Alteration of soil rhizobial populations by rabbit latrines could impair symbiotic nitrogen fixation in the insular alpine ecosystem of Teide National Park

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ABSTRACT

Spartocytisus supranubius (Teide broom) is the dominant legume of the high-mountain ecosystem in Teide National Park (Tenerife, Canary Islands). Herbivory by rabbits is one important cause of its declining populations. Biological nitrogen-fixing of rhizobia in symbiosis with *S. supranubius* is hypothesized to be the main nitrogen (N) input in this alpine ecosystem. Therefore, our main objective was to investigate whether large rabbit dropping aggregations (latrines) affect the *S. supranubius*-rhizobia symbiosis by disturbing the rhizobia populations in the soils. Soils were sampled in the rhizosphere of broom plants without (RS) and with latrines (RLS) as well as in non-rhizospheric bare areas without (BS) and with latrines (BLS), and assessed for N content and number of rhizobia. Rhizobial diversity was evaluated by sequencing three housekeeping genes in root-nodule bacteria isolated from broom seedlings grown in these soils. Results showed that BS had the lowest rhizobia counts and N content, whereas RS had on average the highest, which were not exceeded in soils with N-supply from latrines (BLS and RLS). Phylogenetic analysis revealed that strains from the *Bradyrhizobium canariense/Bradyrhizobium lupini* lineage are the main microsymbionts of *S. supranubius*. *Bradyrhizobium rifense/Bradyrhizobium cytisi* strains were scarce and only detected in latrine influenced soils, where endophytic *Bosea* strains seem a common cohabitant in nodules. It can be concluded that *S. supranubius*-Bradyrhizobia symbiosis is a key N input in Teide soils and that rabbit latrines may affect the N-fixing symbiosis by altering abundance and diversity of the soil rhizobial populations. Moreover, latrines could also interfere in symbiosis by favouring co-entrance of non-nodulating bacteria inside the root nodules.

1. Introduction

Rhizobia are gramnegative soil bacteria that establish a nitrogen (N) fixing symbiosis with leguminous plants. These bacteria trigger the formation of root nodules, where they reduce the atmospheric N_2, making ammonia available to the plant. Symbiotic N-fixation by effective rhizobia-fixers can satisfy plant N requirements and eventually enriches soils. They thus play an important ecological role in soil fertility, plant productivity and maintenance of the whole ecosystem (Thilakarathna et al., 2016; Irisarri et al., 2019). However, as rhizobia differ greatly in their N-fixing ability, legume-specific rhizobial strains, well-adapted to particular environmental conditions are of primary importance.

With a large number of endemic legume species from arid coastal areas to the high-mountain ecosystems, the Canary Islands offer an excellent model to study the symbiosis between rhizobia and legumes. Previous studies by our research group have shown a great diversity of rhizobia nodulating the native Canary legumes, which is reflected by the detection of new rhizobial genotypes (Jarabo-Lorenzo et al., 2000; Donate-Correa et al., 2007; Lorite et al., 2010; Armas-Capote et al., 2014; Pérez-Yépez et al., 2014) and in the description of new species and symbiovars (Vinuesa et al., 2005; León-Barrios et al., 2009; Ramírez-Bahena et al., 2012; Armas-Capote et al., 2014). So far, our studies have focused on rhizobia associated with legumes characteristics of the coast

(León-Barrios et al., 2009; Lorite et al., 2010; León-Barrios et al., 2017) or the pine and cloud laurel forests (Lorite et al., 2010; Armas-Capote et al., 2014; Pérez-Yépez et al., 2014). However, there are no previous studies of rhizobia-legume symbiosis in the high-mountain ecosystem, except a partial characterization of 4 bradyrhizobial isolates (Jarabo-Lorenzo et al., 2000).

In Tenerife (Canary Islands), the alpine ecosystem, a high mountain ecosystem within the supra and oromediterranean thermotype (Del Arco-Aguilar and Rodríguez-Delgado, 2018) extends from 2000 m a.s.l. to 3718 m a.s.l. at the Teide volcano summit, most of this area being part of the Teide National Park (N.P.). The main characteristic vegetation in this high mountain ecosystem is summit shrub vegetation with *Spartocytisus supranubius* (L. f.) Christ ex G. Kunkel, commonly known as 'retama del Teide' (Teide broom), as the most representative element. This is a species endemic to the Canary Islands widely found on Tenerife and scarcely on La Palma. A shrub plant growing to 2–3 m high and up to 6 m in diameter, it has small deciduous leaves and photosynthetic stems (Kyncl et al., 2006). Molecular studies include genus *Spartocytisus* within the *Cytisus* clade (Cubas et al., 2002). This broom is the dominant legume in Tenerife alpine ecosystem, thriving on poorly developed soils, between 2000 and 2900 m a.s.l. on the north side and between 2200 and 3100 m a.s.l. on the southern slopes of the islands occupying a large extension of the Teide N.P. (Del Arco-Aguilar and Rodríguez-Delgado, 2018). Nitrogen content in Teide N. P. soils is very low (Arbelo et al., 2009) and several studies have reported that N availability commonly limits vegetation establishment on newly emplaced volcanic deposits (Díaz et al., 2011). Therefore, the importance of the biological nitrogen fixation (BNF) from the Rhizobium-*S. supranubius* symbiosis as main N input to this volcanic mountain ecosystem is of great importance, as suggested by finding a progressively decreasing N gradient as one moves away from the broom rhizosphere (Wheeler and Dickson, 1990).

Currently, *S. supranubius* is showing a progressive regression in its population (Martín-Esquivel et al., 2020). Many specimens display decreased vitality, damage, decrepitude and dryness. One major problem facing this species is the lack of regeneration due to a high rate of juvenile individual consumption by exotic herbivores, mainly the European rabbit (*Oryctolagus cuniculus*) (Kyncl et al., 2006; Cubas et al., 2018). Rabbits have been present in the Teide N.P since they were introduced by the Castilian conquerors and settlers (16th century) (De Abreu-Galindo, 1977). However, the milder climatic conditions in recent decades –probably related to global warming- have allowed larger populations of rabbits to dwell in and roam all over the park (Martín et al., 2015). Previous studies on the high-altitude ecosystem of the nearby island La Palma have shown that selective grazing by introduced herbivores led to alteration of the richness and diversity of endemic plants and caused dominance of the less palatable legume shrub *Adenocarpus viscosus* (Irl et al., 2012, 2014). In Teide N. P., rabbits also have tragic consequences for the summit shrub vegetation, with a drastic parallel decrease in *S. supranubius* (Cubas et al., 2018, 2019; Ibarrola-Ulzurrun et al., 2019; Martín-Esquivel et al., 2020), allowing the less palatable species *Pterocephalus lasiospermus* (Link ex Buch) to extraordinarily increase its presence in the N. P. As a measure to protect the shrub plants from herbivores, fenced herbivore exclosure areas have been installed within the park. Young broom individuals are rarely found outside these exclosures, which can be interpreted as a strong negative effect of introduced herbivores on this plant species rejuvenation (Cubas et al., 2018). In addition, a progressive deterioration of adult individuals has also been detected, as a consequence of prolonged drought episodes and temperature increase related to climate change (Olano et al., 2017). The extremely dry conditions that occurred in the last autumn and winter 2019–2020 have exacerbated the mortality of brooms, due to almost zero rainfall together with prolonged episodes of dry Sahara winds (Calima).

Despite the obvious importance for the alpine ecosystem of the BNF by rhizobia in symbiosis with Teide broom, research into this is almost nonexistent. Hence, in the present study, one objective was to describe the phylogenetic diversity of the rhizobia nodulating *S. supranubius* in its natural habitat at the east of Teide N. P. to answer: i) Is *Bradyrhizobium* the main microsymbiont of this broom as previously shown for other woody Genisteae? and ii) Which species are their closest phylogenetic relatives?

Furthermore, since rabbit activities were evident as large latrine deposits in the study area, as another main objective we tried to answer whether rabbit latrines may disturb the rhizobial populations, which eventually would affect the N-fixation symbiosis. Apart from their effect on plant community structure, rabbits have also been shown to alter the structure (Eldridge and Simpson, 2002) and composition (Eldridge and Koen, 2008; Cubas et al., 2018) of soils. Cubas et al. (2018) reported a declining in the N content in soils under latrines, but increased N amounts in burrow mounds has also been reported (Dhillion et al., 1994). These changes point to disturbances by rabbits in the N cycle, and alterations in soil physicochemical characteristics disturb the soil microbiota. However, scarce attention has been paid to the effect of rabbits on soil bacterial communities. An interesting recent study by Eldridge et al. (2016) included the characterization of the soil bacterial community composition and the enzyme activities (soil functions) in echidna (*Tachyglossus aculeatus*) and European rabbit pits. Positive effects on soil functions were mainly found in echidna pits, which were microbiologically and enzymatically more active than those of exotic rabbits and differed in the soil microbial community composition. Interestingly, compared to echidna, rabbit pits showed lower relative abundance in Alphaproteobacteria and Bacteroidetes, two highly functional soil microbial groups. To our knowledge, there are no studies on how rabbits specifically affect the soil rhizobia populations. In the present study we hypothesized that i) rabbit excreta (deposition of faeces and urine) in latrines can modify the rhizobia populations in abundance and/or species diversity and if so, ii) it will be reflected in the soil nitrogen contents.

2. Material and methods

2.1. Site description

This study was performed in a location at the east of Teide National Park, close to the Fasnia volcanoes (UTM 28R351796E/3130005 N, 2180 m a.s.l), in the Fasnia municipality, Tenerife (Canary Islands, Spain) (Fig. S1). The soil type at the study area is a Vitrandic Xerorthent (Soil Survey Staff, 2010) or tephric, humic Vitric Leptosol (IUSS Working Group WRB, 2015). Climate in the Teide National Park, including the study area is typified by irregular precipitation, averaging 200–500 mm per year, high annual and daily temperature variation and extremely dry summers (Del Arco et al., 2006). The dry high mountain conditions on islands subject to trade winds lead to highly adapted vegetation. The most characteristic species is *S. supranubius* (Fig. S2), along with other smaller shrubs like *P. lasiospermus* and *Descurainia bourgeauana* (Del Arco et al., 2006).

2.2. Experimental design and description of treatments

The study area, a 6 ha extension located at the northeast of the Fasnia volcanoes, is occupied by a *S. supranubius* broom shrubland greatly affected by rabbit herbivory. This area includes a fenced rabbits-exclosure plot of 400 m² (20m × 20 m). The presence of rabbits is seen from large dropping aggregations, known as latrines (*e.g.* Mutze et al., 2014; Cubas et al., 2018). The experiment followed a simple random sampling design with three replications for the sampling points selection at the four types of soils considered (treatments): *i*) RS: rhizospheric soil from *S. supranubius* plants within an exclosure plot protected from influence of rabbits, *ii*) RSL: the rhizospheric soil in unfenced areas under the influence of rabbit latrine; *iii*) BS: bare soil (non-vegetated, no latrines) and *vi*) BLS: bare soil under latrines. 'Rhizospheric soil' refers to the soil under the influence of the root (within a radius of

0–30 cm from the main root), given that *S. supranubius* is a threatened plant we were not allowed to collect. Only large latrines were considered following the criterion of Cubas et al. (2018), who describe them as agglomerations of more than 200 pellets (droppings) within a single patch. Furthermore, latrines were considered to affect the rhizospheric soil when located at less than one-metre from the main root.

2.3. Soil sampling

Samples were taken in 'early autumn' (20th October 2016 and 28th October 2019), which is a climatically unfavourable period in the Teide N.P. after the very dry summers, and in late spring (24th May 2018 and 4th June 2019), considered a favourable period for plant growth in the Park. Soil samples (2–15 cm depth) were collected in sterile plastic bags using aseptic spades. For each type of soil sample, three replicates separated at least 6–7 m apart from each other were collected. For microbial purpose (the MPN method, see below), soil samples were sieved through a 3 mm mesh to remove larger pebbles and plant remains and kept refrigerated at 4 °C until used within the next two-three days. Prior to physicochemical analysis, soil samples were air-dried for 72 h and passed through a 2 mm soil screen and left at room temperature until analysed within the next two-six weeks.

2.4. Analyses

2.4.1. Soil physicochemical characteristics

Particle size distribution (sand, silt and clay) was determined by the densimeter method (Boyoucos, 1962); pH (pHe) and electrical conductivity (ECe) in saturated paste extracts (Soil Survey Staff, 2010); oxidizable organic carbon (OC) by potassium dichromate oxidation (Skjemstad and Baldock, 2008), total nitrogen (TN) by the Kjeldahl method (Jackson, 1982). Available P was extracted with 0.5 M NaHCO3; available NH_4^+ was extracted with a 2 M KCl solution, as proposed by Maynard and Kalra, 1993, and determined using the Nessler reagent method (Jackson, 1982). Three replicates were assayed separately and the results show (Table 1) the mean of the three replicates.

2.4.2. Rhizobia counts and isolation

The most probable number (MPN) method was used to determine the

Table 1
Chemical and physical characteristics of the soil surface layer (2-15 cm) during the study period (October 2016–October 2019) in four types of soil samples from Teide National Park.

Parameter	Sampling site			
	RS[a]	RSL[b]	BLS[c]	BS[d]
ECe μS cm^{-1}	330 ± 176 b[e]	458 ± 230 b	1156 ± 800 a	220 ± 80 b
pHe	6.4 ± 0.3 ab	6.1 ± 0.3 b	6.3 ± 0.4 b	6.8 ± 0.4 a
Organic C g kg^{-1}	14.8 ± 6.5 a	14.1 ± 3.1 a	6.4 ± 3.5 b	4.3 ± 1.1 b
Total N g kg^{-1}	1.8 ± 0.8 a	1.6 ± 0.4 ab	1.2 ± 0.5 b	0.6 ± 0.4 c
Olsen-P mg kg^{-1}	20.3 ± 12.1 a	17.5 ± 10.6 a	34.1 ± 12.2 a	25.9 ± 7.1 a
NH_4^+ mg kg^{-1}	48.8 ± 17.6 a	44.8 ± 17.4 a	48.8 ± 22.5 a	34.7 ± 11.8 a
Clay g kg^{-1}	201 ± 47 a	215 ± 23 a	264 ± 41 a	190 ± 72 a
Silt g kg^{-1}	341 ± 12 a	366 ± 73 a	355 ± 135 a	270 ± 113 a
Sand g kg^{-1}	458 ± 48 a	419 ± 92 a	382 ± 115 a	540 ± 183 a

Values with the same lower case letters in the same row are not significantly different according to a one-way ANOVA and *post hoc* Tukey test, or a Kruskal–Wallis test and a non-parametric Tukey-type multiple comparisons test; *p* < 0.05.
[a] Rhizospheric soil.
[b] Rhizospheric soil under the influence of rabbit latrines.
[c] Latrines soil.
[d] Bare soil.
[e] Mean ± standard deviation; *n* = 3.

number of viable rhizobia able to nodulate *S. supranubius*, as described in Somasegaran and Hoben (1994). Seedlings were obtained from germinated sterile seeds as follows. Given the hardness of the seed coat, a previous scarification process was necessary. Seed coats were softened in previously boiled water for 3 min, followed by chemical scarification with sulphuric acid (96%) for 35 min. The seeds were then surface-sterilized with ethanol 96% (20 s) and 50%-diluted commercial bleach (NaClO 35 g/l) for 10 further minutes. Seeds were washed six times with distilled water to remove remaining bleach and placed on agar (1%)-water plates for about five days in the dark. Germinated seeds of similar radical length were selected and transferred to conical plastic pots (5 cm × 5 cm × 5 cm) filled with sterile vermiculite. Seedlings were inoculated with 1 ml of tenfold dilutions for each type of soil (combining three soil replicates), ranging from 10^{-1} (10 g soil in 90 ml saline solution) to 10^{-6}. Four replicates were done for each dilution. The seedlings were inoculated with 1 ml of the correspondent dilution. Plants were grown in a growth chamber (16 h light/8 h darkness, room temperature) and watered with N-free Rigaud and Puppo (1975) solution for 10 weeks. After this time, the presence of nodules in each dilution was recorded, and the MPN of rhizobia was estimated following described procedures (Somasegaran and Hoben, 1994).

For the genetic analysis, the rhizobia were isolated from selected nodules from the MPN experiment were used to isolate the root nodule bacteria following standard procedures (Somasegaran and Hoben, 1994). Purified isolates colonies were stored at −80 °C in YM with 20% glycerol (*v/v*).

2.4.3. Genomic diversity and phylogenetic analyses of rhizobia

Bacterial genomic DNA was obtained from 5 to 7 days YM cultures using a commercial kit (AquaPure Genomic DNA isolation kit, Bio-Rad). The extracted DNA was quantified in a NanoDrop 1000 Spectrophotometer and normalized to 10 ng DNA/μl. The genomic diversity of the bradyrhizobial isolates was estimated from the molecular fingerprints obtained from PCR-amplification of ERIC sequences (Enterobacterial Repetitive Intergenic Conesus) following described procedure (Bruijn, 1992). GelComparII 4.0. software (Applied Maths, Belgium) with Jaccard coefficient was used to generate the UPGMA dendrogram. For phylogenetic studies, the nearly full length of the16S rRNA gene and partial sequences of the *recA* and *glnII* genes were amplified (Armas-Capote et al., 2014) and sequenced (Macrogen Inc. Spain). The phylogenetic analyses were conducted using MEGA version 7 (Kumar et al., 2016). The DNA sequences were assembled and then aligned using the Clustal W tool in the MEGA package. Phylogenetic trees were constructed using neighbor-joining (NJ) and maximum likelihood (ML) methods. NJ trees were built using Kimura's 2-parameter model. For ML tree reconstructions, the best-fitting evolutionary model of nucleotide substitutions for each type of sequence was determined using MEGA7. Robustness of the tree topology was calculated from bootstrap analysis with 1000 replications for NJ and with 500 for ML. The *nodC* genes were amplified as described (Laguerre et al., 2001). Sequences generated in this study are deposited under accession numbers MT186794 MT186813 for the 16S rRNA genes sequences; MT210561-MT210567 for *recA;* MT210553-MT210559 for *glnII*, and MT321498-MT321502 for *nodC* genes.

2.4.4. Infectivity tests

The capacity to reinfect the original legume host *S. supranubius* was tested on a selection of strains from the different bradyrhizobial branches, *B. lupini* (SSUT1, SSUT3), *B. canariense* (SSUT18, SSUT20, SSUT31 and SSUT36) and *B. rifense* (SSUT17). *Bosea* strains (SSUT16, SSUT22 and SSUT24) and *Rhizobium rhizogenes* (SSUT6) were also tested. Seeds were sterilized and germinated as explained above in Section 2.4.2. Six or seven separated plants were inoculated for each strain. A bacterial suspension (0.85% NaCl) of 5 U McFarland turbidity was used to inoculate 2–3 cm seedlings and sown in plastic pots (7 × 12 cm) filled with sterile vermiculite. Plant growth conditions were the

same as explained above in Section 2.4.2. Presence of root nodules was checked after 8 weeks, when some control plants were beginning to show decaying symptoms.

2.5. Statistics

Differences in soil parameters and rhizobia MPN between sampling sites were analysed with IBM SPSS Statistics V21.0. The normal distribution (Kolmogorov–Smirnov test) and homogeneity of variance (Levene test) of the data was checked, and statistical tests were chosen accordingly. A Kruskal–Wallis test and a non-parametric Tukey-type multiple comparisons test were used when parameters did not conform to a normal distribution and homogeneity of variance, whereas a One-way ANOVA and a Tukey or Duncan post-hoc test were used when the aforementioned conditions were confirmed. The level of significance for all tests was set to $p \leq 0.05$.

3. Results

3.1. Soil characteristics

The sampling location in an eastern area of the Teide N.P (Fig. S1) hosts a natural population of *S. supranubius* that is severely affected by rabbits (Fig. S2 E, G, H). Table 1 shows average values for chemical and physical properties of the four soil types. Clay plus silt contents account for about 46–62% of soil particles, providing loam and sandy loam textures (the latter under bare soils), with no significant differences between sites ($p > 0.05$). Soil salinity was invariably low to moderate (values comprised between 220 and 1156 μS cm^{-1} at BS and BLS, respectively). Significantly higher EC$_e$ values ($p < 0.05$) were found in BLS, probably due to the joint effect of leaching of soluble ions from rabbit excreta (deposition of faeces and urine) in latrines, as well as higher evaporation rates associated with the lack of vegetation cover. Soil pH was neutral to moderately acidic, ranging from 6.1 (RLS) to 6.8 (BS). It was generally lower under latrine influence.

Organic carbon and total N contents were in general low, with no soils exceeding 14.8 and 1.8 g kg^{-1}, respectively. These levels are typical of arid and semiarid regions with sparse vegetation and low biomass production (Díaz et al., 2011). Both organic C and total N levels were significantly higher in rhizospheric soils, RS and RLS ($p < 0.05$), suggesting the rhizosphere under *S. supranubius* is a soil organic matter source, and probably the associated BNF is a soil N source. Also interesting was the fact that total N in BLS was double that in BS, confirming the significant amounts of N provided by rabbit's depositions. However, total N amounts in RLS were similar to, but did not exceed those in RS soils (Table 1).

Differences in soil ammonium contents among soil types were not statistically significant ($p > 0.05$), probably due to the high variability found in some sample groups, particularly in samples under latrines, where the variation coefficient was as high as 50%, which could be explained by the variability in the unknown number and dates of rabbit depositions at each point. Similarly, soil available P contents showed no significant differences among sites ($p > 0.05$). However, it is worth pointing out that the highest P levels were found in bare soils and especially under latrines, whereas the lowest average values appeared in rhizosphere soils, regardless of the presence of latrines. Plant uptake arises as a probable explanation for this. However, since rhizosphere samples are expected to be biologically more active, as supported by their respective organic C and total N contents, biological immobilization and/or sequestration of phosphorus should not be disregarded (Frossard et al., 2000).

3.2. Rhizobial population sizes

The number of rhizobia able to nodulate *S. supranubius* at several experimental times (different years and seasons) from the four types of soil samples, jointly with soil N content, are displayed in Table S1 and averages of all these experiments in Fig. 1. Generally, MPNs of rhizobia were highly variable for all soil types, with great fluctuations of up to one or two orders of magnitude between spring and autumn. Despite these fluctuations, non-rhizospheric BS had the lowest rhizobial counts, while the largest counts were always found in RS, without latrines. Samples of RS were 30 to 50 fold higher than all the bare soils, both with (BLS) or without (BS) presence of latrines. These results reflect the expected positive effects of the host legume on populations of compatible rhizobia. Regarding RLS, however, the MPN counts were, on average, one order of magnitude smaller than in RS (Table S1, Fig. 1), lowering it on average to levels closer to the BS and BLS. In this respect, latrines seem to neutralize the positive effects of the host plant on rhizosphere-nodulating rhizobial numbers.

3.3. Culture characteristics and genomic diversity of the root nodule bacteria

Nodules from trap plants used in the MPN experiment in autumn 2016 were used to isolate the bacteria. Plants grown in BS had no more than one nodule per plant with ineffective phenotypes (small and white), and hence, were not selected for rhizobia isolation. In the BLS nodulation was also scarce (1, exceptionally 2, nodules per plant) and only two functional nodules (pink) were chosen for rhizobia isolation. Instead, plants grown in RS and RLS produced 1–6 nodules per plant and so, most of the root-nodule bacteria were isolated from these soils (Table S2). A total of 33 isolates were finally obtained, which were morphological differentiated into several groups, according to their colony appearance and growth rates when growing on YMA medium plates at 28 °C. Most of the isolates (22 strains) formed colonies 0.5–1.0 mm in diameter after 7 days of incubation, consistent with slow-growing bradyrhizobia. One strain (SSUT10) showed a moderate-growth rate. Six other (SSUT16, SSUT22, SSUT23, SSUT24, SSUT26 and SSUT38) formed colonies 1 mm in diameter after 3–5 days, which were slightly mucoid, translucent and iridescent. Isolates SSUT6, SSUT21, SSUT25 and SSUT27 grew like fast-growing rhizobia, producing highly mucoid colonies with diameter ranging between 3 and 4 mm after 3–5 days of incubation.

Genomic diversity of the bradyrhizobial isolates estimated from ERIC-PCR fingerprints showed highly diverse patterns (Fig. S3), suggesting that each isolate was a distinct strain. From these profiles, twelve slow-growing strains (bradyrhizobial-like phenotypes) and eight moderate/fast-growing strains (non-bradyrhizobial) were selected for sequencing the 16S rRNA genes.

3.4. Phylogeny analysis from housekeeping gene sequences

By sequencing the 16S rRNA gene, the twenty selected strains were classified at the genus level and their closest species determined. As phylogenies constructed with the NJ and ML methods showed the same groupings, only the NJ tree is included in the text showing at the nodes bootstrap values for NJ/ML, respectively. Results revealed that the root nodules of *S. supranubius* were occupied by bacteria of genera *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* and *Bosea*. However, these genera are not equally represented in the nodules, most of them belonging to genus *Bradyrhizobium*. The 12 slow-growing strains belonged to genus *Bradyrhizobium* (Fig. 2). Ten strains clustered within the *B. canariense/B. lupini* clade, eight of them have sequences more similar to *B. canariense* BTA-1^T (99.8–99.9%) and another two strains to *B. lupini* USDA3051^T (99.8–99.9%). Two other bradyrhizobial strains (Fig. 2) showed sequences highly similar to *B. cytisi* CTAW11^T (99.4–99.5%) and clustered in a branch close to this species. Eight strains belonged to three other genera (Fig. 3). Four strains belong to genus *Bosea* (99.9%–99.3% sequence similarity). The species *B. thiooxidans* DSM 9653^T was the closest species for three *Bosea* strains (Fig. 3) while one strain was highly similar to *B. vestrisi* 34635^T and
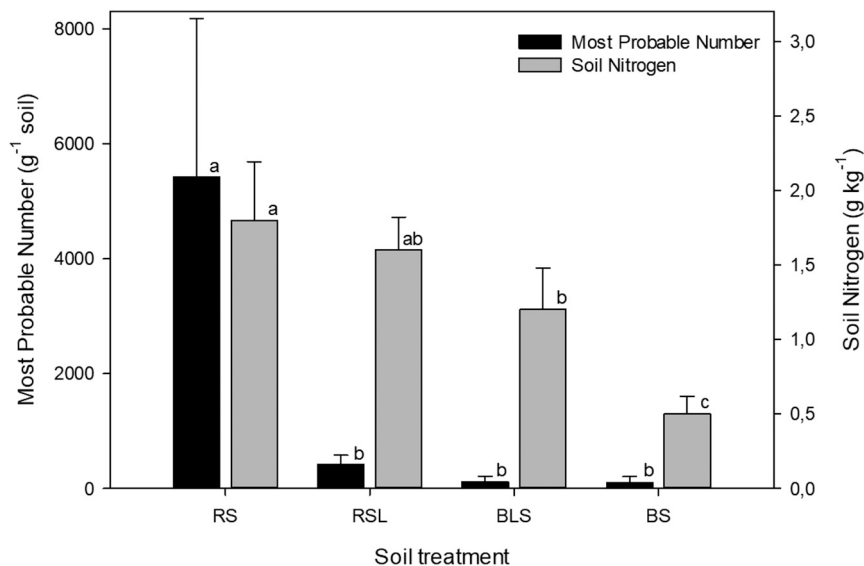
Fig. 1. Averages values of the experiments of the Most probable number (MPN) of *Spartocytisus supranubius* Rhizobia and Nitrogen content of the four type of samples sites in Teide National Park soils. RS, Rhizospheric soil; RLS, Rhizospheric soil under the influence of rabbit latrines; BS, Bare soil; BLS, Bare soil with latrines. Significant groups are indicated by different lowercase letters ($p < 0.05$).

B. eneae 34614[T]. One strain (SSUT10) belonged to genus *Mesorhizobium* and its closest relative was *M. sediminum* YIM M12096[T]. Finally, three strains have almost identical sequences (99.9% similar) to *Rhizobium rhizogenes* IFO 13257[T] (formerly *Agrobacterium rhizogenes*) (Flores-Félix et al., 2020).

Due to the poor taxonomic resolution of the ribosomal phylogeny, seven bradyrhizobial strains, five from the *B. canariense/B. lupini* clade (SSUT1, SSUT3, SSUT18, SSUT20, SSUT36) and the two from the *B. cytisi/B. rifense* group (SSUT17 and SSUT40) were selected for sequencing two protein-coding genes: *recA* and *glnII*. The individual trees of these genes are presented in Fig. S4a and Fig. S4b. The concatenation of these two genes, Fig. 4, produced a more robust phylogeny. A highly supported clade contained five strains within the clade *B. canariense/B. lupini*: two of them (SSUT1 and SSUT3) grouped closer to *B. lupini* (98.6–98.7% similarity) in a 99% supported branch and they are likely to belong to this species. The other three (SSUT18, SSU20 and SSUT36) were closer to the *B. canariense/B. lupini* clade, with which they share sequences similarity values above 97% (97.2–97.4% with *B. lupini* and 97.0–97.3% with *B. canariense*). This makes it difficult to conclude whether they belong to one or another species or represent a sister species within this lineage. In fact, species *B. canariense* and *B. lupini* are hard to distinguish based on multilocus sequence analysis, whereas both species are easily distinguished through genomic DNA–DNA hybridization (Peix et al., 2015). Finally, strains SSUT17 and SSUT40 have *B. cytisi* CTAW11[T] and *B. rifense* CTAW71[T] as their closest species (94.1% and 95.7–96.1%, sequence similarity, respectively). These values are lower than those shared between several already recognized bradyrhizobial species (*e.g. B. americanum* and *B. daqingense* type species are 97.56%; *B. diazoefficiens* USDA 110 and *B. japonicum* USDA 6[T] 97.44% and *B. canariense* BTA-1 and *B. lupini* USDA 3051[T] share 96.78%).

3.5. Symbiotic characteristics

Nodulation was only observed with the seven bradyrhizobial strains tested (SSUT1 SSUT3, SSUT18, SSUT20, SSUT36, SSUT17, SSUT40), which produced 5–8 nodules per plant on average. No differences among the inoculated bradyrhizobial strains were observed in this infectivity test (Fig. S5), though a larger experiment will be needed to determine putative differences in symbiotic effectiveness. No nodules were produced by the *Bosea* strains or *R. rhizogenes* (nor were hairy roots observed in plants infected with this species). This suggests that these

strains are not true rhizobia but are likely endophytes co-inhabiting the root nodule with a true *S. supranubius* rhizobium. Indeed, some of them were isolated from a nodule together with a *Bradyrhizobium*. These were the cases for *Rhizobium* sp. SSUT6 and *Bradyrhizobium* sp. SSUT7, and for *Bosea* sp. SSUT16 and *Bradyrhizobium* sp. SSUT17 (Table S2). However, for the other *Bosea* and *Rhizobium* isolates a true rhizobial strain could not be obtained from the root nodules and only non-nodulating endophytes were recovered on YMA plates.

Amplification of the nodulation *nodC* gene was successful for the five of the bradyrhizobial strains. They have highly similar sequences (96.03–100% similar) that place them within symbiovar genistearum (Fig. S6). However, several attempts to amplify the *nodC* gene for *Bosea* sp. failed, which correlates with its inability to nodulate (De Meyer and Willems, 2012; Sazanova et al., 2019).

4. Discussion

4.1. Effect of rabbit depositions on N-content and rhizobial counts in soils of the Teide high mountain ecosystem

The current floristic composition of the summit shrub vegetation in the Teide National Park is greatly threatened. Changes in the balance of dominant plant species of this alpine ecosystem are evident, resulting in a rapid decrease in the population of the keystone species, *S. supranubius* (Cubas et al., 2018; Ibarrola-Ulzurrun et al., 2019; Martín-Esquivel et al., 2020). In this fragile ecosystem, nitrogen fixation associated with the symbiosis between the rhizobia and that legume is assumed to be the main input of N (Wheeler and Dickson, 1990; Cubas et al., 2018). In addition to affecting ecosystems by altering richness and diversity of plant species (Irl et al., 2014; Cubas et al., 2018, 2019), rabbits may affect the soil structure (Eldridge and Simpson, 2002) and composition (Cubas et al., 2018) as well as the soil microbial community composition (Eldridge et al., 2016). Therefore, we hypothesized that the rhizobial soil populations could also be disturbed, which might eventually affect the BNF associated with the Teide broom. The fact that rhizospheric soils had the largest N and C contents reveals the positive influence of the nitrogen-fixing plant *S. supranubius* on the underlying soil. This is in agreement with Cubas et al. (2018) who showed the positive effect on C and N contents inside exclosure plots with higher density of *S. supranubius*. Latrines had a clear effect, increasing the total amounts of total N in bared, non-vegetated soils. Total N values in latrine soils
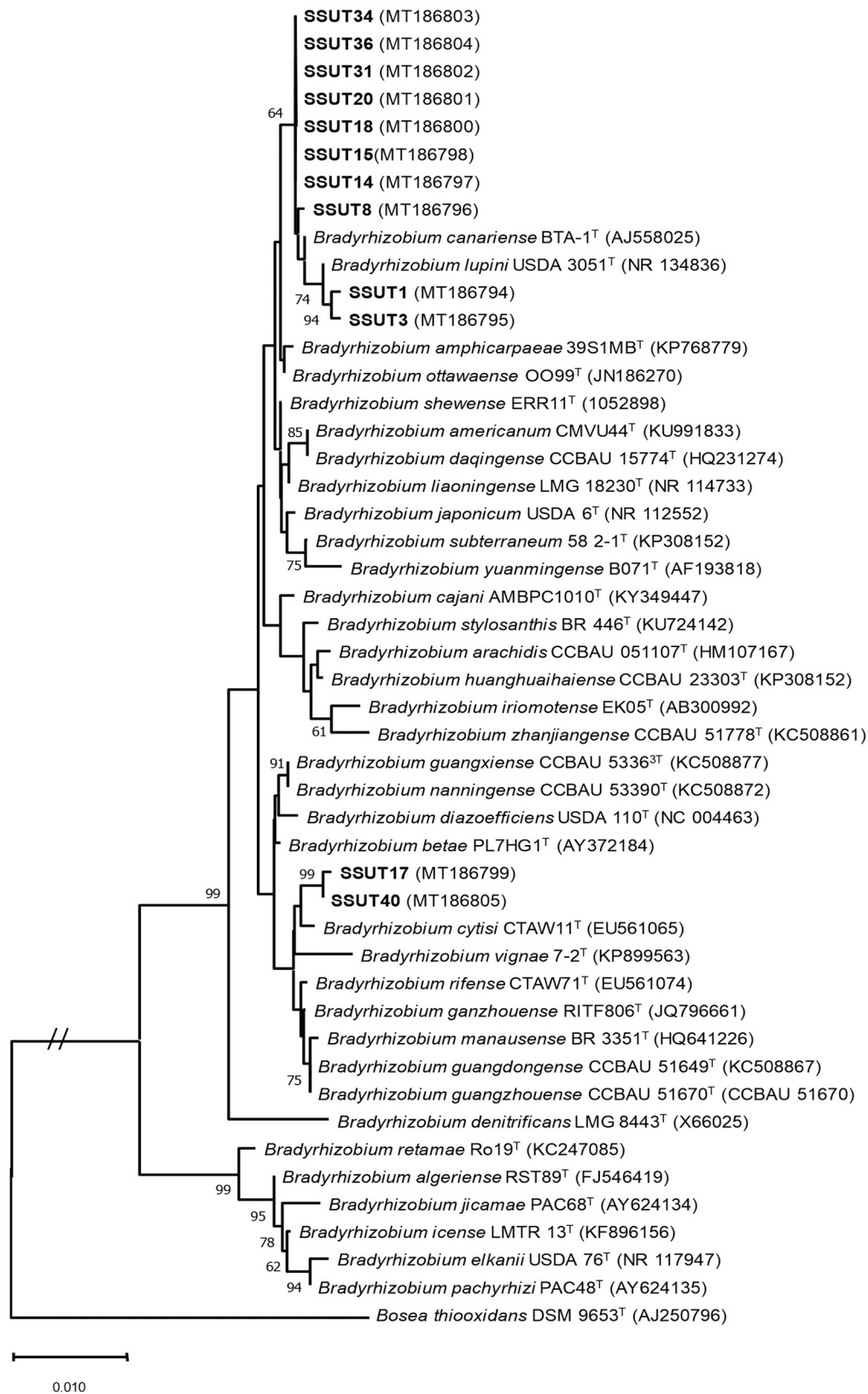
SSUT34 (MT186803)
SSUT36 (MT186804)
SSUT31 (MT186802)
SSUT20 (MT186801)
SSUT18 (MT186800)
SSUT15(MT186798)
SSUT14 (MT186797)
SSUT8 (MT186796)
Bradyrhizobium canariense BTA-1[T] (AJ558025)
Bradyrhizobium lupini USDA 3051[T] (NR 134836)
SSUT1 (MT186794)
SSUT3 (MT186795)
Bradyrhizobium amphicarpaeae 39S1MB[T] (KP768779)
Bradyrhizobium ottawaense OO99[T] (JN186270)
Bradyrhizobium shewense ERR11[T] (1052898)
Bradyrhizobium americanum CMVU44[T] (KU991833)
Bradyrhizobium daqingense CCBAU 15774[T] (HQ231274)
Bradyrhizobium liaoningense LMG 18230[T] (NR 114733)
Bradyrhizobium japonicum USDA 6[T] (NR 112552)
Bradyrhizobium subterraneum 58 2-1[T] (KP308152)
Bradyrhizobium yuanmingense B071[T] (AF193818)
Bradyrhizobium cajani AMBPC1010[T] (KY349447)
Bradyrhizobium stylosanthis BR 446[T] (KU724142)
Bradyrhizobium arachidis CCBAU 051107[T] (HM107167)
Bradyrhizobium huanghuaihaiense CCBAU 23303[T] (KP308152)
Bradyrhizobium iriomotense EK05[T] (AB300992)
Bradyrhizobium zhanjiangense CCBAU 51778[T] (KC508861)
Bradyrhizobium guangxiense CCBAU 5336[3T] (KC508877)
Bradyrhizobium nanningense CCBAU 53390[T] (KC508872)
Bradyrhizobium diazoefficiens USDA 110[T] (NC 004463)
Bradyrhizobium betae PL7HG1[T] (AY372184)
SSUT17 (MT186799)
SSUT40 (MT186805)
Bradyrhizobium cytisi CTAW11[T] (EU561065)
Bradyrhizobium vignae 7-2[T] (KP899563)
Bradyrhizobium rifense CTAW71[T] (EU561074)
Bradyrhizobium ganzhouense RITF806[T] (JQ796661)
Bradyrhizobium manausense BR 3351[T] (HQ641226)
Bradyrhizobium guangdongense CCBAU 51649[T] (KC508867)
Bradyrhizobium guangzhouense CCBAU 51670[T] (CCBAU 51670)
Bradyrhizobium denitrificans LMG 8443[T] (X66025)
Bradyrhizobium retamae Ro19[T] (KC247085)
Bradyrhizobium algeriense RST89[T] (FJ546419)
Bradyrhizobium jicamae PAC68[T] (AY624134)
Bradyrhizobium icense LMTR 13[T] (KF896156)
Bradyrhizobium elkanii USDA 76[T] (NR 117947)
Bradyrhizobium pachyrhizi PAC48[T] (AY624135)
Bosea thiooxidans DSM 9653[T] (AJ250796)

0.010

Fig. 2. Neighbor-joining (kimura 2-p) phylogenetic tree based on the 16S rRNA gene (1235 positions) of *Spartocytisus supranubius* root nodule bradyrhizobia and reference type strains of genus *Bradyrhizobium* (after checking our sequence in the EzBiocloud database, www.ezbiocloud.net, against all currently described *Bradyrhizobium* species, more distant species were eliminated from the final tree). Numbers at the nodes are neighbor-joining and maximum likelihood bootstrap support values from 1000 and 500 replications, respectively. Only BS values greater than 60% are indicated. Bar, 1 substitution per 1000 nucleotides. Accession numbers are shown (in parenthesis).
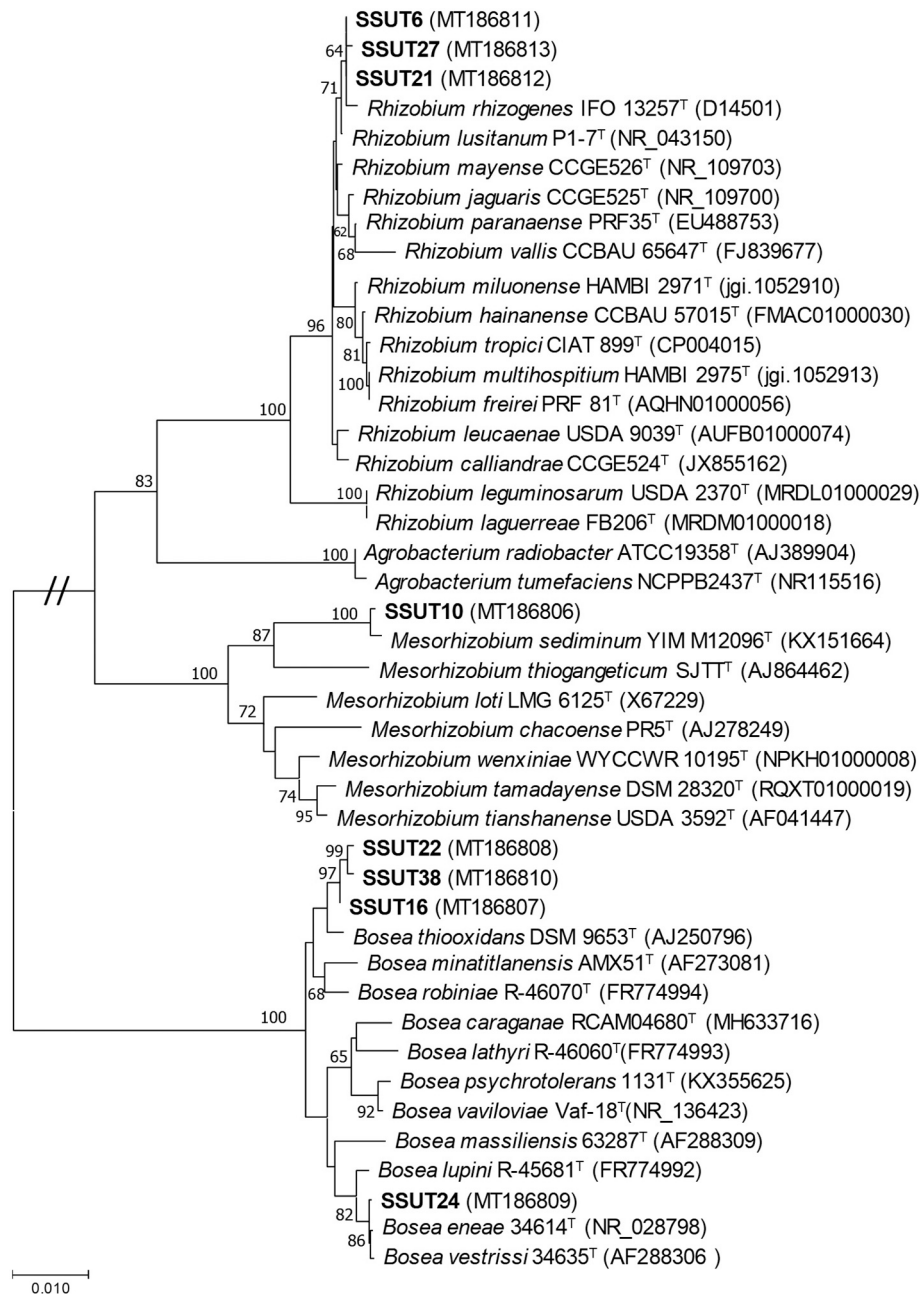
Fig. 3. Neighbor-joining (kimura 2-p) phylogenetic tree based on the 16S rRNA gene (1265 positions) of *Spartocytisus supranubius* root nodule endophytes and type strains of the closest species. Bootstrap confidence levels were derived from 1000 replications (values greater than 60% are indicated at the internodes). Bar, 1 substitution per 1000 nucleotides. Accession numbers are shown in parenthesis.

nearly doubled the amounts in BS (Table 1), which is likely due to urea addition and increases in the C input into the soil from rabbit excreta (Zhang et al., 2019). On the other hand, we measured similar amounts of N in rhizospheric soils RLS and RS. However, this N could come from various sources in these soils. Since RS soils received no rabbit influence, most if not all N in these soils must have originated from the N-fixing legume-rhizobia symbiosis. In contrast, it is reasonable to think that a significant portion of the total N in RLS samples derives from rabbits, similar to LS. It is difficult to discern what portion of the N quantified in RLS actually derives from rabbit depositions, but it would not be a minor amount considering differences between BLS and BS. Thus, it is likely that latrines do have the effect of reducing the amounts of N derived from nitrogen fixation by *S. supranubius*. Fixed nitrogen, particularly nitrates but also ammonia, can inhibit both nodulation and nitrogen

fixation in legumes (Streeter, 1988; Patriarca et al., 2002). Accurate methods like natural ^{15}N abundance would be needed to determine how latrines contribute to the N nutrition of *S. supranubius*, including a possible inhibition of fixation. It will be also interesting for future research to study whether latrines affect root growth and structure, as can be inferred from Cubas et al. (2018). This would indirectly influence development, abundance and nodule number.

Given that rhizobial numbers were counted by the MPN method in three years and two seasons, we consider the average to be quite reliable, even if the MPN of rhizobia was estimated from a combination of three soil replicates. As expected, the lowest numbers of rhizobia were in soils lacking vegetation, both without (BS) and with (BLS) presence of latrines. The opposite was observed for rhizospheric soils, specially protected areas without rabbits (RS), which had the highest rhizobial
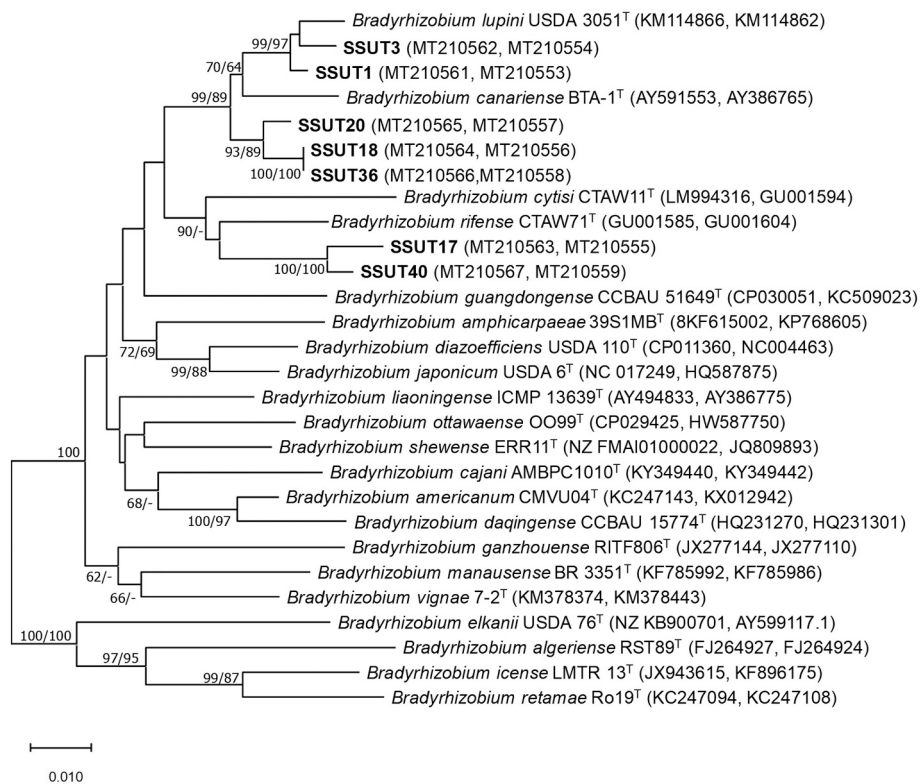
Bradyrhizobium lupini USDA 3051ᵀ (KM114866, KM114862)

SSUT3 (MT210562, MT210554)

SSUT1 (MT210561, MT210553)

Bradyrhizobium canariense BTA-1ᵀ (AY591553, AY386765)

SSUT20 (MT210565, MT210557)

SSUT18 (MT210564, MT210556)

SSUT36 (MT210566, MT210558)

Bradyrhizobium cytisi CTAW11ᵀ (LM994316, GU001594)

Bradyrhizobium rifense CTAW71ᵀ (GU001585, GU001604)

SSUT17 (MT210563, MT210555)

SSUT40 (MT210567, MT210559)

Bradyrhizobium guangdongense CCBAU 51649ᵀ (CP030051, KC509023)

Bradyrhizobium amphicarpaeae 39S1MBᵀ (8KF615002, KP768605)

Bradyrhizobium diazoefficiens USDA 110ᵀ (CP011360, NC004463)

Bradyrhizobium japonicum USDA 6ᵀ (NC 017249, HQ587875)

Bradyrhizobium liaoningense ICMP 13639ᵀ (AY494833, AY386775)

Bradyrhizobium ottawaense OO99ᵀ (CP029425, HW587750)

Bradyrhizobium shewense ERR11ᵀ (NZ FMAI01000022, JQ809893)

Bradyrhizobium cajani AMBPC1010ᵀ (KY349440, KY349442)

Bradyrhizobium americanum CMVU04ᵀ (KC247143, KX012942)

Bradyrhizobium daqingense CCBAU 15774ᵀ (HQ231270, HQ231301)

Bradyrhizobium ganzhouense RITF806ᵀ (JX277144, JX277110)

Bradyrhizobium manausense BR 3351ᵀ (KF785992, KF785986)

Bradyrhizobium vignae 7-2ᵀ (KM378374, KM378443)

Bradyrhizobium elkanii USDA 76ᵀ (NZ KB900701, AY599117.1)

Bradyrhizobium algeriense RST89ᵀ (FJ264927, FJ264924)

Bradyrhizobium icense LMTR 13ᵀ (JX943615, KF896175)

Bradyrhizobium retamae Ro19ᵀ (KC247094, KC247108)

0.010

Fig. 4. Neighbor-joining (kimura 2-p) based on concatenated *recA* (405 positions) and *glnII* (495 positions) sequences showing the relationship of *Spartocytisus supranubius* root nodule bradyrhizobia and reference type strains of genus *Bradyrhizobium*. Numbers at the nodes are neighbor-joining and maximum likelihood bootstrap support values from 1000 and 500 replications, respectively. Only BS values greater than 60% are indicated. Bar: 1 substitution per 200 nucleotide positions.

counts. The numbers of rhizobia in the broom rhizosphere could be considered low if compared to rhizopheric soils from other legumes (Mendes and Bottomley, 1998), while more similar to the rhizosphere of non-legumes (Depret et al., 2004). However, similar or even lower numbers of rhizobia have also been scored for legume rhizospheres (Irisarri et al., 2019). Nevertheless, our MPN data should be taken as minimum numbers of rhizobia in the rhizosphere of *S. supranubius*, as the protected status of this species limits and conditions the sampling. It must be considered that due to its current threat status (Martín-Esquivel et al., 2020) juvenile individuals must not be disturbed. Therefore, we sampled the nearby soil 0-30 cm from main roots, avoiding any damage to the root system. This could have caused a 'rhizosphere dilution effect' in our samples, since part of the soil collected was not attached to a root. As such, our 'rhizospheric soil' is used *sensu lato,* referring to soil in the vicinity of the legume roots. A very interesting result was that the number of nodulating rhizobia in the rhizosphere was significantly decreased by the presence of latrines (RLS), as compared to undisturbed RS samples (Table S1). The nearly 10-fold reduction observed in the rhizobial numbers in RLS may be determinant for the successful establishment and efficacy of the symbiosis, particularly in less favourable periods like autumn when the calculated rhizobial cell numbers can fall to just a few hundred or even less, per g of soil. Moreover, latrines appear not only to have a negative effect on the amounts of nodulating rhizobia but might also affect the genetic diversity of the rhizobial populations, as discussed below.

4.2. The diversity of Spartocytisus supranubius root-nodule bacteria

In the Canary Islands, only bradyrhizobial strains have been found nodulating Genistea of genera *Chamaecytisus*, *Adenocarpus* and *Teline* (syn. *Genista*) (Jarabo-Lorenzo et al., 2000; Vinuesa et al., 2005). More recent studies have also clarified that strains of genus *Bradyrhizobium* are the dominant rhizobia nodulating legumes of the tribe Genisteae (Stepkowski et al., 2018). Our results show that *S. supranubius* is nodulated by species of genus *Bradyrhizobium* and that strains belonging to

the lineage *B. canariense/B. lupini* are the main microsymbionts for *S. supranubius*. Another minor group of strains represents a potential new species close to the *B. cytisi/B. rifense* lineage. Interestingly, these strains were only found in soils under latrine influence, which suggest that bradyrhizobial species diversity is affected by rabbits.

Some changes in soil physicochemical properties under rabbit latrines (Table 1) could positively affect some species, but others negatively. Zhang et al. (2019) found that urea addition, even though improving microbial biomass, significantly decreased the soil bacterial diversity. Hydrolysis of urea is known to increase soil pH (Cabrera et al., 1991), but the subsequent nitrification reactions act as a sink for alkalinity (Tarre and Green, 2004) and therefore may induce medium- or long-term decreases in soil pH. Dorta-Almenar et al. (2015) observed sharp, linear increases in water-soluble nitrate concentrations nine months after a wildfire in Teide NP with a heavy NH_4^+ load from ashes, together with a decrease in soil pH values. In our case, soil pH values were generally lower under latrines, and these changes could negatively affect acidophilic species like *B. canariense* (Vinuesa et al., 2005; Stepkowski et al., 2018). Additionally, higher saline concentrations (EC_e) at latrines sites (significantly higher in BLS), can also (and negatively) affect bradyrhizobia from the *B. canariense/B. lupini* lineage, which are unable to grow at 1% NaCl or higher (Vinuesa et al., 2005; Peix et al., 2015). A larger survey including several zones of the NP will be needed to reach more definitive conclusions.

Although the nodules from the various soils selected to isolate the rhizobia had similar appearance (pink nodules), bradyrhizobia strains were scarcely isolated from areas under the influence of rabbits. Instead, several non-nodulating bacteria were also recovered from the crushed-nodule on the YMA plates. The isolation of non-nodulating bacteria from root nodules is a quite common event. Great diversity of bacteria belonging to several genera have been detected with and without the co-presence of a rhizobium (Wang et al., 2006; Lorite et al., 2010; Murugesan et al., 2010; Sánchez et al., 2014; Zgadzaj et al., 2015; Martínez-Hidalgo and Hirsch, 2017; Kalita et al., 2020; Soares et al., 2020). In our study, strains of genus *Bosea* stood out with up to six isolations. *Bosea* is a

member of family *Bradyrhizobiaceae* (now family *Nitrobacteriacea*, Tindal, 2019). Five out of the eleven species currently described, *Bosea lupini, Bosea lathyri, Bosea robiniae* (De Meyer and Willems, 2012), *Bosea vaviloviae* (Safronova et al., 2015) and *Bosea caraganae* (Sazanova et al., 2019), have been isolated from root nodules of diverse legumes. From some nodules only non-nodulating endophytes could be recovered. Since these nodules had a functional appearance (pink colouration), our unsuccessful recovery of the N-fixing bradyrhizobia could have been due to the faster growth on the plates of the endophyte, overgrowing the slow-growing bradyrhizobia and complicating their isolations, as reported for other legumes nodulaed by bradyrhizobia (Soares et al., 2020). It is also possible that these endophytes co-exist together with non-culturable rhizobia as demonstrated in wild legumes (Muresu et al., 2008). Further work will be needed to investigate this possibility by using culture independent methods based on the use of high-throughput sequencing technologies (Lu et al., 2017; Muresu et al., 2019). It is notable that our *Bosea* strains were only isolated from root nodules of plants growing in soils under the influence of latrines, which would support a positive effect of latrines on endophyte populations, favouring nodule invasion. This influence might have been caused by changes in the relative abundance between rhizobia and other opportunistic soil bacteria (Sánchez et al., 2014).

5. Conclusion

In conclusion, the results from this study suggests that in addition to the direct effect of rabbits by herbivory on Teide brooms, there might be an indirect effect by rabbit latrines perturbing the soil rhizobia populations and eventually the capacity of this species to fix atmospheric nitrogen. This would be another serious, additional consequence of rabbit overpopulation on this native broom's competence to thrive in this harsh environment. Given the pioneering role of *S. supranubius* as a nitrogen fixer, either a decline in the population or a decline of its nitrogen fixing capacity would be detrimental to the persistence of the whole ecosystem. Seedling inoculation with symbiotically-efficient rhizobia has proved to be very successful in the conservation of the endangered legume *Lupinus mariejosephae* (Navarro et al., 2014). A similar microbiological strategy would be required in current conservation campaigns in the Teide alpine ecosystem. Our rhizobial collection of *S. supranubius* microsymbionts is a starting point for further symbiotic effectiveness studies, in order to select highly efficient strains for field trials.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could influence the work reported in this paper.

Acknowledgments

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.apsoil.2020.103850.

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