

Characterization of bacteria associated with nodules of two endemic legumes of Algeria, *Hedysarum naudinianum* and *H. perrauderianum*

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Received: 11 August 2013 / Accepted: 24 October 2013
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Abstract The root nodules of two wild legume species endemic to Algeria, *Hedysarum naudinianum* and *He. perrauderianum*, were investigated with regard to their anatomy and histology, and the identity of the associated bacteria. Both plants were found to form root nodules with regular features and well infected by rod-shaped bacteria. The culturable fraction of bacteria that could be obtained from surface-sterilized nodules included a prevailing presence of Enterobacteriaceae having 100 % 16S rDNA sequence identity with both *Enterobacter cloacae* and *E. ludwigii*. In *H. perrauderianum*, this taxon was the sole cultured isolate, while from *H. naudinianum* we also found *Bacillus*, *Lactobacillus*, *Staphylococcus*, *Rothia*, and isolates that were 100 % identical to *Corynebacterium pseudodiphthericum*, which is known to be an agent of

respiratory and cardiac infections in humans. Whereas no culturable rhizobia and alike could be obtained on plates, PCR-based culture-independent approaches revealed in both plants the presence of a *Mesorhizobium* sp., which in *H. perrauderianum* was identical to isolates nodulating other legumes from Africa, European Mediterranean countries, and Asia, while in *H. naudinianum* it bore a single nucleotide polymorphism which is so far unique for any observed mesorhizobia. Data from the microsymbionts appear to suggest interesting clues to interpret the evolutionary ecology of their host plants.

Keywords *Hedysarum naudinianum* · *Hedysarum perrauderianum* · *Mesorhizobium* · Root nodules · Algeria · Endophytic bacteria · Endemic legumes · SNP

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Introduction

Many legume plants rely on the symbiosis with nitrogen-fixing rhizobia, and an increasing number of studies is nowadays addressing the bacterial associations of spontaneous leguminous species. *Hedysarum* is a genus of the Hedysareae tribe featuring about 309 species distributed worldwide. The taxon is divided in two main groups: the first includes the Alpine, Arctic, and Asiatic species (2n=14 chromosomes), while the second harbours the Mediterranean species (2n=16) (Trifi-Farah and Marrakchi 2001), some of which have been repositioned in the new genus *Sulla* (Choi and Ohashi 2003). Within the Mediterranean set of *Hedysarum*, our earlier interest has been devoted to *Hedysarum coronarium*, which exists both as a cultivated forage crop and as wild stands. For this species, we described the new species of

host-specific nitrogen-fixing symbiont, *Rhizobium sullae* (Squartini et al. 2002). Subsequently, we have investigated the bacteria in Algeria within root nodules of *Hedysarum spinosissimum* ssp. *capitatum*, *H. pallidum* and *H. carnosum* (Benhizia et al. 2004), whose occupants are dominated by endophytic gammaproteobacteria. Further, we covered two other Mediterranean wild species of *Hedysarum* by studying *H. spinosissimum* and *H. glomeratum* in Sardinia (Muresu et al. 2008). Subsequently, we investigated the nodule content of the relictual *H. confertum* (= *H. humile*) (Tondello et al. 2011). In these studies, we showed the consistent non-culturability of the rhizobial symbionts and the occurrence of a vast array of opportunistic endophytes.

Within Algeria, the genus *Hedysarum* includes ten species, some of which are endemic, such as *H. naudinianum* and *H. perrauderianum*. The latter is moreover reported as very localized (Abdelguerfi-Berrekia et al. 1991). These two taxa are rather rare. *H. naudinianum* is found in the tell constantinois (mount of Bibans), in Algiers (Littoral, Tell Atlas), in Boghar, and the north of Setif (Bougaa, Ain Roua). *H. perrauderianum* occurs in the region of Batna and south of Setif (Abdelguerfi-Berrekia et al. 1988; Quezel and Santa 1962).

From ecological and conservational perspectives, the entire genus *Hedysarum* is regarded as a wealthy phylogenetic resource which is, however, increasingly endangered by progressive genetic erosion due to overgrazing, irregular rainfall, and range land contraction, particularly in dry and semi-arid areas (Trifi-Farah et al. 2002). In such scenarios, it is of primary importance to determine whether the rarity of given species could be due to their dependence on symbionts to ensure their nutritional needs. The abundance or exiguity of the suitable interactive microorganisms deeply affects soil capability to host and support plants. Both productivity and health of the crops as well as the distribution of natural vegetation are conditioned by interactions with ground microbiota, with the most significant correlation being the presence or absence of symbiotic or pathogenic microorganisms (Klironomos 2002). For these reasons, we deemed it important to cast light on the presence and identity of root nodule bacteria associated with the above-mentioned two endemic legume species thriving exclusively in Algeria and enduring harsh conditions.

Materials and methods

Plant and nodule collection

The biological material was collected in the region of Sétif (36°12'0"N, 5°24'0"E) in the East of Algeria (Fig 1); *Hedysarum naudinianum* whole plants were excavated in

the three sites of Bougaa (00°00'00"N, 5°04'60"E), Ain Roua (36°19'60"N, 5°10'60"E) and Maoklane (36°23'50"N, 5°04'31"E), while *H. perrauderianum* was gathered from Ouled Tebbene (35°48'46"N, 5°06'05"E). All specimens were green and healthy plants. Nodules were in non-senescent, fully developed stage.

Nodule excision was carried out as described by Vincent (1970) and Beck et al. (1993). Roots were carefully cleaned and washed free of soil. Dry portions of roots with nodules were stored with CaCl₂ until nodule sterilization. Plants and their root apparatus are shown in Fig. 2.

Nodule microscopy

Nodules were fixed in 3 % glutaraldehyde in 0.1 M cacodylate buffer (pH 6.9) for 24 h at 4 °C and postfixed for 2 h at 4 °C in 1 % osmium tetroxide in 0.1 M cacodylate buffer, dehydrated in a graded ethanol series, and then embedded in araldite resin. Thin sections (1 µm), obtained with a Reichert-Jung ultramicrotome, were stained with 1 % toluidine blue for light microscopy.

Bacterial isolation from the nodules

Root portions bearing nodules were washed free of soil under running water, engaged in a fine-mesh steel holder and surface-sterilized by immersion in 95 % ethanol for 20 s, followed by 5 % sodium hypochlorite for 3 min, and then washed 7 times with sterile distilled water. Finally, nodules were transferred aseptically into sterile Petri dishes and crushed in a drop of sterile distilled water.

The nodules suspension was plated on YMA-congo red, YMA-bromo-thymol blue, and PCA- and GPA-bromo-cresol purple (Vincent 1970).

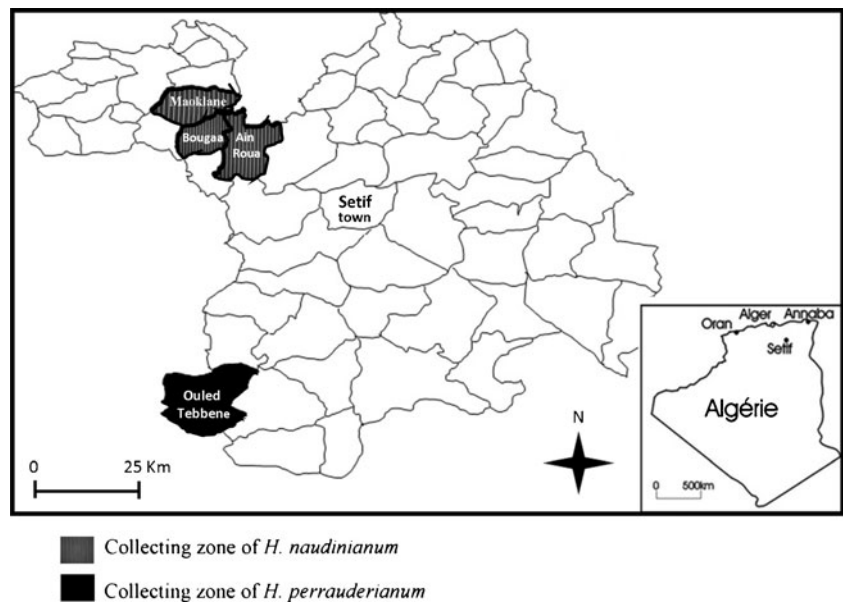
Tests to validate surface-sterilization of plant tissues, including root nodules, were performed by touching them several times on the surface of plate count agar (PCA, Difco) plates prior to isolation of the interior microbiota. The absence of colonies on these plates was a requirement to ascertain the disinfection from residual external bacteria.

CaCl₂-stored dry nodules were rehydrated for 6 h in distilled water prior to the surface-sterilization treatment.

DNA extraction from culturable bacteria

Cells were lysed by resuspending a loopful of plate-grown isolated colonies in 50 µL of lysis buffer (0.25 % sodium dodecyl sulphate, SDS 0.05 M NaOH), followed by stirring for 60 s on a vortex and heating at 95 °C for 15 min. The lysate was centrifuged for 15 min at 13,400 g and 10 µL of the supernatant were mixed with 90 µL of sterile water. Lysates were stored at 4 °C prior to PCR.

Fig. 1 Algeria showing the collection sites



DNA extraction from nodules

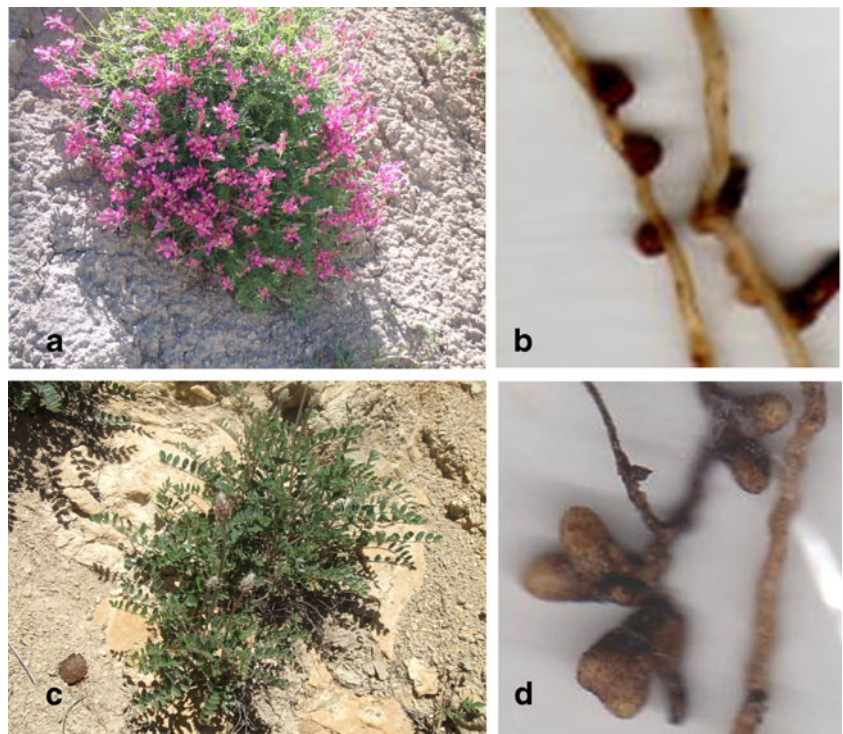
For direct PCR analysis, aliquots of nodule suspensions (10 μ L, irrespectively of original volume) were transferred to 50 μ L of lysis buffer, [0.25 % sodium dodecyl sulphate (SDS) 0.05 M NaOH] in a 1.5-mL polypropylene tube, followed by stirring for 60 s on a vortex and heating at 95 $^{\circ}$ C for 15 min. The lysate was centrifuged for 15 min at 13,400 g and 10 μ L of the supernatant were

mixed with 90 μ L of sterile water. Lysates were stored at 4 $^{\circ}$ C prior to PCR.

PCR amplification of the 16S rDNA gene

One μ L of the lysate containing the total DNA extracted from nodules was treated in a PCR BioRad I-Cycler using the two 16S rRNA gene-targeted universal bacterial primers, 63F (5'CAGGCCTAACACATGCAAGTCC3') (Marchesi et al.

Fig. 2 *Hedysarum naudinianum* plant (a) and root nodules (b); *H. perrauderianum* plant (c) and root nodules (d)



1998) and 1389R (5'ACGGGCGGTGTGTACAAGG3') (Osborn et al. 2000) in a 25- μ L reaction volume, using the following program: initial denaturation at 95 °C for 2 min; 40 cycles at 95 °C for 30 s, 55 °C for 1 min, 72 °C for 2 min; and a final extension at 72 °C for 10 min. The PCR reaction mixture contained 67 mM Tris-HCl (pH 8.8), 16 mM (NH₄)₂SO₄, 0.01 % Tween-20, 2 mM MgCl₂, 0.2 mM of each dATP, dCTP, dGTP and dTTP, 0.2 μ M of each primer and 0.5 U Taq DNA Polymerase (EuroTaq, EuroClone). To verify the positive reaction amplicons were loaded on a 1.5 % agarose gel and run electrophoretically for 1 h at 110 V. The Sybergreen-stained gel was visualized over an UV transilluminator and photographed by a Kodak DC290 digital camera.

ARDRA Analysis

Amplicons were digested overnight at 37 °C upon mixing 5 μ L from the 25- μ L reaction volume with 1 μ L of *Cfo*I enzyme (Pharmacia, Uppsala, Sweden) and 2 μ L of 10 \times reaction buffer. Digested DNA was loaded on a 1.5 % agarose gel, run electrophoretically for 3 h at 110 V. The stained gel was visualized over an UV transilluminator and photographed by a Kodak DC290 digital camera. Upon ARDRA analysis, the isolates were sorted and selected for sequencing.

DNA Sequencing and bacterial molecular taxonomical analysis

One μ L of the amplicon resulting from the above-described PCR amplification was mixed with 1 μ L containing 6.4 pmol of the primer 63 F in a 0.2-mL polypropylene tube and then dried by incubating the tube open for 15 min at 65 °C in an I-Cycler thermal cycler. The template and primer mix was directly used for di-deoxy-cycle DNA sequencing with fluorescent terminators (Big Dye; Perkin-Elmer/Applied Biosystems, Foster City, CA, USA) and run in an Applied Biosystems ABI Prism 3730XL automated DNA sequencer. Chromatograms were analyzed by Chromas 2.23 software (Technelysium, Tewantin, Australia). BLAST analysis (Altschul et al. 1990) against nucleotide databases was performed via the NCBI website (<http://www.ncbi.nlm.nih.gov/>).

DNA extraction from nodules for amplicon library cloning

Sterile nodules were resuspended in TE 1 \times (100 μ L), smashed using a sterile pipetman tip and incubated at 95 °C for 10 min. Lisozyme (1 mg/mL) and SDS (1 % final concentration) were added and the solution was incubated at 37 °C for 15 min. A proteinase-K treatment (100 μ g/mL) was carried out at 55 °C for 1 h.

Subsequently, a protocol adapted from the PowerSoil® DNA Isolation Kit (MO BIO, Solana Beach, CA, USA) was developed in order to increase DNA yield and purity, and after the C3 solution treatment, the supernatant was transferred in a new Eppendorf tube. Two extractions with phenol:chloroform:isoamyl alcohol (25:24:1) and one chloroform extraction were carried out, followed by an overnight precipitation using ammonium acetate (2 M f.c.) and absolute ethanol (2.5 volumes) at -20 °C.

After incubation, samples were centrifuged at 4 °C for 40 min and 12,000 rpm, washed twice with 70 % Et-OH, and resuspended in 50 μ L of sterile distilled H₂O.

PCR amplification for library cloning

16S rRNA genes amplification was carried out using the universal primers described above.

A 20- μ L reaction for each sample was prepared using 0.2 U of Phusion High-Fidelity DNA Polymerase (NEB) and the thermal cycler (iCycler, Bio Rad) was set as follows: initial denaturation at 98 °C for 2 min, 30 cycles at 98 °C for 20 s, 61 °C for 45 s, 72 °C for 1 min, and a final extension at 72 °C for 6 min.

To verify the positive reaction, amplicons were loaded on a 1 % agarose (SeaKem LE), run electrophoretically and purified by Agencourt AMPure XP-PCR Purification (Beckman Coulter).

PCR products were phosphorylated by a T4-Polynucleotide-Kinase (NEB) treatment 37 °C for 30 s, inactivated at 65 °C for 20 min.

Amplicon cloning

One μ g of pGEM®-3Zf(+) (Promega) was digested in a 20- μ L reaction by 40U of *Sma*I restriction enzymes (NEB), at 25 °C for 1,5 h and inactivated at 65 °C for 20 min. Vector dephosphorylation was carried out in a 25- μ L reaction using 2.5 U of Antarctic Phosphatase (NEB) at 37 °C for 30 min followed by inactivation at 65 °C for 10 min. Phosphorylated-PCR products and dephosphorylated-pGEM were purified by an Agencourt AMPure XP-PCR Purification (Beckman Coulter) treatment. pGEM and PCR-products (1:6 ratio) were ligated using 1 U of a T4-DNA ligase (NEB) in a 20- μ L reaction, incubated at 16 °C for 16 h followed by inactivation at 65 °C for 20 min. Products were cloned in *E. coli* DH10B. Plasmids were extracted using a FastPlasmid Mini kit (Eppendorf). A vector-screening was carried out to select clones for sequencing; a double-digestion *Eco*RI-*Hind*III (10U each) in a 20- μ L reaction was performed at 37 °C for 90 min and inactivated at 65 °C for 20 min. Results were verified by a 1.5 % agarose (SeaKem LE) gel electrophoresis. Sanger sequencing was carried out as described above.

Results and discussion

Nodule histology

Nodules were inspected by brightfield and transmission electron microscopy (Fig. 3). Toluidine-stained thin sections indicated that the root structures formed by *H. naudinianum* and *H. perrauderianum* are consistent with the histology of genuine legume nodules, displaying an uninfected cortex, a peripheral vascular system, and a central tissue of host cells infected to various degrees with bacteria; TEM images allow observation of details of bacterial shapes within the infected tissues. This evidence demonstrates that both species are nodulated and that their nodules bear normally invasive bacteria.

Characterization of culturable microbiota

Upon plating the content of the surface-sterilized nodules on YMA and PCA media, colonies of different kinds were observed from *H. naudinianum*, while only one morphology stemmed from *H. perrauderianum*. The recurring types were purified and characterized by DNA-based identity analyses; ARDRA profile sorting and 16S rDNA sequencing allowed the assignment of the species identities that are reported in Table 1. As already typically observed in previous studies in other wild legumes (Benhizia et al. 2004; Muresu et al. 2008; Tondello et al. 2011), no culturable rhizobia could be isolated; instead, different bacteria populated the inner tissues of these nodules. In both *H. naudinianum* and *H. perrauderianum*, we found a recurring member of the Gammaproteobacteria displaying 100 % identity with both *Enterobacter cloacae* and *E. ludwigii*. Although this was the sole culturable type that we could observe in *H. perrauderianum*, the other legume, *H. naudinianum*, appears to harbor a wider spectrum of

endophytes (Table 1) featuring lactobacilli, Gram-positives including actinobacteria, among which it is worth signaling the presence of an isolate which is 100 % identical to *Corynebacterium pseudodiphthericum*. This taxon is classically known as the Hoffmann bacillus, a resident of the human throat not proficient in causing diphtheria but reported as involved in clinical cases of pneumonia and other respiratory infections (Gorricho et al. 1996; Gutiérrez-Rodero et al. 1999; Aspiroz Sancho et al. 2002) and even suspected of being the agent of endocarditis (Dijken et al. 1950). Such occurrences of potential human or animal pathogens concealed within wild legume nodules of semiarid habitats is not new to our experience. In prior reports, we disclosed the occurrence of species known to human medicine within the nodules of *H. spinosissimum* subsp. *capitatum*, *H. pallidum*, and *H. carnosum*, including *Enterobacter cloacae*, *E. kobei*, *Escherichia vulneris*, *Pantoea agglomerans*, *Leclercia adecarboxylata*, and *Pseudomonas* sp. (Benhizia et al. 2004). In a subsequent report, we ascertained that some of the above isolates bore phenotypic and genotypic determinants of virulence that were investigated using human cultured cells, which tested positive for the traits of cytotoxicity, vital stain exclusion, and adhesion to epithelia (Muresu et al. 2010). Antibiogram analyses also revealed a complex pattern of multiple antibiotic resistances. The data unfolded a scenario in which legume root nodules could act as a site of survival and of active multiplication for populations of mammalian pathogens whose cycle might alternate between the target animal and a number of neutral plant hosts. The worldwide diffusion of as yet uninvestigated legumes raised the concern for a general niche that could enhance the hazards posed by microorganisms of a clinical nature. The present finding of *Corynebacterium pseudodiphthericum*, again occurring in the nodules of a north African spontaneous legume, further extends the case history and emphasizes the issue.

While at least six different culturable taxa could be observed to inhabit nodules of *H. naudinianum*, the same analysis on its companion species *H. perrauderianum* did not yield such a variety of species, as the sole gammaproteobacterial enterics identical to *Enterobacter cloacae*/*Enterobacter ludwigii* was found to grow from these nodules. The 16S sequence of the isolate is equal to that also occurring in *H. naudinianum*. These kind of bacteria of the *Pantoea*–*Enterobacter* group are knowingly proficient as endophytes in a number of plants (Elvira-Recuenco and Van Vuurde 2000), and are also shared in both of the *Hedysarum* species analyzed here, as well as in others within the Mediterranean checklist of our former studies (Muresu et al. 2008). As regards the culturable array of diversity, none of the above-described isolates belonged to the Rhizobiaceae family or to other genera of ascertained nitrogen-fixing symbionts or legumes. This led to us considering that, as observed in prior

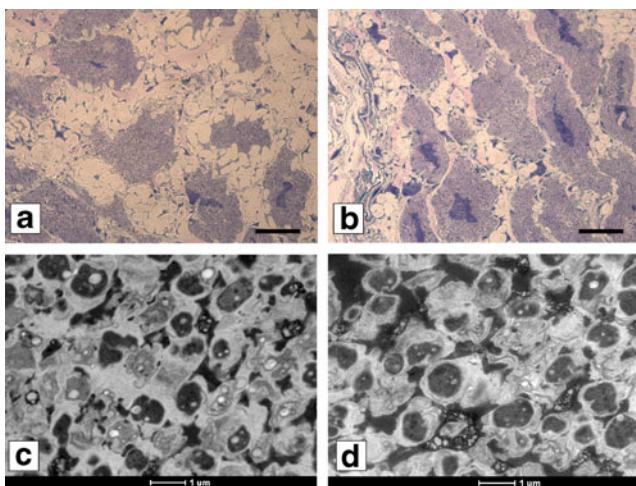


Fig. 3 Histological observations of nodule sections of *H. naudinianum* (a, c) and *H. perrauderianum* (b, d) in optical (a, b) and electron microscopy (c, d). Scale bars (a, b) 30 μ m, (c, d) 1 μ m

Table 1 Identities of bacteria associated with nodules of *Hedysarum naudinianum* and *H. perrauderianum* as inferred by 16S rDNA sequencing and BLAST analysis in GenBank

Identifier ^a	GenBank code	Best match homologies to GenBank taxa and % similarity
Culture-dependent method (plate-cultured colonies)		
HnA1	KF147863	<i>Enterobacter cloacae</i> LRC85 (JF772064) 100 % <i>Enterobacter ludwigii</i> YPB10-1 (JQ308612) 100 %
Hn33Ya	KF147864	<i>Staphylococcus</i> sp. DG9 (JN208200) 99 %
Hn34Ya	KF147865	<i>Bacillus</i> sp. NH1 (JN208177) 100 %
Hn35Ya	KF147866	<i>Lactobacillus casei</i> PC_CA (JN133441) 100 %
Hn36Ya	KF147867	<i>Rothia amarae</i> R-36507 FR682692 99 %
Hn37Ya	KF147868	<i>Corynebacterium pseudodiphthericum</i> CIP103420T (AJ439343) 100 %
HpRA1	KF147863	<i>Enterobacter cloacae</i> LRC85 (JF772064) 100 % <i>Enterobacter ludwigii</i> YPB10-1 (JQ308612) 100 %
Culture-independent method (16S amplicon cloning and bank screening)		
HnNod1	KF147869	<i>Mesorhizobium</i> sp. (JF273844) <i>Coronilla varia</i> , China, 99 % <i>Mesorhizobium</i> sp. (EU697961) <i>Ammopiptanthus mongolicus</i> , China, 99 % <i>Mesorhizobium</i> sp. (AM748949) <i>Anagyris foetida</i> , Sicily, 99 % <i>Rhizobium</i> sp. H-4 (AF279889) <i>Hedysarum spinosissimum</i> , Israel, 99 %
HnNod4	KF147870	<i>Flavobacterium</i> sp. TP-Snow-C28 (HQ327137) 98 %
HnNod5	KF147871	<i>Caulobacter</i> sp. HWE-A01 (JF722654) 100 %
HnNod6	KF147872	<i>Afipia</i> sp. BAC312 EU130951 98 %
HnNod7	KF147873	<i>Moraxella osloensis</i> DSM 6998 AB643599 100 %
HpNod2	KF147875	<i>Mesorhizobium</i> sp. (JF273844) <i>Coronilla varia</i> , China, 100 % <i>Mesorhizobium</i> sp. (EU697961) <i>Ammopiptanthus mongolicus</i> , China, 100 % <i>Mesorhizobium</i> sp. (AM748949) <i>Anagyris foetida</i> , Sicily, 100 % <i>Rhizobium</i> sp. H-4 (AF279889) <i>Hedysarum spinosissimum</i> , Israel, 100 %
HpNod1	KF147874	<i>Acinetobacter</i> sp. uncultured, clone ASC753 (JQ775355) 99 %
HpNod3	KF147876	<i>Phyllobacterium brassicacearum</i> MR4 (EF581126) 99 %

^a Isolates from *H. naudinianum* are coded with the prefix *Hn*; those from *H. perrauderianum* with the prefix *Hp*

reports (Muresu et al. 2008), these two legumes could also harbor non-culturable rhizobia along with various culturable endophytes. As, when dealing with other Mediterranean wild species, we had been successful in the direct 16S rDNA amplification of rhizobium (Muresu et al. 2008, 2011; Tondello et al. 2011; Ourarhi et al. 2011), we performed several attempts with nodules of the two species described here but in no case could we get any readable sequence with this approach, the PCRs resulting in either no amplification or mixed chromatograms. Such limitation led to a different strategy and, in order to circumvent the problem, we cloned amplicons in plasmid vectors, transforming *E. coli* and screening the libraries. This approach led to us uncovering another portion of diversity in which we found frequent clones whose sequence was consistent with members of the rhizobiaceae, namely *Mesorhizobium* sp. which was found in both plants. Under the existing evidence, these are to be considered the putative true symbionts of both plants, and show identities to other mesorhizobia reported in different legumes from Mediterranean to Far East locations (Table 1).

Different taxa including *Caulobacter*, *Afipia* and *Moraxella* were present within *H. naudinianum* nodules, while *Acinetobacter* and *Phyllobacterium* were in *H. perrauderianum*.

As regards the two sequences of *Mesorhizobium* found in *H. naudinianum* and *H. perrauderianum*, it is worth reporting that these differ from each other by a single nucleotide. For this reason, the identities with other mesorhizobia are in one case 99 % and in the other 100 %. As these cases of single mismatching could be suspected of being artifacts due to sequencing errors in nucleotide attribution for poorly resolved fluorescence peaks, we repeated the sequencing twice and inspected the corresponding regions of the chromatograms whose output was clear, and consistently carried a genuine difference in a nucleotide (G for T) which corresponds to position 238 of the reference *E. coli* 16S rRNA gene.

Interestingly, the presence of a G in that position for the bacterium in *H. perrauderianum* is conserved not only among the four examples of mesorhizobia from *Anagyris foetida*, *Coronilla varia*, *Ammopiptanthus mongolicus*, and

Hedysarum spinosisimum reported in Table 1 but also in those found in Sardinia (Muresu et al. 2008) within *Hedysarum glomeratum*, (DQ457611), *Hippocrepis unisiliquosa* (DQ457615), *Scorpiurus muricatus* DQ457619, *Tetragonolobus purpureus* DQ457620, and *Psoralea bituminosa* (DQ457618), all of which carry the G at position 238. Therefore, the sequence of the *Mesorhizobium* within *H. naudinianum* bears a mutation which is unique and originates a single nucleotide polymorphism (SNP) which stands out as indicative of an interesting separation from other mesorhizobia occurring throughout different continents, which instead all share the alternative version of the sequence. The uniqueness of the bacteria associated to this Algerian botanical endemism suggests that its origin could be that of a neo-endemism (originating locally possibly by hybridization with other species) rather than a paleo-endemism (formerly more extended but presently relictual and extinct elsewhere), which could instead be the case of *H. perrauderianum* whose symbiont is still also present in legumes of far distant ranges and different tribes. This finding and the possible speculations exemplify an additional element of usefulness in studying bacterial symbionts as keys to interpret their host plants' evolutionary biology.

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