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Research Article

RESEARCH ARTICLE

# Endophytic microbial diversity of the halophyte *Arthrocnemum macrostachyum* across plant compartments

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**One sentence summary:** We present the microbial characterization of the endosphere of the halophyte *Arthrocnemum macrostachyum* in three different compartments (roots, green and red stems) detecting a reduction of diversity from root to the more mature stems. We reported also, species never reported before as endophytic.

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## ABSTRACT

In this study, the microbial community structures of the endosphere of the halophyte *Arthrocnemum macrostachyum* were evaluated from two locations in Mallorca, Spain, focusing on three plant compartments (roots, green and red stems) compared to the rhizospheric soil where the plants grew. The physicochemical parameters of the rhizospheric soils differed between locations, and the soils were characterized by different microbial community structures. Accordingly, the endophytic community composition, mainly composed of putatively halophilic organisms, was highly influenced by the rhizospheric soil microbiota, as revealed by the co-occurrence of the major endophytic taxa in the endosphere and the rizospheric soils. Moreover, the reduction of diversity from the endorhizosphere towards the red leaves may support the fact that part of colonization of the plant by bacteria could have an origin in the rhizospheric soils through the roots and subsequent migration to the aerial parts of the plant. Finally, there were certain relevant ubiquitous taxa, such as *Chromohalobacter canadensis*, *Rudaea cellulosilytica* (never reported before as endophytic), *Psychrobacter* sp., *Bradyrhizobium* sp. and *Halomonas* sp., that, due their moderate halophilic nature, seemed to find an optimal environment inside the plants. Some of these relevant endophytes were not always detectable in their respective soils, and were probably part of the soils' rare biosphere, which would gain preponderance in a favorable endophytic environment.

**Keywords:** halophytes; endophytes; roots; green and red stems; rhizospheric soil

## INTRODUCTION

Coastal salt marshes are inhospitable environments for the majority of plants; nevertheless, a few highly salt-tolerant plants (halophytes) are able to survive and flourish under such conditions. Halophilic plants are common in coastal ecosystems around the world and present diverse adaptations to

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hypersaline environments. Furthermore, some of them show no evidence of inhibition in response to salinity stress, and normally exhibit a pronounced salt requirement for optimal growth (Qiu and Lu 2003; Parida and Das 2005; Redondo-Gómez *et al.* 2010).

The halophyte *Arthrocnemum macrostachyum* is particularly interesting because it is one of the few species proven to be extremely well adapted to hypersaline conditions (Redondo-Gómez *et al.* 2010). Even though the optimum salinity in the substrate ranges from 171 to 540 mM NaCl, this species can grow on substrates with salinities up to 1030 mM NaCl (Redondo-Gómez *et al.* 2010). As we have already reported (Mora-Ruiz *et al.* 2015), the internal tissues of this plant can serve as a suitable environment for the colonization of moderately halophilic bacteria. These are known to represent important and complex communities associated with halophytes (Ruppel, Franken and Witzel 2013) and, as in other plants, they might affect (positive or negatively) the fitness of *A. macrostachyum*. The colonization of bacteria in the internal tissues of the plants occurs in both phyllospheric and root compartments, but both differ importantly in the environmental conditions that may influence the structure of the colonizing microbial communities. In this regard, the phyllosphere and rhizosphere have rather different environmental fluctuations. The first is under constant fluctuations of temperature, humidity, UV radiation and wind, whereas the rhizosphere can suffer strong fluctuations of water soil content and salinity. However, not much is known about the microbial communities associated with halophytes (Simon, Abeliovich and Belkin 1994). In general, studies have focused on *Arabidopsis thaliana* (Bulgarelli *et al.* 2012; Bodenhausen, Horton and Bergelson 2013) or plants with commercial interest (Wulff, van Vuurde and Hockenhull 2003) and a description of the community structure with no information about the presence or absence of spatial patterns of species diversity. Additionally, in comparison with rhizosphere and green leaf habitats, little is known about microbial colonization in other plant areas (Bulgarelli *et al.* 2013; Jin *et al.* 2014), and even less on specific endophytic microorganisms and their biogeography at this microscale (Hoffman and Arnold 2010).

*Arthrocnemum macrostachyum* has very small, scale-shaped leaves and the phyllosphere is in fact composed of succulent and articulate stems. In addition, besides the roots and green stems, during the dry hot seasons stems exhibit large areas with a red-colored pigmentation. This color is generated by production of photopigments, such as anthocyanin and betacyanin (Chang-Quan *et al.* 2006) among others, which protect the integrity of the photosynthetic apparatus by acting as an osmotic buffer against the osmotic and ionic challenges of the saline environment (Kaya *et al.* 2001; Renault *et al.* 2001; Parida and Das 2005; Song *et al.* 2011; Flowers, Munns and Colmer 2015). The anthocyanin helps in the elimination of oxygen radicals and inhibits the peroxidation of lipids (Chalker-Scott 1999; Khavari-Nejad, Najafi and Khavari-Nejad 2008). We hypothesized that the endophytic community in the halophyte *A. macrostachyum* could exhibit variations related to different compartments of its plant structures (endorhizosphere, green and red stems) and it would be different from the rhizospheric soil microbial communities.

Therefore, in this study, we aimed to determine whether the three different compartments contained distinct microbial communities, and whether two different locations shared the same community structures. For this purpose, 454-pyrosequencing of 16S rRNA amplicons was applied following the operational phylogenetic unit (OPU) approach (Mora-Ruiz *et al.* 2015; Fig. S1, Supporting Information). The OPU approach allows the comparison of sequences from different length and 16S rRNA gene regions due to the nature of phylogenetic inference algorithm. Moreover, it minimizes the overestimation of richness measures calculated with OTUs as one OPU may group distinct OTUs that would not be recognized of being of the same unit due to differences in length or slight sequence dissimilarities (Vidal *et al.* 2015). The physicochemical characteristics of the soil from where the samples were collected were also recorded in order to evaluate possible environmental factors that would determine the structure of the internal bacterial communities and identify how these environmental variables could help predict the microbial community.

## MATERIALS AND METHODS

### Plant sample collection and surface sterilization

Six halophytes, identified as *Arthrocnemum macrostachyum*, were collected from Campos (39°21′03.3″ N, 3°00′44.3″ E, Spain) and Albufera d'Alcúdia (39°47′49″ N, 3°6′24″ E, Spain) in April 2014. Both locations are in Mallorca and are separated by a distance of approximately 60 km. Three plants were chosen from each location and all plants exhibited green and red (mature) stems (Fig. S2, Supporting Information). Individual plants were collected whole and stored in zip-lock plastic bags using sterile gloves for immediate processing on arrival at the laboratory. Green and red fractions and roots were excised from each halophyte, obtaining three samples in triplicate for each location. Excision areas were first hot cauterized using an incandescent metal loop to avoid the loss of sap.

Approximately 40 g of each section of plant were selected (fragments of 7–10 cm). Once the plant material had been excised and removed, it was sterilized and macerated. Plant material surfaces were washed with the following sequential steps: initial incubation for 5 min in 0.2% Triton X-100 with sterile distilled water, followed by 5 min in sterile distilled water alone, 5 min in 2% bleach (NaClO) and finally 5 min in sterile distilled water. The root samples were rinsed six times with sterile water (5 min each step) in order to completely remove the soil attached to their surfaces. After the last wash, the plant material was dried using sterile paper towels. To disaggregate the plant tissues, 5–25 mL of PBS 1X were added to the plant material and the mixture was macerated using a sterile mortar and pestle (Mora-Ruiz *et al.* 2015).

### Separation of the microbial fraction from vegetal debris, and microbial DNA extraction

Fifteen milliliters of macerated biomass were subjected to differential centrifugation and sucrose density gradient centrifugation, as previously reported (Mora-Ruiz *et al.* 2015). All layers recovered from the centrifugation gradient were numbered according to their density and stored at −20°C. The layers with higher density were used for DNA extraction, which was performed as previously reported (Mora-Ruiz *et al.* 2015).

### Soil collection and microbial DNA extraction

For each plant, its respective rhizospheric soil was collected by shaking the roots vigorously in order to separate the loosely adhered soil. The soil tightly adhered to the roots was also collected and the samples were immediately transferred to the

laboratory for bacterial DNA extraction following an existing protocol (Nogales et al. 1999).

### PCR amplification and 454-pyrosequencing

PCR amplification with the bacterial primers GM3 and S (Table S1, Supporting Information) was performed as previously described (Lane et al. 1986). A second PCR was performed using 5 μL of the product as template, in triplicates of 25 μL, using the primers GM3-PS and a variant of 907-PS (from position 8 to 907: V1–V4 regions of 16S rRNA; Table S1, Supporting Information) to tag and link the amplicon, with the same annealing temperature. The products were observed in 1% agarose gel electrophoresis run in 1X TAE buffer (at 25 V for 50 min). The band with a size of ∼960 bp was excised and eluted using the Zymoclean Gel DNA Recovery Kit (Zymo Research, California, USA) following the manufacturer's instructions. The NanoDrop Spectrophotometer (Thermo Fisher Scientific Inc., Massachusetts, USA) and MassRuler Express Forward DNA Ladder Mix (Thermo Scientific) were used to measure the concentration of the barcoded amplicons. An equimolar mix of the amplicons was sent to Macrogen Inc. (Seoul, Korea) for sequencing using 454 GS-FLX+ Titanium technology. Trimmed sequences have been deposited in the ENA sequence repository under the study accession number SAMEA3928333–SAMEA3928356.

### Sequence trimming, OTU grouping, phylogenetic affiliation and OPU design

Sequence data were processed using Mothur (Schloss et al. 2009) software to trim and remove chimeras. Low-quality and short sequences were discarded (<300 nucleotides). Trimming was performed with a window size of 25, and average quality score of 25, and no mismatches or ambiguities between primer pairs and barcodes were allowed. Chimeras were removed using Chimera Uchime (Mothur) and sequences were clustered in OTUs at 99% using the UCLUST tool included in QIIME (Caporaso et al. 2010). The longest read of each OTU was selected as representative and was added to the non-redundant SILVA REF115 database using the ARB software package (Ludwig et al. 2004). Sequences were initially aligned with SINA (Pruesse, Peplies and Glöckner 2012) and inserted using the parsimony tool (ARB package) in a default tree. Chloroplast and mitochondrial sequences were discarded. The closest relative sequences of adequate quality were selected and merged with the LTPs115 database (Yarza et al. 2010). A phylogenetic reconstruction was performed using the neighbor-joining algorithm and the Jukes–Cantor correction with the selected closest relative sequences and type strain representatives. Finally, the OTU representatives were inserted into the final reconstruction using the parsimony tool, and then clustered in OPUs (França et al. 2015) based on the visual inspection of the final tree. An OPU is the smallest monophyletic group of sequences containing OTU representatives together with the closest reference sequence, including the sequence of a type strain when possible.

### Soil sample collection and physicochemical parameters

Six soil samples were collected from locations Campos and Alcúdia where the sampled plants were growing. Physicochemical parameters were analyzed at the Conselleria d'Agricultura, Medi Ambient i Territori (Govern de les Illes Balears, Mallorca, Spain). The analyzed physicochemical parameters were pH, electrical conductivity (at 25°C), oxidizable organic matter (OM), total nitrogen, carbonates ($CO_3^{2-}$), available phosphorus and changeable potassium ($K^+$), sodium ($Na^+$) and magnesium ($Mg^{2+}$).

### Ecological indices and statistical analysis

Rarefaction curves and equitability-J index were calculated from OPUs using PAST *v* 3.01 software (Hammer, Harper and Ryan 2001). Jost index ($q = 1$) was computed by a Monte Carlo resampling (1000 simulations) the OPU matrix to the minimum sequences. Non-parametric Kruskal–Wallis tests were performed in order to compare ecological indices and soil physicochemical parameters in each location and internal fraction, as they did not satisfy assumptions of normality and homogeneity of variances. The multivariate community structure in relation to sampling location, plant compartment and interindividual variability of OPUs was statistically analyzed using non-metric multidimensional scaling (NMDS; Kruskal and Wish 1978). The goodness of the NMDS was evaluated according to a stress value smaller than 0.263, which is considered acceptable for 18 samples (Sturrock and Rocha 2000). Fitted vectors were represented as arrows pointing in the direction of the most rapid change in the environmental variables (Díaz-Gil et al. 2014; Oksanen 2016). Additionally, a permutational multivariate analysis of variance (Anderson 2001) was used to test the statistical significance of the differences between bacterial communities and the environmental parameters, in order to determine their influence on the variability in the microbial communities. Hierarchical cluster analysis was performed using the Sørensen distance (Sørensen 1948) and Ward's linkage (Ward 1963). An indicator species analysis (ISA) (De Cáceres and Legendre 2009) was used to identify the indicator taxa responsible for the differences between groups identified with the cluster analyses. The correlation matrixes were obtained by comparing the diversity at the phylum level in the endophytic and rhizospheric community with their respective soil. Analyses were performed with the packages *vegan* (Oksanen 2016) and indicspecies in R *v* 3.1.1. (www.r-project.org).

## RESULTS

### Analysis of 454-pyrosequencing data

This study included plant biomass in triplicate and their respective rhizospheric soil collected in Campos and Alcúdia, both located in Mallorca, Spain. A total of 97 872 sequences were generated that, after trimming and removing short, bad or chimera sequences, resulted in a dataset of 58 517 sequences with a mean of 2438 ± 1825 per sample (Table S1, Supporting Information). The clustering into OTUs at 99% rendered a total of 5393 with a mean of 225 ± 296 representatives that after phylogenetic inference were grouped into a total of 657 OPUs (78 ± 70; Table 1). From these, 249 OPUs were present in the endosphere of the plants studied, and 408 were exclusive to soils.

### Soil characterization

Soil physicochemical parameters indicated that the pH was alkaline and nearly the same in both locations (8.28 ± 0.39 and 8.29 ± 0.18; $P > 0.05$). In addition, carbonates (%), exchangeable $Na^+$ and $Mg^{2+}$ (%) displayed similar values ($P > 0.05$) in both places. However, the values of salinity (%), exchangeable $K^+$, oxidizable OM (%), total N (%) and available P (%) (Figs 1 and

**Table 1.** Raw data and sequence distribution in OTUs and OPUs from pyrosequencing. Jost indices in q = 0 and q = 1 are shown per sample. SD = standard deviation. Eqt = Equitability-J.

| | Raw seqs | Removed seqs | Final seqs | OTUs | q = 0 (OPUs) | q = 1 | Eqt. |
|---|---|---|---|---|---|---|---|
| | | | *Endosphere* | | | | |
| **Alcúdia** | | | | | | | |
| Green | 2656 | 320 | 2336 | 31 | 23 | 14.9 | 0.86 |
| Green | 2304 | 64 | 2240 | 27 | 18 | 10.8 | 0.83 |
| Green | 1704 | 288 | 1416 | 34 | 22 | 8.9 | 0.72 |
| Red | 2120 | 44 | 2076 | 63 | 27 | 5.0 | 0.49 |
| Red | 1672 | 40 | 1632 | 54 | 29 | 7.2 | 0.59 |
| Red | 1740 | 24 | 1716 | 68 | 27 | 6.2 | 0.56 |
| Root | 2240 | 32 | 2208 | 114 | 56 | 19.3 | 0.73 |
| Root | 1600 | 8 | 1592 | 70 | 39 | 12.1 | 0.69 |
| Root | 1966 | 30 | 1936 | 115 | 57 | 14.5 | 0.67 |
| **Campos** | | | | | | | |
| Green | 1224 | 15 | 1209 | 31 | 22 | 9.4 | 0.73 |
| Green | 2035 | 34 | 2001 | 269 | 79 | 8.1 | 0.49 |
| Green | 3251 | 2245 | 1006 | 58 | 40 | 14.4 | 0.72 |
| Red | 18 679 | 15 266 | 3413 | 187 | 72 | 10.6 | 0.55 |
| Red | 14 878 | 12 330 | 2548 | 175 | 84 | 11.0 | 0.55 |
| Red | 12 898 | 5916 | 6982 | 211 | 74 | 4.2 | 0.34 |
| Root | 3027 | 75 | 2952 | 237 | 93 | 16.3 | 0.62 |
| Root | 4993 | 835 | 4158 | 187 | 94 | 19.0 | 0.66 |
| Root | 8659 | 85 | 8574 | 358 | 90 | 10.0 | 0.51 |
| | | | *Rizospheric soil* | | | | |
| Alcúdia | 1654 | 275 | 1379 | 778 | 218 | 81.8 | 0.81 |
| Alcúdia | 2506 | 499 | 2007 | 1286 | 300 | 127.1 | 0.85 |
| Alcúdia | 1358 | 368 | 990 | 683 | 212 | 114.1 | 0.87 |
| Campos | 2022 | 490 | 1532 | 120 | 82 | 18.5 | 0.66 |
| Campos | 1429 | 415 | 1014 | 97 | 60 | 34.1 | 0.86 |
| Campos | 2048 | 448 | 1600 | 140 | 70 | 11.7 | 0.56 |
| **Total** | **98 663** | **40 146** | **58 517** | **5393** | **657** | | |
| **Mean** | 4110.96 | 1672.75 | 2,438.21 | 224.71 | 78 | | |
| **SD** | 4729.69 | 3955.98 | 1825.03 | 295.52 | 70 | | |

2) showed differences depending on the location, with salinity and $K^+$ being higher in C ($P < 0.05$), whereas an inverse pattern ($P < 0.05$) was observed for the remaining measured parameters (Figs 1 and 2).

## Analysis of the total bacterial endophytic community structures

The majority of sequences in the endosphere represented 249 OPUs affiliated with *Proteobacteria* (86.7% of the total sequences integrated in 151 OPUs). The major proteobacterial taxa affiliated with the classes *Alphaproteobacteria* (48 OPUs = 7.7% sequences) and *Gammaproteobacteria* (74 OPUs = 76.3% sequences). *Firmicutes* (35 OPUs = 8.8% sequences) and *Bacteroidetes* (28 OPUs = 1.8% sequences) followed *Proteobacteria* in abundance. Minor taxa were detected in 11 additional phyla (35 OPUs = 2.6% sequences; Table 2).

The top most abundant OPUs affiliated with *Proteobacteria*. OPUs 1 (*Rudaea cellulosilytica*), 6 (*Psychrobacter* spp.), 12 (*Chromohalobacter canadensis*), 13 (*Halomonas* spp.) and 109 (*Bradyrhizobium* spp.) were present in all fractions of all plants (Fig. 3; Table 2), and in high sequence numbers. However, a high number of OPUs were exclusively detected in single compartments (green = 10, red = 26 and root = 79), all of which, except OPU 163 (*Bhargavaea* spp. Fig. 3; Table 2), were detected in very low abundances of less than 0.48%. The results were similar for the beta-diversity analyses where the Campos compartments did not show differences (*F-statistic* = 1.034, $P = 0.413$) and the Alcúdia compartments showed results very close to the significance level $\alpha = 0.05$ (*F-statistic* = 1.78, $P = 0.049$; Table S2, Supporting Information). In the same way, richness estimations ($P > 0.05$; $q = 0$) and rarefaction curves (Table 1 and Fig. S3, Supporting Information) did not exhibit differences by compartment. However, the Jost $q = 1$ and equitability-J indices were different between compartments, and were always lower in the red fraction (Fig. 3). On the other hand, when comparing the beta-diversity between both locations the differences were significant (*F-statistic* = 1.91, $P < 0.001$; Table S2, Supporting Information) and similar to the richness estimations ($P < 0.05$).

Regarding the community structures in relation to the soil physicochemical properties, salinity and OM were the parameters that better correlated with the endophytic differences between locations (Fig. 1). However, only OM showed a significant value ($P < 0.05$) for the effect of discriminating the communities by location. Additionally, it was notable that the correlation analyses (data not shown) indicated that none of the soil chemical parameters measured were good predictors of the bacterial diversity indices.

## Description of the bacterial communities by plant compartment and location

The most abundant OPUs 1 (*R. cellulosilytica*), 6 (*Psychrobacter* sp.), 12 (*C. canadensis*), 13 (*Halomonas* spp.) and 109 (*Bradyrhizobium* sp.) were present in all fractions (Table 2). *Psychrobacter* sp. (OPU 6; up to 37.5%) was especially abundant in red and *R. cellulosilytica* (OPU 1; up to 12.1%) was remarkably abundant in green and root (Fig. 3). In addition, there were compartment-exclusive OPUs, especially noticeable in the root fraction dominated principally by *Enterobacter* species, and uncultured *Sodalis* sp. in red. The 10 OPUs exclusive to green were homogeneously distributed between *Bacteroidetes* (*Hymenobacter* sp.) and *Actinobacteria* (*Corynebacterium* sp.) (Table 2).

The differences between locations were marked by 45 exclusive OPUs in Alcúdia and 120 in Campos. The latter showed the highest richness values, which were principally observed in the root compartments (Table 1). In general, the abundances of the exclusive OPUs were low and ranged between 0.01% and 1.70%, with the exception of *Enterobacter amnigenus* (OPU 57) that accounted for 8.67% of the total sequences in Alcúdia roots (Fig. 3). In the Campos location, the most common families were *Moraxellaceae*, *Bradyrhizobiaceae*, *Sphingomonadaceae* and *Flavobacteriaceae*, whereas in Alcúdia they were *Enterobacteriaceae* and *Planococcaceae*. Among these, according to the ISA, there were 27 indicator species for Campos and 3 for Alcúdia ($P < 0.05$; Table S3, Supporting Information). Additionally, all the representatives of *Epsilonproteobacteria* (OPUs 101 and 102), *Tenericutes* (OPUs 180 and 181) and *Gemmatimonadetes* (OPU 229) were only detected in Alcúdia, with all *Planctomycetes* (OPUs 224 and 225), *Armatimonadetes* (OPU 244) and *Chloroflexi* (OPU 249) only in Campos.

## Rhizospheric soil microbial community structures

Alcúdia samples exhibited higher richness than those in Campos, contrary to that observed for the endophytic community (Table 1), and both locations showed different community

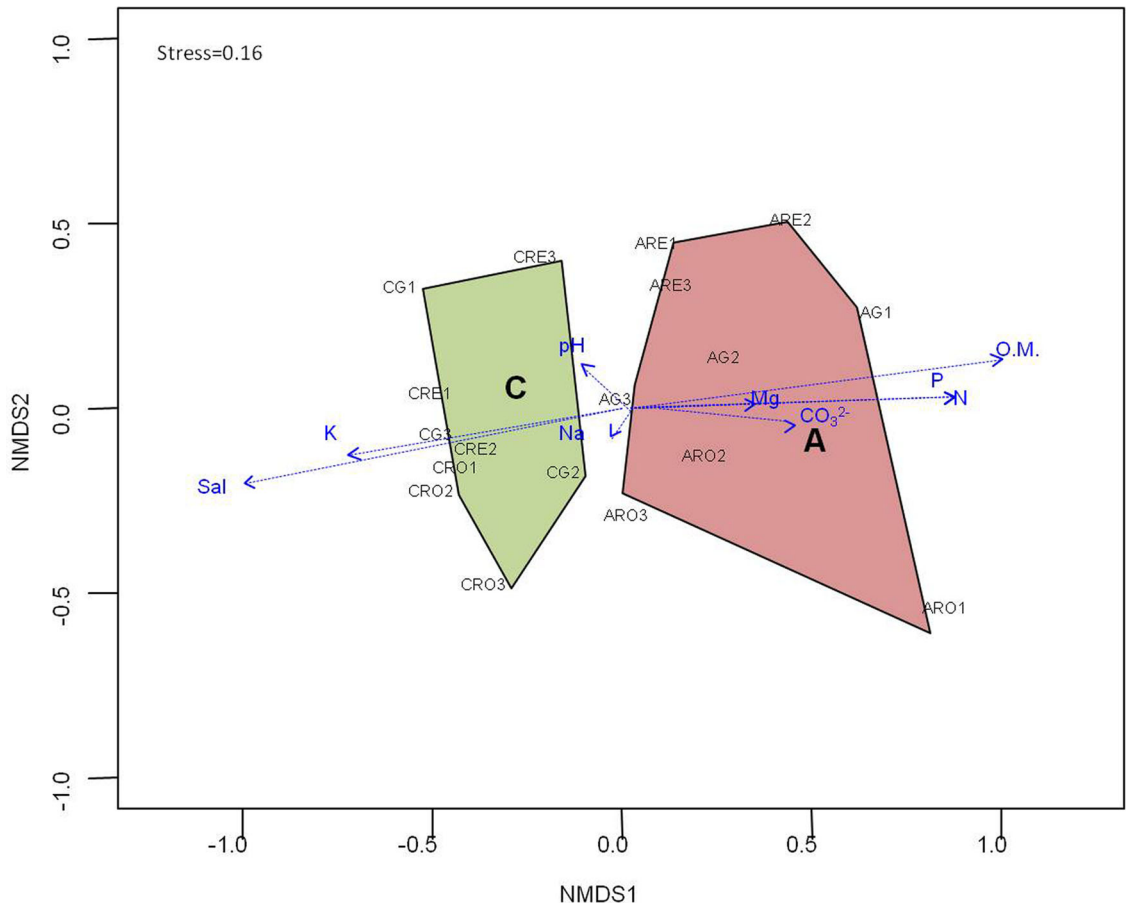


**Figure 1.** Two dimensional NMDS of endophytic bacterial communities associated with *A. macrostachyum*. C = Campos, A = Albufera d'Alcúdia. Dotted lines show the gradient for the soil environmental parameters.

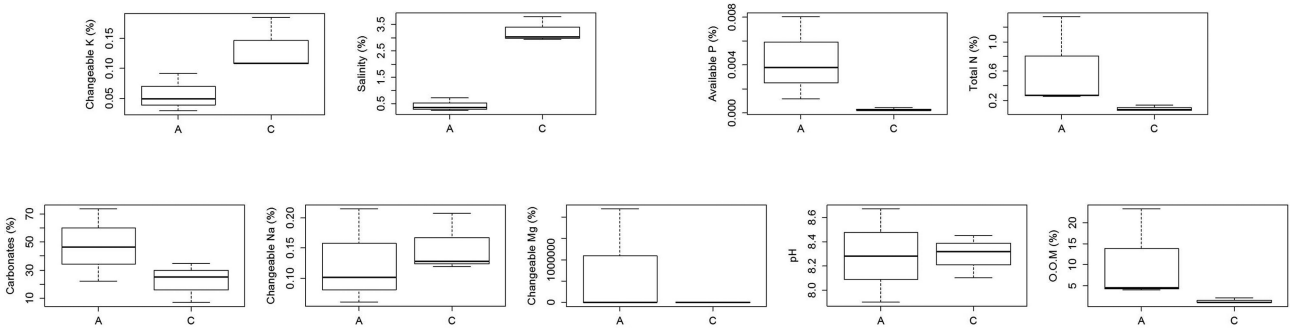


**Figure 2.** Boxplot of analyzed physicochemical parameters in each location. A = Alcúdia and C = Campos.

structures (Fig. S4 and Table S2, Supporting Information). Rhizospheric soils from Alcúdia and Campos shared 17 (4.8 ± 2.5% of the total sequences) and 39 (20.2 ± 3.3% of the total sequences) OPUs with the respective endospheres. On the other hand, these shared OPUs represented approximately 8.9 ± 5.0% and 53.5 ± 17.3% in Campos of the sequences belonging to the endophytic communities (Fig. 4). No special abundance of the shared OPUs was detected in any specific fraction of the plant. *Halomonas* sp. and *Pseudomonas* sp. (OPUs 13 and 16) were the most important OPUs shared between rhizospheric soil and endosphere in both locations.

Despite the fact that the rhizospheric communities were distinct in both locations, they were both principally represented by *Proteobacteria* (52.7% of the total sequences for Alcúdia, and 33.9% for Campos), with *Gammaproteobacteria* being the most abundant class. Another 21 phyla were additionally detected but, of these, only *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Gemmatimonadetes*, *Firmicutes*, *Planctomycetes* and *Saccharibacteria* exhibited abundances higher than 2% in Alcúdia or Campos (Table 2).

The most abundant OPUs in Alcúdia soil were *Rhodovibrio salinarum* (OPU 145; representing up to 3%–18% of the sequences), *Halomonas* sp. (OPU 13), uncultured *Ruminococcaceae* sp. (OPU 190), *Acidobacteria* Subgroup 6 sp. (OPU 254), *Draconibacteriaceae* sp. (OPU 408) and uncultured *Azospira* sp. (OPU 432) (these latter five clades together represented between 10% and 16% of

**Table 2.** Most relevant OPUs of the endosphere and the rizospheric soil. First column (from left to right): OPU number; second column: information about the affiliation with the closest relative sequence and the accession number. Other columns contain the percentage mean of relative abundance of each OPU referenced to the total sequences for each kind of sample.

| # OPU | Affiliation/Accession number | CAMPOS | | | | ALCÚDIA | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Green | Red | Root | Soil | Green | Red | Root | Soil |
| 1 | *Xanthomonadaceae, Rudaea cellulosilytica* (EU741687) | 8.74 | 5.69 | 12.14 | 0.85 | 16.36 | 1.54 | 6.26 | 0 |
| 6 | *Moraxellaceae, Psychrobacter* sp. (AY513646/ AJ748266) | 1.52 | 21.73 | 1.85 | 0.04 | 1.79 | 28.62 | 0.9 | 0 |
| 12 | *Halomonadaceae, Chromohalobacter canadensis* (AJ295143) | 36.21 | 30.76 | 17.55 | 3.27 | 21.81 | 23.11 | 19.73 | 0 |
| 13 | *Halomonadaceae, Halomonas* sp. (AM945689/ GQ903435) | 3.11 | 6.11 | 5.93 | 1.58 | 7.88 | 4.84 | 6.2 | 1.81 |
| 15 | *Halomonadaceae, Kushneria indalinina* (JF820663) | 0.72 | 0.61 | 0.2 | 0.13 | 1.69 | 5.12 | 0.59 | 0 |
| 16 | *Pseudomonadaceae, Pseudomonas* sp. (AB060135/D84019) | 0.51 | 0.21 | 12.11 | 1.39 | 1.58 | 0.51 | 4.8 | 0.28 |
| 18 | *Alteromonadaceae, Marinobacter persicus* (HQ433441) | 0.02 | 0.04 | 2.06 | 0 | 0.91 | 0.08 | 0.22 | 0.13 |
| 29 | *Oceanospirillaceae, Marinomonas* sp. (DQ492749/AJ843079) | 0.29 | 0.25 | 0.22 | 0 | 1.37 | 0 | 0.12 | 0 |
| 30 | *Oceanospirillaceae, Marinomonas* sp. | 0 | 0 | 3.66 | 0.35 | 0 | 0 | 1.75 | 0 |
| 31 | *Pseudoalteromonadaceae, Pseudoalteromonas* sp. (FJ200652/AY682201) | 0 | 0.36 | 0.85 | 0 | 0 | 3.18 | 0 | 0 |
| 32 | *Shewanellaceae, Shewanella* sp. (AF500078/FM887037) | 0 | 0 | 0.18 | 0.61 | 0 | 0 | 1.84 | 0 |
| 38 | *Vibrionaceae, Vibrio* sp. (AB562592/AY332401) | 0.46 | 0.03 | 0.13 | 1.35 | 1.37 | 0 | 0.23 | 0 |
| 40 | *Vibrionaceae, Vibrio fortis* (AB257333) | 0 | 2.85 | 0.01 | 0 | 9.27 | 19.3 | 0.06 | 0 |
| 42 | *Vibrionaceae, Vibrio rumoiensis* (AB013297) | 0 | 0.01 | 10.99 | 0.22 | 0 | 0 | 0.96 | 0 |
| 45 | *Enterobacteriaceae, Erwinia* sp. (HM008943/FJ611860) | 0 | 0 | 0.01 | 0.61 | 0 | 0 | 1.93 | 0 |
| 50 | *Enterobacteriaceae, Enterobacter hormaechei* (JN645954) | 1.76 | 0.08 | 0.7 | 0 | 0.75 | 0.3 | 1.32 | 0 |
| 57 | *Enterobacteriaceae, Enterobacter amnigenus* (AM062693) | 0 | 0 | 0 | 2.29 | 0 | 0 | 8.67 | 0 |
| 65 | *Enterobacteriaceae, Serratia marcescens* (FM213391) | 0.02 | 0 | 0 | 2.09 | 0 | 0 | 0 | 0 |
| 66 | *Enterobacteriaceae, Serratia* sp. (HQ326819/JF431270) | 0.02 | 0 | 0 | 1.05 | 0 | 0 | 12.38 | 0 |
| 68 | *Enterobacteriaceae, Rahnella aquatilis* (JX867757) | 0 | 0 | 0 | 0.74 | 0 | 0 | 0.06 | 2.4 |
| 76 | *Burkholderiaceae, Burkholderia* sp. (EU219865/HQ698908) | 2.21 | 0.42 | 0.35 | 0.21 | 0.48 | 0.48 | 0.62 | 0 |
| 109 | *Bradyrhizobiaceae, Bradyrhizobium* sp. (FJ025111/AY039016) | 5.44 | 4.5 | 7.68 | 0 | 4.56 | 0.6 | 2.34 | 0 |
| 128 | *Sphingomonadaceae, Sphingomonas rhizogenes* (AY962684) | 5.29 | 2.06 | 2.2 | 0 | 0 | 0.06 | 0.03 | 0 |
| 139 | *Rhodobacteraceae, Falsirhodobacter halotolerans* (HE662814) | 0 | 0 | 0.07 | 0 | 2.74 | 0 | 0 | 0 |
| 145 | *Rhodospirillaceae, Rhodovibrio salinarum* (FM177506) | 0.22 | 0.16 | 0.08 | 3.29 | 0 | 0 | 0 | 9.06 |
| 156 | *Bacillaceae, Alkalibacillus salilacus* (EU377478) | 3.26 | 1.81 | 1.75 | 0.24 | 3.28 | 1.65 | 3.25 | 0 |
| 157 | *Bacillaceae, Marinococcus halotolerans/M. tarijensis* (AY817493/JQ413413) | 0.82 | 1.8 | 1.62 | 0.47 | 1.87 | 0.33 | 3.78 | 0 |
| 165 | *Planococcaceae, Sporosarcina* sp. (AB243859/JX840395) | 0.25 | 0.01 | 0.01 | 0.35 | 4.96 | 3.79 | 1.8 | 0 |
| 171 | *Carnobacteriaceae, Marinilactibacillus psychrotolerans* (AB083413) | 3.31 | 6.88 | 0 | 0 | 0 | 0.29 | 0 | 0 |
| 176 | *Streptococcaceae, Streptococcus* sp. (HG315101/JX861483) | 8.38 | 0.45 | 0.23 | 0.84 | 0 | 0.16 | 0 | 0 |
| 190 | *Ruminococcaceae,* Uncultured *Ruminococcaceae* (HQ716315) | 0 | 0.03 | 0.01 | 2.4 | 0 | 0 | 0 | 2.6 |
| 204 | *Flavobacteriaceae,* Uncultured *Flavobacteriaceae* (U87104) | 0.15 | 0 | 0 | 0.07 | 1.41 | 0.71 | 1.44 | 0.03 |
| 219 | *Chitinophagaceae, Gracilimonas* sp. (JN038257) | 0 | 0 | 0 | 2.02 | 0 | 0.08 | 0 | 1.43 |
| 246 | *Deinococcaceae, Deinococcus* sp. (Y11331/CP002536) | 0.12 | 0 | 0 | 0 | 3.67 | 1.27 | 0.6 | 0.03 |
| 251 | *Acidobacteria,* ABS-19 (JQ801025) | 0 | 0 | 0 | 5.11 | 0 | 0 | 0 | 1.5 |
| 278 | OM1 clade (KF964596/GQ263220) | 0 | 0 | 0 | 32.96 | 0 | 0 | 0 | 0.79 |
| 297 | *Hyphomicrobiaceae,* Uncultured *Cucumibacter* (JQ800771) | 0 | 0 | 0 | 1.76 | 0 | 0 | 0 | 0.14 |
| 408 | Uncultured *Draconibacteriaceae* (KJ817690/DQ899885) | 0 | 0 | 0 | 1.35 | 0 | 0 | 0 | 3.85 |
| 432 | *Rhodocyclaceae,* Uncultured *Azospira* (DQ337003) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1.96 |
| 536 | *Staphylococcaceae, Salinicoccus carnicancri* (ANAM01000001) | 0 | 0 | 0 | 2.03 | 0 | 0 | 0 | 0 |
| 559 | *Ectothiorhodospiraceae*, Uncultured *Thioalkalispira* (FR828704) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1.48 |
| 638 | Uncultured *Saccharibacteria* (AB015558) | 0 | 0 | 0 | 2.21 | 0 | 0 | 0 | 0.09 |

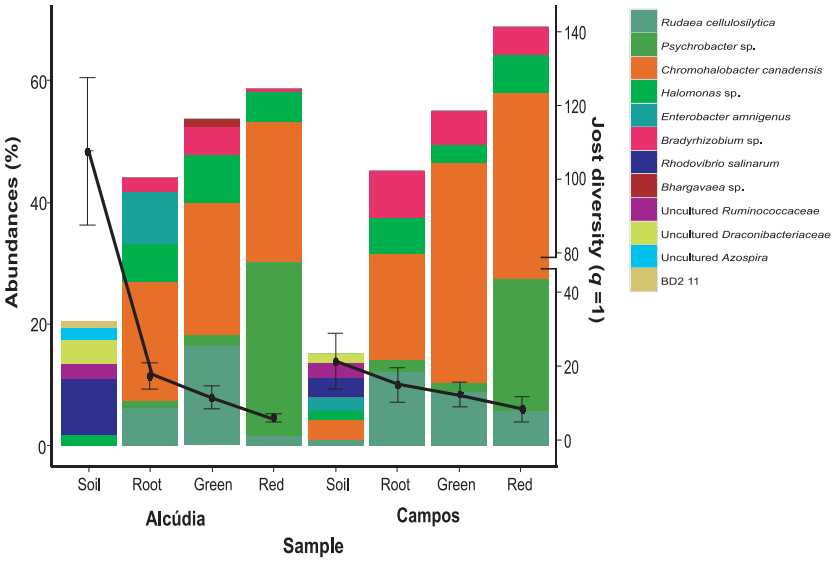


**Figure 3.** Most abundant OPUs in each sample. Rhizospheric soils included Alcúdia and Campos. Numbers inside the bar charts are the average percentages of sequences detected in each OPU per sample; abundances under 0.5% are not represented. The Y axis and black squares represent the relationship of the values for the Jost diversity index q = 1 per group of samples.

the total sequences; Table 2). Only the first OPUs co-occurred in their respective endophytic fraction, and *Halomonas* sp. (OPU 13) occurred in all fractions.

On the other hand, Campos soils were characterized by high abundances of OM1 clade sp. (OPU 278; between 11% and 48% of the total sequences), *C. canadensis* (OPU 12), *E. amnigenus* (OPU 57), *Rh. salinarum* (OPU 145; uncultured *Ruminococcaceae* sp. (OPU 190) and ABS-19 (OPU 251) (these latter five clades together represented between 10% and 19% of the total sequences). From these, only *C. canadensis* and *Rh. salinarum* co-occurred in some of their respective endophytic fractions (Table 2; Fig. 3). In addition, *R. cellulosilytica* also co-occurred but with abundances lower than 1.4%, whereas *C. canadensis* occurred in all fractions from plants and soil, which was similar to *Halomonas* sp. for the Alcúdia location.

## DISCUSSION

To our knowledge, this is the first report where compartmentalization of endophytic bacterial communities from the phyllosphere and rhizosphere of halophytes has been analyzed at a microscale and mesoscale. OPUs could be identified as taxa at the species and genus levels because the sequences were of higher quality (lengths of over 800 bp) compared to other similar studies (Gottel *et al.* 2011; Bodenhausen, Horton and Bergelson 2013), and also because the OPU approach by means of phylogenetic inferences produced more accurate identifications (92.8% of the OPUs could be affiliated at the genus level; Vidal *et al.* 2015).

We are aware that the sample number may be low, but our results appear consistent with our previous studies (Mora-Ruiz *et al.* 2015), where differences in endosphere composition were dependent on the location, but the major key players were common in all plants. Most of the principal elements in the endosphere were moderate halophiles. The presence of *Chromohalobacter canadensis* was previously reported in *Arthrocnemum macrostachyum* and the other halophyte *Allenrolfea vaginata* (Mora-Ruiz *et al.* 2015).

The differences detected between the locations may respond to the combination of the soil physicochemical properties and microbial composition, and the host genotype (Bulgarelli *et al.* 2012). However, relevant host genotype differences were discarded as the plants were only 60 km apart. In this regard, the soils studied here exhibited considerable physicochemical differences that could condition their bacterial diversity (Fierer and Jackson 2006), despite the fact that pH was alkaline and similar to other reported values (Pereira, Vicentini and Ottoboni 2014; Gao *et al.* 2015). However, the soil in Campos exhibited 10-fold higher salinity (considered to be a strongly saline soil ≥ 2%; U.S. Regional Salinity Laboratory Staff 1947) and differences in other parameters depending on the location were observed. These physicochemical parameters might directly influence the microbial composition of soils (Gao *et al.* 2015), and the rhizospheric soil of Alcúdia (more mesophilic environment and organic rich) showed nearly 3-fold more OPUs than Campos. In addition, a direct correlation was detected between $CO_3^{2-}$ and salinity concentrations with the presence of *Bacteroidetes*, *Deltaproteobacteria* and *Chlorobi* (higher in Alcúdia), and $K^+$ concentrations with *Actinobacteria* (higher in Campos). $K^+$ has been considered as one of the principal chemical parameters influencing distinct abundances of phyla in soil bacterial communities (Pereira, Vicentini and Ottoboni 2014). However, there may be other environmental traits (e.g. aeration, pore size, temperature and water) not registered in this study that could control microbial diversity patterns (Nemergut *et al.* 2011; Bokulich *et al.* 2014).

Accordingly, the soils from the two locations were different in their taxa compositions. For both locations, only *Rhodovibrio salinarum* and uncultured *Ruminococcaceae* sp. were part of the most abundant OPUs in Campos and Alcúdia. The former OPU, already reported in hypersaline sediments (Mack *et al.* 1993), was shared by both soils in similar quantities. However, it was remarkable that *C. canadensis* remained undetectable in the Alcúdia soils, this OPU being one of the most abundant in endosphere. In the same way, *Bradyrhizobium* sp. was abundant in the endosphere from the two locations and not detected in their corresponding soils, contrary to previously reported in *Populus deltoides*, where it was principally detected in rhizosphere (Gottel *et al.* 2011).

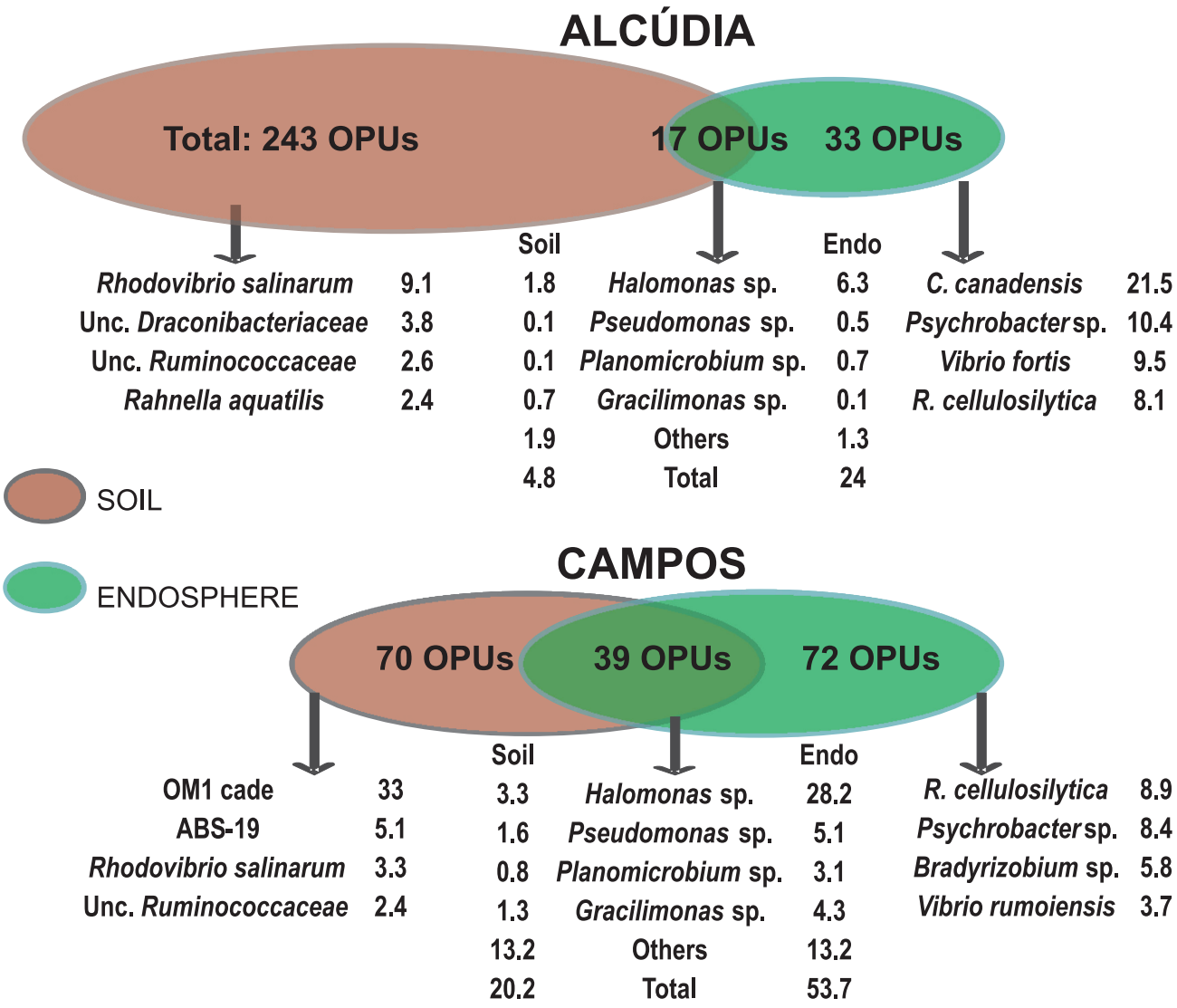


**Figure 4.** Diagram showing the comparisons between the bacterial communities of the rizhospheric soils and the endospheres of the halophytes in Alcúdia y Campos. The size of the ellipses represents their total number of OPUs. The percentages of abundances of the most relevant OPUs in each group of samples (rhizospheric soil and endosphere) and shared OPUs are given below.

The soil salinity also influences the halophytes colonizing them. As other halophytes, *A. macrostachyum* accumulates distinct ion concentrations in its vacuoles in amounts directly correlated with the increase of external (soil) salt concentrations due to increased uptake by osmoregulation (Redondo-Gómez *et al.* 2010). As Campos soil was more saline than Alcúdia soil, the plants may have had different saline contents in their endospheres, which would influence the composition of each endosphere. There were some major ubiquitous organisms in the endosphere, and some were also detected in the soils of both locations, such as *Halomonas* sp., or only in the Campos soils. Their presence in the endosphere could originate after root colonization due to chemotaxis (Bulgarelli *et al.* 2013) from the soil communities (Wulff, van Vuurde and Hockenhull 2003; Chi *et al.* 2005), followed by migration to the aerial parts. However, some relevant taxa were undetectable in Alcúdia soils, although they probably formed part of the seed bank or rare biosphere of these soils (Pedro-Aliós 2006), and would gain preponderance in a favorable endophytic environment. These were not isolated cases, as other organisms (not observed as ubiquitous), such as *Marinomonas* sp. (also endophytes of marine plants; Espinosa *et al.* 2010) or *Sphingomonas* sp., were not detected in the soils. An alternative origin could also be related to aerosol transmission through the aerial parts (Fahlgren *et al.* 2010), or by vertical transmission (bacteria already occurring in the seeds; Truyens *et al.* 2015). However, the fact that between 9% (for Alcúdia) and 53% (for Campos) of the total endospheric taxonomic composition was shared with their corresponding rhizospheric soil communities led us to believe that root colonization may be the major contributor to the endosphere composition (Bulgarelli *et al.* 2013). The lower salinity of the Alcúdia soils may explain the low abundances of the ubiquitous and relevant endophytic, halophilic bacteria, such as *C. canadensis* that would gain preponderance once colonizing the plant with higher saline levels in its endosphere.

A clear diversity gradient was observed between the three compartments in each plant, being higher in the rhizosphere (as already reported for *Arabidopsis thaliana*; Bodenhausen,

Horton and Bergelson 2013) and decreasing towards the more mature aerial parts, with the latter having less equitability. In general, the diversity values detected were very similar to previous studies of leaves (Emiliani *et al.* 2014; Jin *et al.* 2014). No clear trend could be detected in the distinct occurrence of specific OPUs in the different compartments, some of which (generally a minority) seemed to be specifically associated with one fraction of the plant. Others, like most of the representatives of the *Enterobacteriaceae* (especially *Enterobacter amnigenus*), were notably abundant in the root's endosphere, some of which have been reported as having plant growth-promoting effects principally associated with roots (Whipps 2001). Additionally, two of the most important OPUs, *Psychrobacter* sp. and *Rudaea cellulosilytica,* had notable abundance variations in the red fractions, being higher and lower (respectively) in these more mature parts of the plant in comparison with other areas of the endosphere. The reduction of diversity from the roots towards the more mature parts of the plant, apart from reinforcing a rhizospheric origin of the endogenous microbiota, may respond to factors such as passive mobility following water fluxes (Taghavi *et al.* 2010); specific selection due to plant–microbe interaction, such as chemotaxis (Bulgarelli *et al.* 2013) or plant defensive systems (Jones and Dangl 2006); and specialization in niche colonization (Bulgarelli *et al.* 2013).

In summary, the results presented here indicated that the endosphere of the halophyte *A. macrostachyum* may be strongly influenced by the microbial composition of the soils where this plant grows, and the soils themselves by their environmental physicochemical parameters. In this regard, the higher saline conditions of the Campos soil led to reduced taxa richness in accordance with what occurs in extreme saline environments (Hollister *et al.* 2010). The influence of the soil on plant microbial colonization was supported by its sharing of taxa with the plant compartments, and with reduction of diversity from the roots towards the mature aerial parts. On the other hand, there were several ubiquitous major microbial components, moderately halophilic in their metabolism, which seemed to be plant related and not always detectable in the rhizospheric soils. Their presence may indicate a probable relevant role in the plant–microbe interaction, and the absence of detection in some soil samples could point to other alternative origins (e.g. presence in plant seeds prior to germination). Finally, the endophytic lifestyle of some taxa is reported here for the first time, such as the known cellulose-degrading microorganism *R. cellulosilytica*, which although present in some soils has not yet been reported as part of the endomicrobiota of plants (Weon *et al.* 2009). The study provides an insight into understanding the possible mechanisms of dispersion and colonization in different plant lifecycle stages (seeds, seedlings and adults) and the endosphere microbial community dynamics associated with halophytes.

## SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

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***Conflict of interest.*** None declared.

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