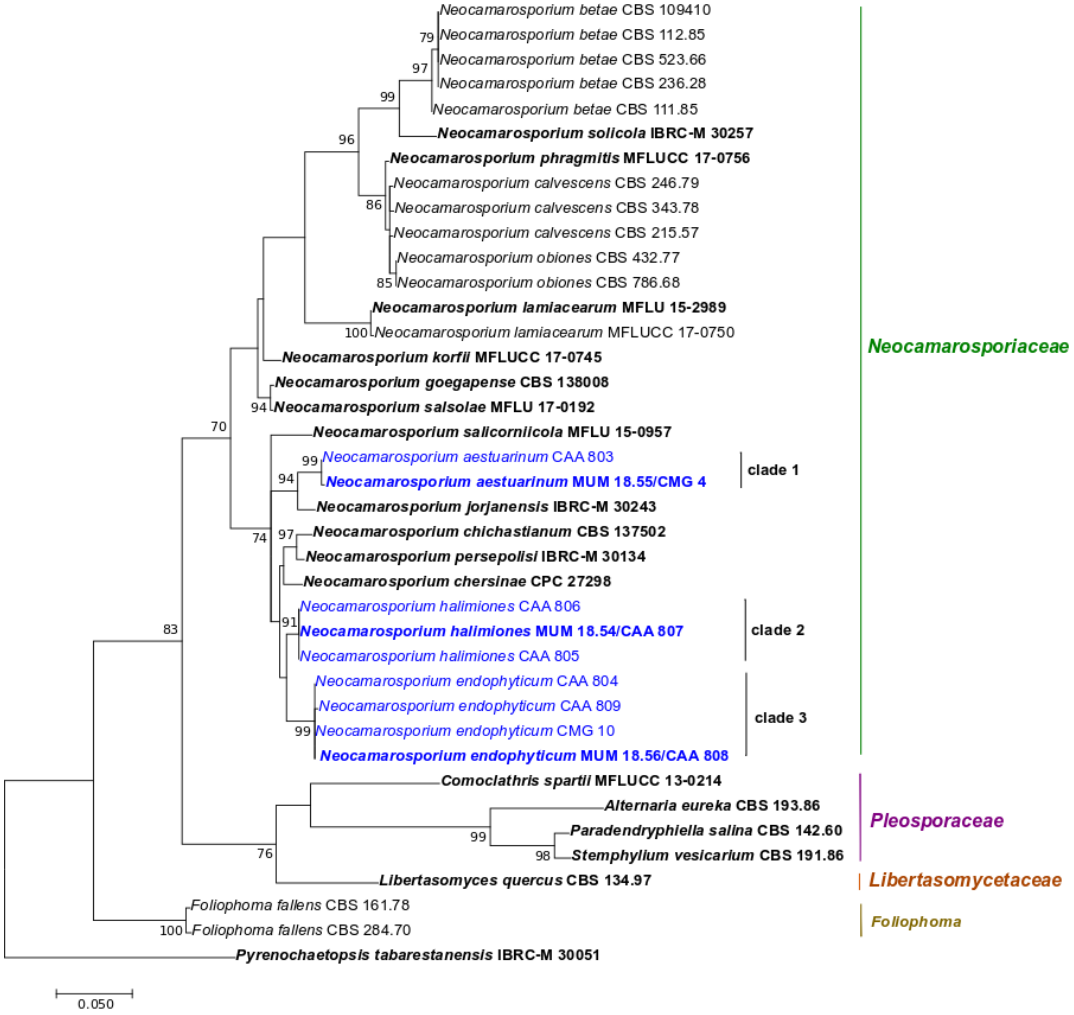


**Figure 1** – Phylogenetic relationships of *Neocamarosporium* species based on ITS sequence data and inferred using the Maximum Likelihood method under the Kimura 2-parameter model. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site and rooted to *P. tabarestanensis* (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. Fig. 2

Mycobank: MB829965; Facesoffungi number: FoF06133

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

Holotype − MUM-H 18.55

*Endophytic* in marine habitats. Sexual morph: not observed. Asexual morph: coelomycetous. *Conidiomata* solitary, pycnidial, immersed to erumpent and globose. *Paraphyses* absent. *Conidia* (mean ± S.D. = 10.7 ± 2.5 × 12.1 ± 2.0 μm, 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 tuffs 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 tuffs of dirty white mycelium. Colonies growing slower on PDA and MEA, reaching 20 mm in 14 days at 25 ºC, in darkness. On OA the colonies grew fast reaching 90 mm in the same time. At 35º C, there was only growth on OA with 50 mm in 14 days.

Known distribution – Portugal.

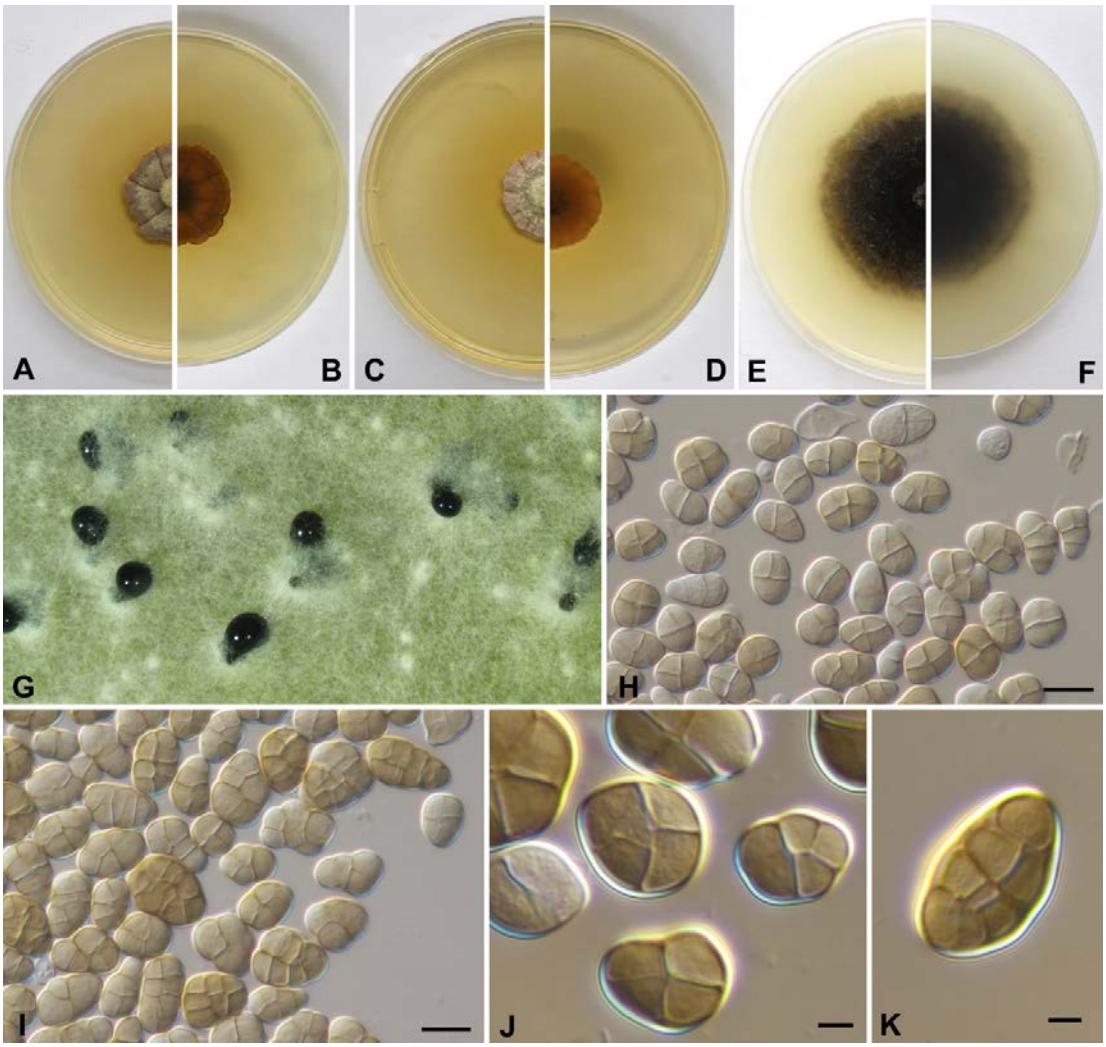


**Figure 2** – *Neocamarosporium aestuarinum* (MUM 18.55). a-b Colony after 2 weeks at 25 ºC on PDA (B reverse). c-d Colony after 2 weeks at 25 ºC on MEA (D reverse). e-f Colony after 2 weeks at 25 ºC on OA (F reverse). g Conidiomata developed on PDA. h-k Conidia. Scale bars: h–i = 10 μm, j–k = 2.5 μm.

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 ± S.D. = 7.8 ± 1.2 × 3.2 ± 0.4 μm, 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.

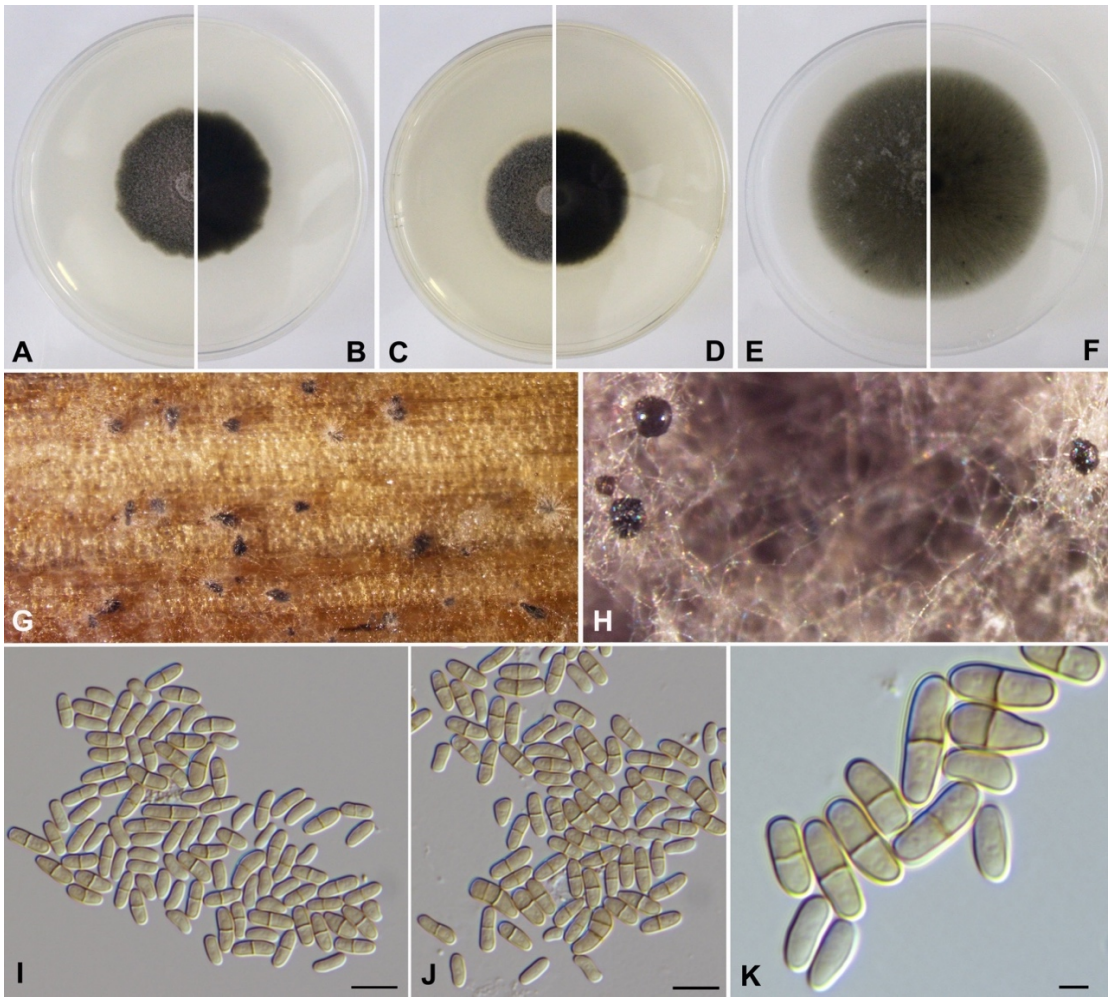


**Figure 3** – *Neocamarosporium endophyticum* (MUM 18.56). a-b Colony after 2 weeks at 25 ºC on PDA (B reverse). c-d Colony after 2 weeks at 25 ºC on MEA (D reverse). e-f Colony after 2 weeks at 25 ºC on OA (F reverse). g Conidiomata developed on fennel stems. h Conidiomata developed on PDA. i-k Conidia. 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 ºC, under dark. On OA the colonies grew fast reaching 70 mm in the same time. At 35º 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

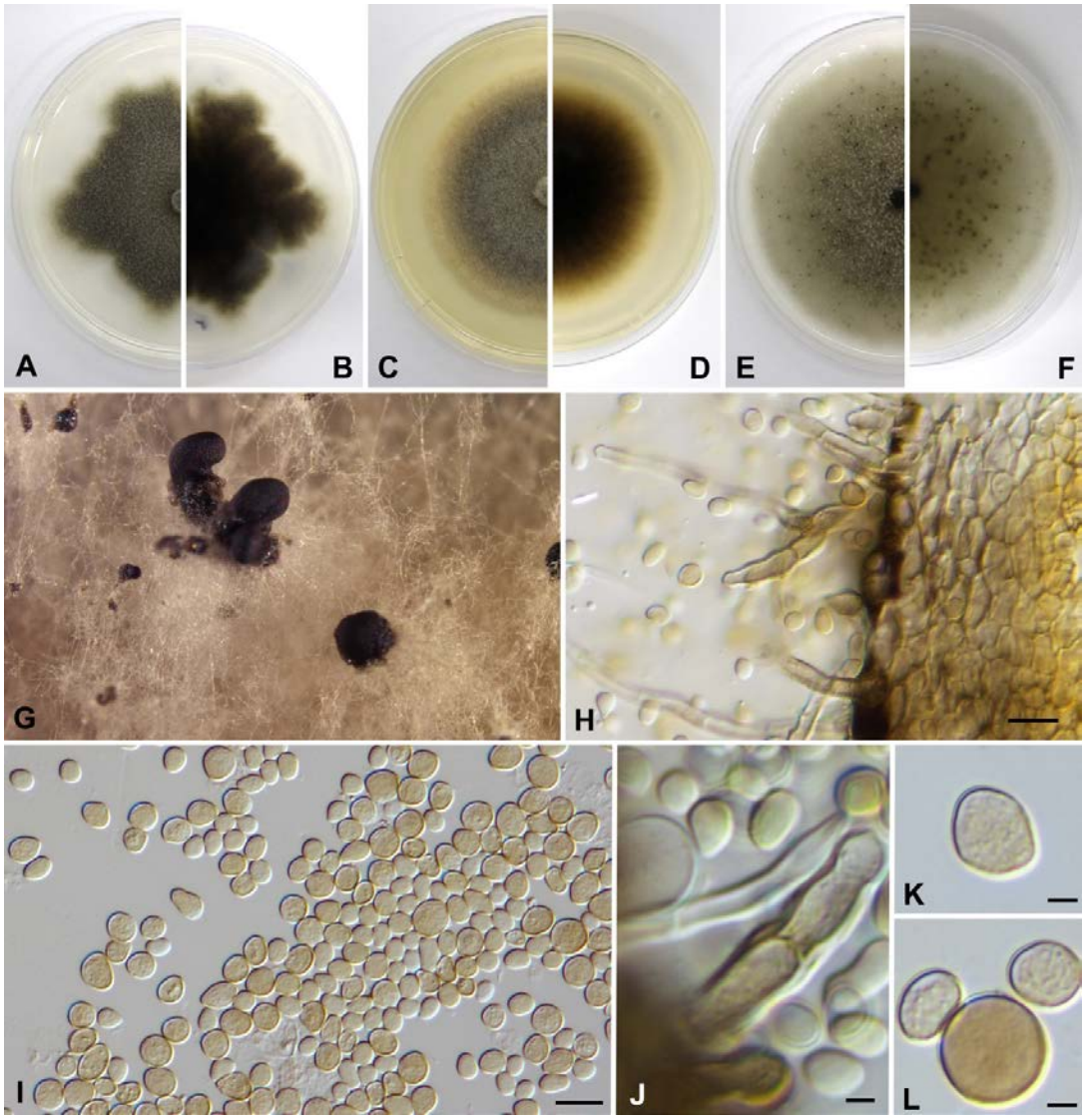


**Figure 4** – *Neocamarosporium halimiones* (MUM 18.54). a-b Colony after 2 weeks at 25 ºC on PDA (B reverse). c-d Colony after 2 weeks at 25 ºC on MEA (D reverse). e-f Colony after 2 weeks at 25 ºC on OA (F reverse). g Conidiomata developed on PDA. h Conidiogenous layer with conidiogenous cells and 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 ± S.D. = 4.5 ± 0.8 × 4.0 ± 0.7 µm, 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 ºC, under dark. On OA the colonies grew faster reaching 65 mm in the same time. At 35º 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 |
|---|---|---|---|---|
| *N. aestuarinum* | 10.7 ± 2.5 × 12.1 ± 2.0 | Muriform | Golden-brown | Present study |
| *N. betae* | (2.5–)4–6.5(–9.5) × (1.5–)2.5–4(–5.5) | 0 | - | Boerema 1993 |
| *N. calvescens* | - | 1 | Brown | Wijayawardene et al. 2016 |
| *N. chersinae* | (10–)12–13(–15) × (5–)6(–7) | Muriform | Golden-brown | Crous & Groenewald 2017 |
| *N. chichastianum* | (11–)15–19(–22) × (6–)8–9(–11) | Muriform | Brown | Crous et al. 2014b |
| *N. endophyticum* | 7.8 ± 1.2 × 3.2 ± 0.4 | 1 (–2) | Golden-brown | Present study |
| *N. goegapense* | (15–)20–22(–24) × 15–17(–19) | Muriform | Golden-brown | Crous et al. 2014a |
| *N. halimiones* | 4.5 ± 0.8 × 4.0 ± 0.7 | 0 | Golden-brown | Present study |
| *N. jorjanensis* | (9–)11–12(−13) × 4–7(−8) | Muriform | Golden-brown | Papizadeh et al. 2017 |
| *N. korfii* | 12–18 × 8–10 | Muriform | Dark brown | Wanasinghe et al. 2017 |
| *N. lamiacearum* | 14–20 × 8–11 | Muriform | Pale brown | Wanasinghe et al. 2017 |
| *N. obiones* | 8.5–9 × 3–4 | 1 | Pale-yellow to brownish | Dickinson & Jones 1966 |
| *N. persepolisi* | (8–)14–18(−19) × 6–9 | Muriform | Golden-brown | Papizadeh et al. 2017 |
| *N. phragmitis* | 12–16 × 6–8 | Muriform | Pale brown | Hyde et al. 2018 |
| *N. salicorniicola* | 8–12 × 4–6 | Muriform | Dark brown | Wanasinghe et al. 2017 |
| *N. salsolae* | 18–25 × 12–20 | Muriform | Dark brown | Wanasinghe et al. 2017 |
| *N. solicola* | (8–)12(−16) × (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 |
|---|---|---|
| *N. aestuarinum* and *N. jorjanensis* | 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 |
| *N. endophyticum* and *N. halimiones* | 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 |
| *N. endophyticum* and *N. chichastianum* | 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 |
| *N. endophyticum* and *N. chersinae* | 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 |
| *N. halimiones* and *N. chichastianum* | 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 |
| *N. halimiones* and *N. chersinae* | 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 $MgCl_2$. 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.

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