Ecoregion—Rather Than Sympatric Legumes—Influences Symbiotic Bradyrhizobium Associations in Invasive Scotch Broom (Cytisus scoparius) in the Pacific Northwest

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Ecoregion—Rather Than Sympatric Legumes—Influences Symbiotic *Bradyrhizobium* Associations in Invasive Scotch Broom (*Cytisus scoparius*) in the Pacific Northwest

Abstract

Plant-microbe mutualisms can determine the success of invasive plants. Legumes (Fabaceae) are particularly successful invaders in a variety of habitats. This is partly due to their ability to access atmospheric nitrogen through microbial mutualists (rhizobia) in their root systems, which allow them to colonize a wide variety of disturbed or nutrient-poor habitats. While many plant-rhizobia mutualisms are highly species-specific, plant promiscuity with different species of rhizobia can significantly enhance the success of invasive legumes, since the availability of suitable rhizobial mutualists in a new geographic area may serve as a limiting factor. Scotch broom (Fabaceae: *Cytisus scoparius*) is one of the most problematic invasive legumes in the Pacific Northwest (PNW), yet very little is known about the Scotch broom-rhizobia system. We explored the rhizobial communities of root nodules of Scotch broom and sympatrically occurring legumes across three major ecoregions (coast, valley, and mountain) in the western PNW (Washington, Oregon, and California) to better understand the Scotch broom-rhizobia system in nature. We found that bradyrhizobia are the exclusive rhizobial mutualists of Scotch broom but that there is promiscuity at the species level. While there was very little overlap with rhizobial communities of sympatric native and naturalized legumes, ecoregion did influence the species composition of Scotch broom-associated rhizobial communities. Our findings suggest that Scotch broom is not reliant on sympatric legumes to provide a source of suitable rhizobial mutualists, but instead forms spatially variable associations with a range of other bradyrhizobia.

Keywords: Plant-microbe interactions, rhizobia, invasion ecology, mutualism, biodiversity

Introduction

Invasive species are one of the greatest threats to biodiversity and ecosystem stability worldwide (Orth et al. 2006, Butchart et al. 2010, Vila et al. 2011). When a plant species becomes invasive, trophic interactions in the invaded range can be disrupted (Bennett 2013, Kautz et al. 2017) and ecosystem functions may be altered, resulting in widespread cascading effects (Ehrenfeld 2003, Brooks et al. 2004, Godschalx et al. 2015). In addition to ecological consequences, such changes in ecosystem function can have dramatic negative economic effects (Pimentel et al. 2005). Apart from physiological, morphological, or life history traits that directly determine competitiveness, plant-microbe mutualisms are often critical factors determining the success of an invasive plant (Traveset and Richardson 2014, Klock et al. 2015). These mutualisms bolster invader success by providing the host plant with increased access to limiting nutrients (Abelson 1985, Biswas et al. 2000), by increasing the frequency of pollinator visits (Gange and Smith 2005), or by affecting host plant physiology in competitively advantageous ways, namely by increasing growth rate and improving resistance to pathogens (Berg 2009).

Scotch broom (Fabaceae: *Cytisus scoparius* (L.) Link) is a highly invasive legume in western North America that is native to western and central Europe (Lee 2010). First introduced to the North American west coast as an ornamental in the 1850s, it was subsequently used by the United States Department of Agriculture for sand dune stabilization and the prevention of soil erosion in the 1940s, and has since established an alarming foothold across the Pacific Northwest (PNW; Washington, Oregon, California, and Idaho) (Hulting et al. 2008, Lee 2010). Scotch broom

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is a strong competitor in novel ranges where it spreads swiftly, suppressing native vegetation and forming dense monospecific stands (Peterson et al. 1998, Hulting et al. 2008). Scotch broom causes significant economic losses by outcompeting planted seedlings of Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) and other commercially important conifers in the PNW (Zielke et al. 1992, Peterson et al. 1998, Slesak et al. 2016). Due to its strong performance on heavily disturbed soils, Scotch broom readily invades a variety of anthropogenically affected habitats such as logged forests, roadsides, and over-grazed pastures (Hulting et al. 2008). The highly invasive habit of Scotch broom has earned it noxious weed status across the PNW and Hawaii, such that effective management and removal of the species currently requires a costly, labor-intensive arsenal of tactics that range from wrench-pulling and high-temperature controlled burns, to chemical applications and biological control via introduced granivorous insects. The state of Oregon alone spends an estimated $40 million annually on management costs and lost timber revenue, while Washington State loses an estimated $58.7 million to direct impacts on livestock and timber, as well as a projected $142.8 million in business activities per year (according to the Economic Impact of Invasive Species Report [Community Attributes Inc., 2017]).

A number of known factors contribute to the success of Scotch broom. Among them are its rapid growth rate, year-round photosynthesis, extreme fecundity, and the long-term survivability of its seeds in the seed bank (Hulting et al. 2008). However, comparably little is known about the belowground microbial associates of Scotch broom and the potential ecological outcomes that such associations might underpin. Like other members of the family Fabaceae, Scotch broom has a competitive advantage in nutrient-poor and degraded soils due to its ability to fix atmospheric nitrogen via symbiotic bacteria (rhizobia) in root nodules (Allen and Allen 1981, Andrews et al. 2013). The biological process of transforming free-living soil bacteria into root nodule-forming, nitrogen-fixing symbionts involves a staggeringly complex array of signal transduction pathways and specificity factors, which lead to a high degree of host specificity among plant-rhizobia interactions (Hirsch et al. 2001, Rogel et al. 2011, Andrews and Andrews 2017). Bacteria in the genus *Bradyrhizobium* are considered the primary symbionts of Papilionoideae legumes, but genera in the tribe Genisteae—including Scotch broom—show a propensity for promiscuity at the species level (Allen and Allen 1981, Andrews and Andrews 2017). Subsequently, symbiotic promiscuity has been hypothesized to be important for their colonization of new territory (Parker 2001).

The availability of suitable microbial mutualists in an exotic range (or lack thereof) can limit the spread of invasive legumes (Richardson et al. 2000, Callaway et al. 2011) regardless of symbiotic promiscuity. Rodriguez-Echeverria et al. (2012) showed that the source of symbiotic rhizobia for legumes growing in new geographic areas might come from the co-introduction of exotic rhizobia with their exotic host plants. Alternatively, Parker et al. (2006) demonstrated that Scotch broom can take advantage of novel rhizobia strains that are provided by native or sympatric legumes in the vicinity, but it appears that symbiont-overlapping with neighboring legumes might not be the norm (La Pierre et al. 2017). Additionally, geographic variation—and its associated selection effects on the evolution of symbiosis—has long been hypothesized to be a driver of the observed biogeographic patterns of legume–rhizobia mutualisms (Lie et al. 1987, Parker 1999, Martinez-Romero 2009). Nevertheless, much of the information regarding Scotch broom–rhizobia mutualisms has been derived from field studies and greenhouse experiments in countries other than the United States (Weir et al. 2004, Lafay and Burdon 2006, Zurdo-Pineiro et al. 2007, Rodriguez-Echeverria et al. 2009, Chahboune et al. 2011), leaving the status of suitable rhizobia symbionts and potential spatial patterns in the PNW unexplored.

Here, we conducted a field study in the westernmost extent of the PNW, from Washington State to northern California. This portion of the PNW is dominated by three primary ecoregions: the marine coast and Coast Range, valleys and lowlands, and the montane forests of the Cascade Range. Among these regions, elevations range from

sea level to 3,050 m, and precipitation regimes vary from 89 cm to 610 cm annually. Considering that soil bacterial communities can be influenced by abiotic factors such as elevation (Bryant et al. 2008), precipitation, and pH (Richter et al. 2018, Wang et al. 2018), as well as by anthropogenic pressure (Regar et al. 2019) and community-level biotic interactions (Nielsen et al. 2012), such ecoregional factors may contribute to the structure of Scotch broom-rhizobia mutualisms in the PNW. Since mutualistic interactions can determine the invasion success of exotic species in their introduced range (Richardson et al. 2000, Parker et al. 2006, van der Putten et al. 2007, Nunez et al. 2009, Pringle et al. 2009, Rodrigues-Echeverria et al. 2009, Traveset and Richardson 2014), and we currently lack a clear understanding of why certain species are more invasive than others (Parker 2001, Lockwood et al. 2007, Richardson et al. 2000), we sought to characterize the rhizobia symbionts found in root nodules of invasive Scotch broom across the PNW. Specifically, we asked the following questions: 1) does Scotch broom show promiscuity with rhizobia mutualists; 2) is there overlap between Scotch broom-associated rhizobia genotypes and those associated to sympatric legumes; and 3) do nodulating rhizobia exhibit ecoregional spatial patterns?

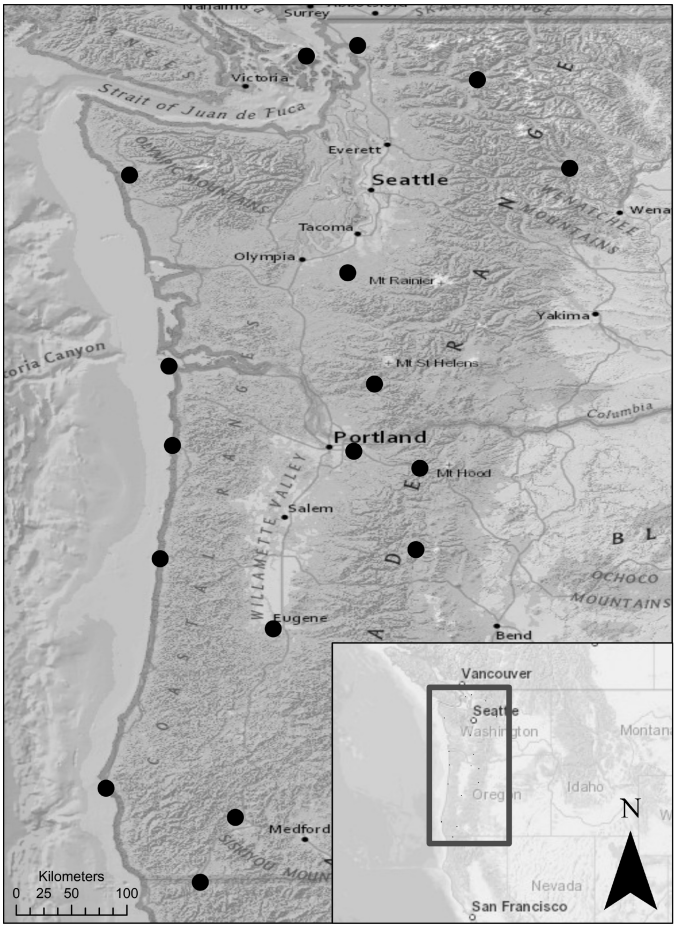

Figure 1. Locations of the 17 sample sites for Scotch broom in the Pacific Northwest, from northern Washington to northern California (north to south: 48°47'30"N, 122°26'46"W to 41°58'43"N, 123°43'25"W), and the coast to the Cascade Range (west to east: 45°31'35"N, 123°57'10"W to 47°47'27"N, 120°43'16"W).

Methods

Study Site

Nodulated root segments were sampled 14 May to 04 November 2018 from 17 sites across western Washington, Oregon, and California (Figure 1 and Table 1). Each site was categorized as one of three PNW ecoregions: coast, valley, and mountain (US Environmental Protection Agency 1986). Characterized by variable topography and precipitation (152 to 610 cm annually), the coast region is dominated by dense conifer forests of the Olympic Mountains, marine coast, and coastal plain. The relatively dry (89 to 127 cm annual rainfall) valley region, generally bordered by the Coast Range in the west and the Cascade Range in the east, is comprised of the Puget Lowlands in Washington and the Willamette Valley in Oregon. Situated in a rain shadow, and supporting an agricultural mosaic of croplands and livestock, the topography is dominated by low hills (< 610

TABLE 1. GPS coordinates and ecoregion categories of sample sites in the western Pacific Northwest.

Site location	Site location code	Ecoregion	Coordinates (lat, long)
Portland, OR	A	valley	45°29'16"N, 122°28'32"W
Bay Ocean Peninsula, OR	B	coast	45°31'35"N, 123°57'10"W
Mt. Hood, OR	C	mountain	45°20'56"N, 121°56'22"W
Olympic Peninsula, WA	D	coast	47°44'08"N, 124°18'01"W
Grants Pass, OR	E	mountain	42°30'25"N, 123°26'24"W
Hwy 199, CA	F	mountain	41°58'43"N, 123°43'25"W
Port Orford, OR	G	coast	42°44'36"N, 124°29'30"W
Astoria, OR	H	coast	46°10'53"N, 123°58'51"W
Cougar, WA	I	mountain	46°04'01"N, 122°11'59"W
Roy, WA	J	valley	46°56'26"N, 122°31'31"W
Bellingham, WA	K	valley	48°47'30"N, 122°26'46"W
Marblemount, WA	L	mountain	46°56'26"N, 122°31'31"W
Leavenworth, WA	M	mountain	47°47'27"N, 120°43'16"W
Orcas Island, WA	N	valley	48°42'15"N, 122°51'45"W
Newport, OR	O	coast	44º37'08"N, 124º02'54"W
Eugene, OR	P	valley	44º03'07"N, 123º05'12"W
Detroit, OR	Q	mountain	44º41'12"N, 121º58'15"W

m elevation) in the north and generally flat (30 to 91 m elevation) floodplains in the south. The mountain ecoregion is comprised primarily of the western Cascade Mountain Range and includes the Klamath Mountains at the southern reach of the study area. Average annual precipitation of the west Cascade Range (sea level to 3,050 m elevation) varies from 127 to 254 cm, and the densely forested region supports timber production. The Klamath Mountains of southern Oregon and northern California lie to the southwest of the Cascade Range and have a mild Mediterranean climate (Sleeter and Calzia 2012).

Sampling

Root segments were collected from at least five Scotch broom plants at each site (excluding Orcas Island where only one plant was found; $n = 84$). Individuals sampled were no closer than 10 m apart. When available, as many as three native or naturalized sympatric legumes per Scotch broom sample (up to 5 m distance from the Scotch broom plant) were also collected ($n = 41$). Sympatric legumes included deervetch (*Hosackia crassifolia* Benth. var. *crassifolia*), peavines (*Lathyrus latifolius* L. and *Lathyrus japonicus* Willd.), lupines (*Lupinus bicolor* Lindl., *Lupinius littoralis* Douglas ex Lindl. var. *littoralis,* and *Lupinus polyphyllus* Lindl.), medic (*Medicago lupulina* L.), clovers (*Trifolium pratense* L., *Trifolium repens* L. and *Trifolium wormskioldii* Lehm.), and vetches (*Vicia sativa* L., and one other *Vicia* sp.) (Table 2). Hitchcock and Cronquist (2018) was used for plant identification and nomenclature. Root segments (10 to 30 cm) were excavated and pruned using a gardening knife and scissors. Samples were stored in plastic sandwich bags (Ziploc®) in a cooler at 4 °C for up to 72 hours before processing.

Surface Sterilization

Root segments with nodules still attached were rinsed under ddH_2O for 20 seconds. Nodules were excised and placed into scintillation vials. Surface sterilization was conducted with a series of 2-minute incubations on a shaker at 160 rpm, and consisted of the following wash steps: two consecutive washes in 5-mL 0.625% NaClO, followed by three consecutive rinses in 5-mL sterile ddH_2O. Surface-sterilized nodules were transferred to 1.5-mL Eppendorf tubes and stored at –80 °C until DNA extraction.

DNA Extraction and Sequencing

DNA was extracted from nodules using the Sigma Extract-N-Amp kit (Sigma-Aldrich, Germany) with the following modifications to the manufacturer's protocol. Nodules were ground until homogenous with 50 µL of the kit's extraction solution in 1.5-mL centrifuge tubes using sterile micropestles; 50 µL of the resulting homogenate

TABLE 2. Sympatric legumes of Scotch broom in the Pacific Northwest, by sample site and accession number, and the operational taxonomic units (OTUs) of their associated rhizobia.

Site location	Site location code	Sample accession	Legume species	OTU	Rhizobia species
Bay Ocean Peninsula, OR	B	B1N1	*Lathyrus japonicus*	6	*Rhizobium* sp.
		B2N1	*Lathyrus japonicus*	25	*Rhizobium* sp.
		B3N1	*Lathyrus japonicus*	27	*Rhizobium leguminosarum*
		B4N1	*Vicia* sp.	20	*Rhizobium leguminosarum*
		B5N1	*Lupinus littoralis* var. *littoralis*	9	*Bradyrhizobium* genosp.
		B6N1	*Trifolium wormskioldii*	21	*Rhizobium leguminosarum*
Mt. Hood, OR	C	C5N1	*Medicago lupulina*	28	*Rhizobium leguminosarum*
		C5N2	*Vicia sativa*	6	*Rhizobium* sp.
		C6N1	*Medicago lupulina*	14	*Rhizobium leguminosarum*
		C6N2	*Trifolium wormskioldii*	5	*Rhizobium leguminosarum*
		C6N3	*Vicia sativa*	30	*Rhizobium* sp.
		C7N1	*Vicia sativa*	31	*Rhizobium* sp.
		C7N2	*Medicago lupulina*	32	*Rhizobium* sp.
Hwy 199, CA	F	F4N1	*Hosackia crassifolia* var. *crassifolia*	35	*Bradyrhizobium* sp.
Port Orford, OR	G	G5N1	*Lupinus littoralis* var. *littoralis*	8	*Bradyrhizobium canariense*
Astoria, OR	H	H1N1	*Vicia sativa*	19	*Rhizobium leguminosarum*
		H2N1	*Vicia sativa*	19	*Rhizobium leguminosarum*
		H3N1	*Vicia sativa*	7	*Rhizobium leguminosarum*
		H4N1	*Vicia sativa*	14	*Rhizobium leguminosarum*
		H4N2	*Trifolium repens*	7	*Rhizobium leguminosarum*
		H5N1	*Vicia sativa*	7	*Rhizobium leguminosarum*
		H5N2	*Lathyrus japonicus*	38	*Rhizobium* sp.
Cougar, WA	I	I3N1	*Lathyrus latifolius*	5	*Rhizobium leguminosarum*
		I4N1	*Lathyrus latifolius*	12	*Bradyrhizobium canariense*
Bellingham, WA	K	K1N2	*Lathyrus latifolius*	7	*Rhizobium leguminosarum*
		K2N1	*Lupinus bicolor*	3	*Bradyrhizobium* sp.
		K3N1	*Trifolium pratense*	20	*Rhizobium leguminosarum*
		K4N1	*Lupinus bicolor*	40	*Mesorhizobium* sp.
		K4N2	*Lathyrus latifolius*	7	*Rhizobium leguminosarum*
		K5N1	*Lupinus bicolor*	41	*Mesorhizobium* sp.
Marblemount, WA	L	L3N1	*Trifolium repens*	14	*Rhizobium leguminosarum*
Leavenworth, WA	M	M1N1	*Lupinus polyphyllus*	8	*Bradyrhizobium canariense*
		M1N2	*Medicago lupulina*	42	*Sinorhizobium medicae*
		M2N1	*Lathyrus japonicus*	6	*Rhizobium* sp.
		M2N2	*Medicago lupulina*	5	*Rhizobium leguminosarum*
		M3N1	*Medicago lupulina*	6	*Rhizobium* sp.
		M4N1	*Trifolium pratense*	10	*Bradyrhizobium* sp.
		M4N2	*Medicago lupulina*	5	*Rhizobium leguminosarum*
		M5N1	*Trifolium pratense*	6	*Rhizobium* sp.
		M5N2	*Lathyrus japonicus*	5	*Rhizobium leguminosarum*
Newport, OR	O	O5N1	*Trifolium pratense*	5	*Rhizobium leguminosarum*

was transferred to 0.2-mL tubes and heated at 65 °C for 10 min, then at 95 °C for 10 min, then cooled to 10 °C using a BIO RAD T100™ (Hercules, CA) thermal cycler; 50 µL of the kit's neutralization solution was added to each sample and the samples were vortexed and centrifuged; 60 µL of the supernatant was reserved as polymerase chain reaction (PCR) template. Primers TSglnIIf (AAGCTCGAGTACATCTGGCTCGACGG) and TSglnIIr (SGAGCCGTTCCAGTCGGTGTCG) were used on the glnII locus (Vinuesa et al. 2005) with the following PCR protocol: 95 °C (2 min); 34 cycles at 94 °C (45 sec), 58 °C (30 sec), 72 °C (1.5 min); and 72 °C (7 min). PCR reaction mixtures were based on the GoTaq® (Promega Corp., Madison, WI) master mix with a final ratio of 1.00:0.05:0.50:0.50 (master mix: BSA:TSglnIIf:TSglnIIr); 1 µL of 1:100 DNA template was added to 24 µL of the reaction mixture. Amplicons were Sanger sequenced by Functional Biosciences (Madison, WI). Forward and reverse reads were visually inspected, trimmed by hand, and assembled using Geneious v10.2.3 (Auckland, New Zealand). Assemblies were clustered into operational taxonomic units (OTUs) and BLAST searched in the National Center for Biotechnology Information (NCBI) database with 97% similarity used to determine rhizobia species.

Operational Taxonomic Units and Phylogenetics

Operational taxonomic units were created in Geneious v10.2.3 using the MUSCLE aligner on forward and reverse contigs (≈ 636 bp [base pairs]) and de novo assembling with 99% overlap. They were aligned using MAFFT v.7.388 and a maximum likelihood tree was created using the GTR GAMMA model of RAxML v8.2.1 (1000 bootstrap replicates). The final tree was produced in R v3.6.0 using the package "ggtree" (Yu et al. 2017).

Statistics

All statistical analyses were performed using R v3.5.1 (R Core Team 2019). Data were analyzed with the "vegan" and "indicspecies" packages (De Cáceres and Legendre 2009, Oksanen et al. 2017). Non-metric multidimensional scaling (NMDS) ordinations with Bray-Curtis distances were used to visually compare community composition between host plants (*C. scoparius* vs. sympatric legumes) and among ecoregions. Permutational multivariate analyses of variance (PERMANOVA) with Bray-Curtis distances and 999 permutations were used to determine statistical differences in community composition. One extreme outlier (OTU35) was excluded from analyses as it was a singleton isolated from one nodule of a sympatric legume at the single California sample site. Indicator species analysis was performed to determine whether particular taxa were significantly associated with *C. scoparius* or with specific ecoregions.

Results

Consistent with our expectations, we found that bacteria in the genus *Bradyrhizobium* were the exclusive rhizobial symbionts of Scotch broom, but that Scotch broom was promiscuous at the species level. While symbiont community composition was not significantly different between Scotch broom plants and sympatrically occurring legumes, ecoregion classification did affect community composition (PERMANOVA $F = 1.5789$, $r = 0.12072$, $P = 0.028$).

Overall, a total of 45 rhizobia operational taxonomic units (OTUs) were isolated from root nodules of Scotch broom and sympatric legumes, with 23 found only in nodules of Scotch broom, 18 found only in nodules of sympatric legumes, and four found in both (Table 3). Of the OTUs that associated exclusively with Scotch broom, 16 were identified (NCBI) as *Bradyrhizobium* sp. or genosp., three as *Bradyrhizobium japonicum*, three as *Bradyrhizobium lupini*, and one as *Bradyrhizobium canariense* (found in a total of 67, 5, 6, and 6 plants respectively; Table 4). OTUs 1 and 2 (*Bradyrhizobium* genosp.) were the most commonly occurring and widespread Scotch broom-associated genotypes, found in all three ecoregions and at nine and eight of the 17 sample sites, respectively (Figure 2). Scotch broom-associated OTUs were represented by diverse lineages of the genus *Bradyrhizobium*, clustering into five distinct clades (Figure 3).

TABLE 3. Rhizobia operational taxanomic units (OTUs) isolated from *Cytisus scoparius* (c), sympatric legumes (s) or both (b) with site location(s)[a] in the western Pacific Northwest. Rhizobia species identities are according to the top BLAST hit.

OTU	Rhizobia sp.	BLAST hit	Site location code	Host association (c/s/b)
1	*Bradyrhizobium* genosp.	FJ970382	B,D,F,H,I,J,O,P,Q	c
2	*Bradyrhizobium* genosp.	FJ970382	B,C,D,H,I,J,L,O	c
3	*Bradyrhizobium* sp.	LN907826	A,K,L,N,P	b
4	*Bradyrhizobium* sp.	LN907826	A,C,K,L,M	c
5	*Rhizobium leguminosarum*	KX486956	C,I,M,O	s
6	*Rhizobium* sp.	KX891819	B,C,M	s
7	*Rhizobium leguminosarum*	KJ923112	H,K	s
8	*Bradyrhizobium canariense*	AY386764	E,F,G,M	b
9	*Bradyrhizobium* genosp.	FJ970382	B,D,G,Q	b
10	*Bradyrhizobium* sp.	FJ391055	D,M	b
11	*Bradyrhizobium* genosp.	FJ970382	C,K,P,Q	c
12	*Bradyrhizobium canariense*	AY599104	A,F	c
13	*Bradyrhizobium japonicum*	CP017637	C,J,L	c
14	*Rhizobium leguminosarum*	KY587958	C,H,L	s
15	*Bradyrhizobium lupini*	LR027502	E	c
16	*Bradyrhizobium lupini*	LR027504	E	c
17	*Bradyrhizobium* sp.	LN907826	G	c
18	*Bradyrhizobium* sp.	MG014289	C	c
19	*Rhizobium leguminosarum*	CP001622	H	s
20	*Rhizobium leguminosarum*	KJ923104	B,K	s
21	*Rhizobium leguminosarum*	KY587958	B,I	s
22	*Bradyrhizobium* sp.	AM168365	G,K	c
23	*Bradyrhizobium lupini*	LR027502	M,Q	c
24	*Bradyrhizobium* sp.	KP830234	B	c
25	*Rhizobium* sp.	KX891821	B	s
26	*Bradyrhizobium* sp.	KP830234	B	c
27	*Rhizobium leguminosarum*	CP016286	B	s
28	*Rhizobium leguminosarum*	KY587958	C	s
29	*Bradyrhizobium japonicum*	LR027512	C	c
30	*Rhizobium* sp.	KX891820	C	s
31	*Rhizobium* sp.	KX891820	C	s
32	*Rhizobium* sp.	KX891794	C	s
33	*Bradyrhizobium* sp.	MH182980	D	c
34	*Bradyrhizobium* sp.	KY607953	F	c
35	*Bradyrhizobium* sp.	LN901633	F	s
36	*Bradyrhizobium japonicum*	CP017637	G	c
37	*Bradyrhizobium* sp.	LN901633	H	c
38	*Rhizobium* sp.	CP013643	H	s
39	*Bradyrhizobium* sp.	KM194841	J	c
40	*Mesorhizobium* sp.	HG323920	K	s
41	*Mesorhizobium* sp.	JQ885923	K	s
42	*Sinorhizobium medicae*	KP765345	M	s
43	*Bradyrhizobium* sp.	KY607937	O	c
44	*Bradyrhizobium* sp.	LN901633	O	c
45	*Bradyrhizobium* sp.	AJ891294	Q	c

[a] A = Portland, OR, B = Bay Ocean Peninsula, OR, C = Mt. Hood, OR, D = Olympic Peninsula, WA, E = Grants Pass, OR, F = Hwy 199, CA, G = Port Orford, OR, H = Astoria, OR, I = Cougar, WA, J = Roy, WA, K = Bellingham, WA, L = Marblemount, WA, M = Leavenworth, WA, N = Orcas Island, WA, O = Newport, OR, P = Eugene, OR, and Q = Detroit, OR.

TABLE 4. Sampled Scotch broom plants, by site and accession number, and the operational taxonomic units (OTUs) of their associated rhizobia.

Site location—code	Sample accession	OTU	Rhizobia species
Portland, OR—A	A1	12	*Bradyrhizobium canariense*
	A2	4	*Bradyrhizobium* sp.
	A3	3	*Bradyrhizobium* sp.
	A4	3	*Bradyrhizobium* sp.
	A5	4	*Bradyrhizobium* sp.
Bay Ocean Peninsula, OR—B	B1	24	*Bradyrhizobium* sp.
	B2	1	*Bradyrhizobium* genosp.
	B3	26	*Bradyrhizobium* sp.
	B4	2	*Bradyrhizobium* genosp.
	B5	1	*Bradyrhizobium* genosp.
	B6	2	*Bradyrhizobium* genosp.
Mt. Hood, OR—C	C1	2	*Bradyrhizobium* genosp.
	C2	11	*Bradyrhizobium* genosp.
	C3	13	*Bradyrhizobium japonicum*
	C4	4	*Bradyrhizobium* sp.
	C5	18	*Bradyrhizobium* sp.
	C6	29	*Bradyrhizobium japonicum*
	C7	18	*Bradyrhizobium* sp.
Olympic Peninsula, WA—D	D1	1	*Bradyrhizobium* genosp.
	D2	33	*Bradyrhizobium* sp.
	D3	9	*Bradyrhizobium* genosp.
	D4	10	*Bradyrhizobium* sp.
	D5	2	*Bradyrhizobium* genosp.
Grants Pass, OR—E	E1	8	*Bradyrhizobium canariense*
	E2	15	*Bradyrhizobium lupini*
	E3	16	*Bradyrhizobium lupini*
	E4	16	*Bradyrhizobium lupini*
	E5	15	*Bradyrhizobium lupini*
Hwy 199, CA—F	F1	8	*Bradyrhizobium canariense*
	F2	12	*Bradyrhizobium canariense*
	F3	1	*Bradyrhizobium* genosp.
	F4	34	*Bradyrhizobium* sp.
	F5	12	*Bradyrhizobium canariense*
Port Orford, OR—G	G1	9	*Bradyrhizobium* genosp.
	G2	22	*Bradyrhizobium* sp.
	G3	17	*Bradyrhizobium* sp.
	G4	36	*Bradyrhizobium japonicum*
	G5	17	*Bradyrhizobium* sp.
Astoria, OR—H	H1	37	*Bradyrhizobium* sp.
	H2	1	*Bradyrhizobium* genosp.
	H3	2	*Bradyrhizobium* genosp.
	H4	2	*Bradyrhizobium* genosp.
	H5	2	*Bradyrhizobium* genosp.
Cougar, WA—I	I1	1	*Bradyrhizobium* genosp.
	I2	1	*Bradyrhizobium* genosp.
	I3	1	*Bradyrhizobium* genosp.
	I4	1	*Bradyrhizobium* genosp.
	I5	2	*Bradyrhizobium* genosp.

Continued on next page

TABLE 4. – *Cont.*

Site location—code	Sample accession	OTU	Rhizobia species
Roy, WA—J	J1	2	*Bradyrhizobium* genosp.
	J2	39	*Bradyrhizobium* sp.
	J3	2	*Bradyrhizobium* genosp.
	J4	13	*Bradyrhizobium japonicum*
	J5	1	*Bradyrhizobium* genosp.
Bellingham, WA—K	K1	4	*Bradyrhizobium* sp.
	K2	4	*Bradyrhizobium* sp.
	K3	22	*Bradyrhizobium* sp.
	K4	3	*Bradyrhizobium* sp.
	K5	11	*Bradyrhizobium* genosp.
Marblemount, WA—L	L1	3	*Bradyrhizobium* sp.
	L2	2	*Bradyrhizobium* genosp.
	L3	13	*Bradyrhizobium japonicum*
	L4	3	*Bradyrhizobium* sp.
	L5	4	*Bradyrhizobium* sp.
Leavenworth, WA—M	M1	10	*Bradyrhizobium* sp.
	M2	8	*Bradyrhizobium canariense*
	M3	23	*Bradyrhizobium lupini*
	M4	10	*Bradyrhizobium* sp.
	M5	4	*Bradyrhizobium* sp.
Orcas Island, WA—N	N1	3	*Bradyrhizobium* sp.
Newport, OR—O	O1	1	*Bradyrhizobium* genosp.
	O2	2	*Bradyrhizobium* genosp.
	O3	43	*Bradyrhizobium* sp.
	O4	44	*Bradyrhizobium* sp.
	O5	2	*Bradyrhizobium* genosp.
Eugene, OR—P	P1	1	*Bradyrhizobium* genosp.
	P2	3	*Bradyrhizobium* sp.
	P3	1	*Bradyrhizobium* genosp.
	P4	1	*Bradyrhizobium* genosp.
	P5	11	*Bradyrhizobium* genosp.
Detroit, OR—Q	Q1	1	*Bradyrhizobium* genosp.
	Q2	45	*Bradyrhizobium* sp.
	Q3	11	*Bradyrhizobium* genosp.
	Q4	23	*Bradyrhizobium lupini*
	Q5	9	*Bradyrhizobium* genosp.

Rhizobia community composition did not differ significantly between Scotch broom and sympatric legume hosts (PERMANOVA, $P = 0.384$; Figure 4). However, Scotch broom associated to the same rhizobial genotypes as sympatric legumes at only two of the ten sites where both were collected (Bellingham and Leavenworth, WA). Of the OTUs that were found associated to both Scotch broom and sympatric legumes, three were *Bradyrhizobium* sp. or genosp. and one was identified as *Bradyrhizobium canariense*. At the Bellingham site, OTU3 (*Bradyrhizobium* sp.) associated to one *Lupinus bicolor* individual and one Scotch broom individual, but they were not immediate neighbors (i.e., *L. bicolor* was more than 5 m away from the Scotch broom plant). At the Leavenworth, WA site, OTU8 (*B. canariense*) associated to one *Lupinus polyphyllus* individual

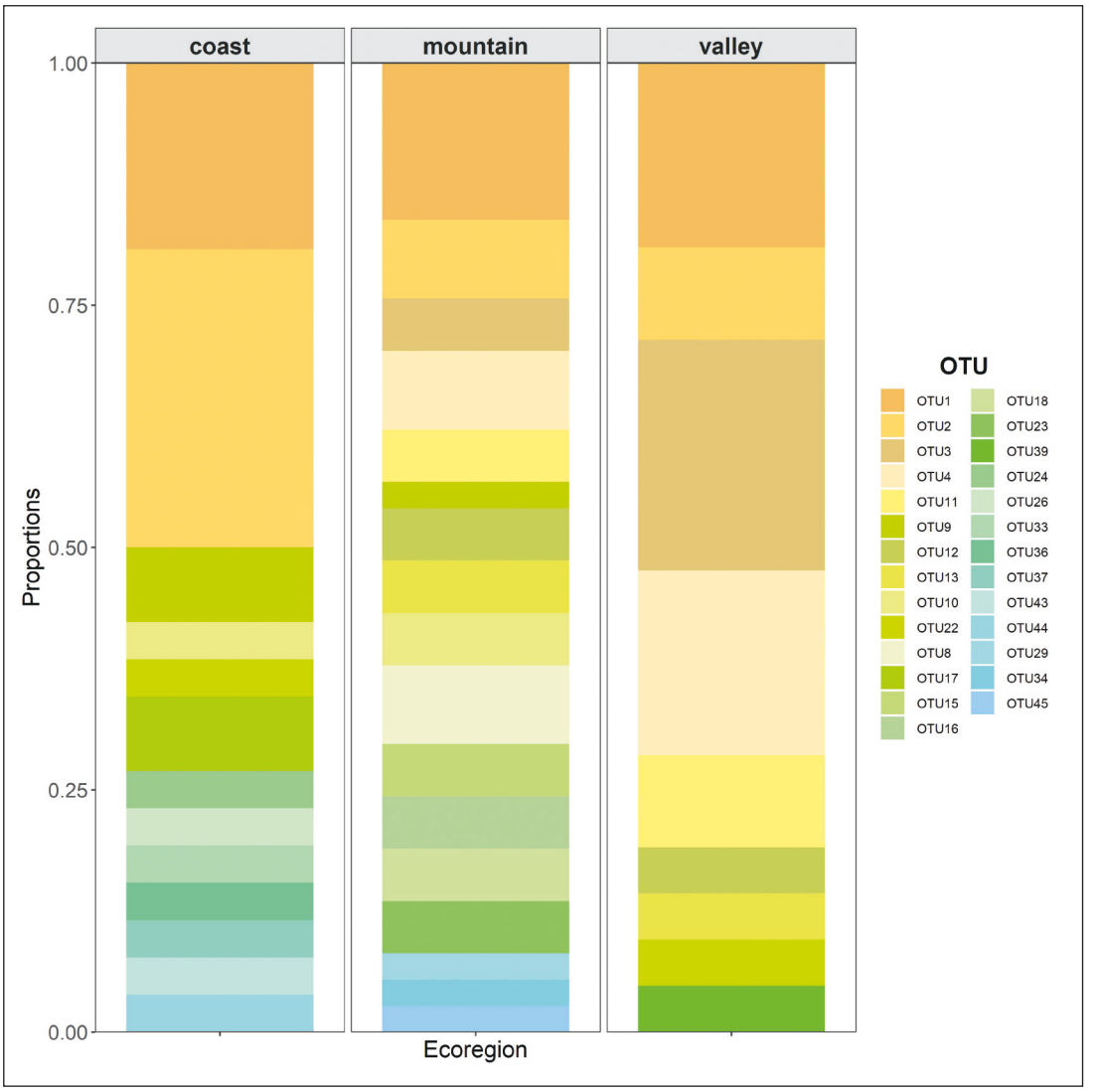

Figure 2. Proportion of Scotch broom-associated rhizobia genotypes (OTUs) by ecoregion (coast, mountain, and valley) in the western Pacific Northwest.

and one Scotch broom individual (also not immediate neighbors), and OTU10 (*Bradyrhizobium* sp.) associated to one *Trifolium pratense* individual and two Scotch broom individuals (one was an immediate neighbor and one was not; Figure 3).

While rhizobial community composition did not differ between host plants, ecoregion had a significant effect overall (PERMANOVA F = 1.5789, r = 0.12072, P = 0.028) (Figure 5). Further, there were significant differences in Scotch broom-associated rhizobia communities by ecoregion (PERMANOVA, F = 1.8633, r = 0.21023, P = 0.037) (Figure 6). OTU3 was a strong indicator for the valley ecoregion (Indicator value [IV] = 0.8397, P = 0.010), and two indicator genotypes were associated with the southern range of the study area (California): OTU12 (IV = 0.9826, P = 0.030) and OTU34 (IV = 1.000, P = 0.045).

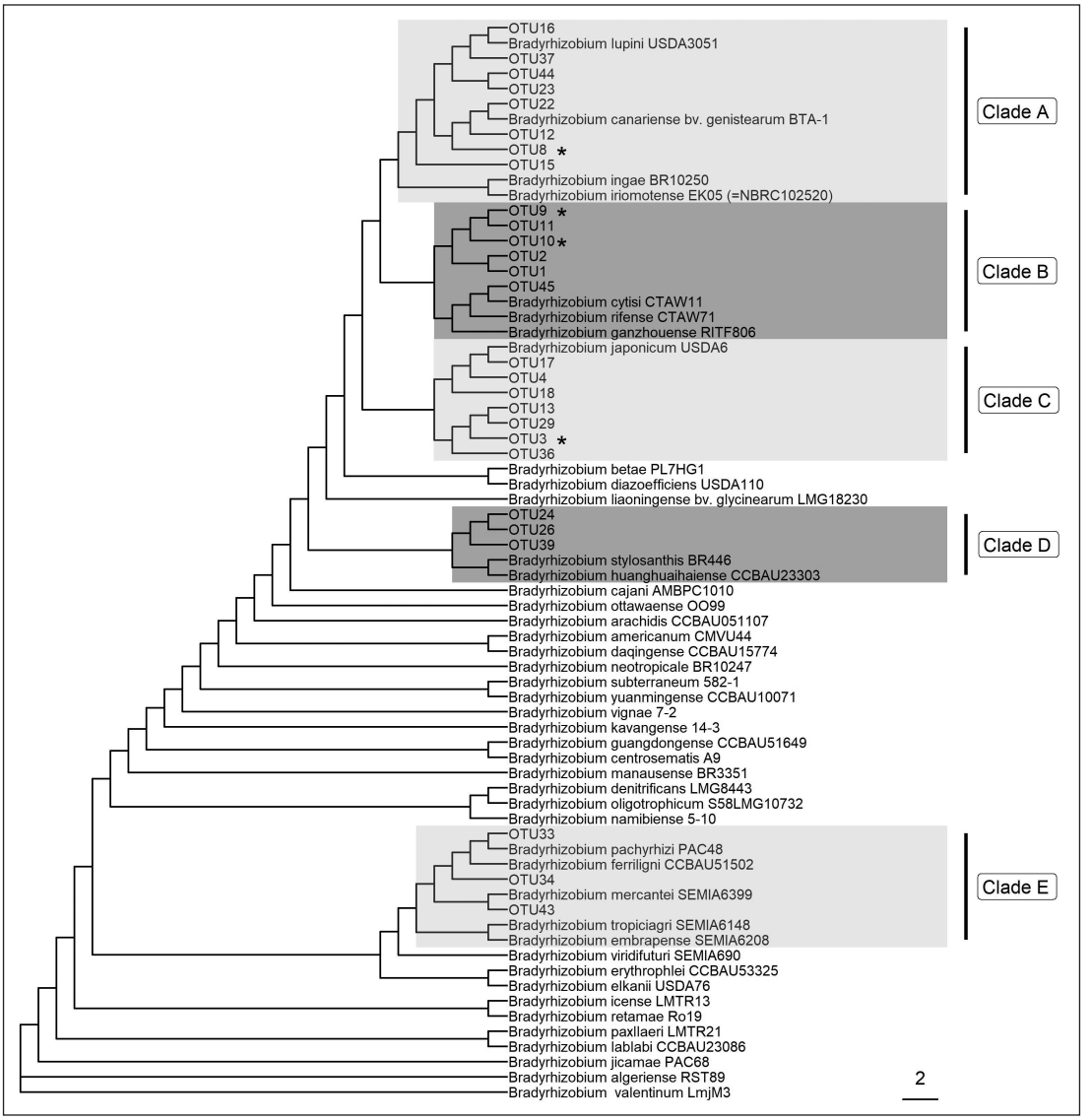

Figure 3. Maximum likelihood phylogenetic analysis of root nodule forming Scotch broom-associated rhizobia isolates (OTUs) from the western Pacific Northwest. Shaded areas show the five clades into which OTUs were grouped. Asterisks designate OTUs that were found in nodules of both Scotch broom and sympatrically occurring legumes. Numeric codes following downloaded sequences are strain designations (i.e., culture collection numbers).

Discussion

The primary objectives of our study were to characterize the rhizobial genotypes associated to Scotch broom in the PNW, to uncover whether or not Scotch broom utilizes the same rhizobia as its leguminous neighbors, and to elucidate any potential rhizobial community spatial patterns across the three primary ecoregions of the western PNW (coast, valley, and mountains).

In line with previous studies, we found that Scotch broom associated exclusively with rhizobia from the genus *Bradyrhizobium* (Allen and Allen

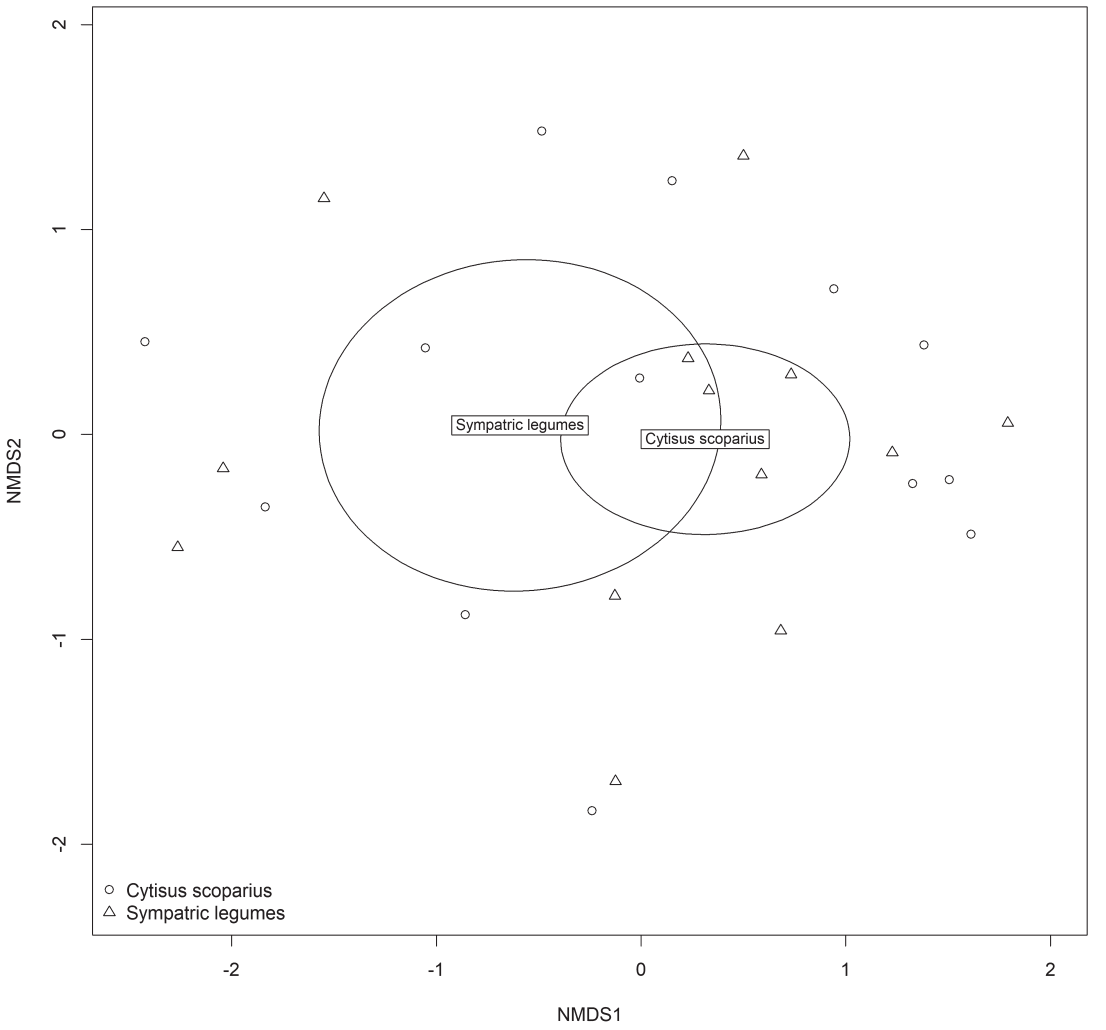

Figure 4. Non-metric multidimensional scaling ordination of rhizobial communities of Scotch broom and sympatric legumes in the western Pacific Northwest; ellipses represent 95% confidence intervals based on the standard error.

1981, Andrews and Andrews 2017, Stepkowski et al. 2018) while showing promiscuity at the species level. We identified two predominant Scotch broom-associated bradyrhizobia genotypes (OTUs 1 and 2) that occurred in all three ecoregions. Current understanding of the legume-rhizobia relationship suggests that plants are able to be selective and, when presented with options, they will establish symbioses with the microbial mutualist that confers the most benefit at the lowest cost (i.e., more atmospheric nitrogen in exchange for less photosynthate; Denison 2000, Simms et al. 2006, Heath and Tiffin 2008, Kiers and Denison 2008). This could explain our finding of two dominant rhizobial genotypes. However, at almost every site where OTUs 1 and 2 were found, additional rhizobial genotypes were observed in root nodules of individual Scotch broom host plants (with the exception of the site in Cougar, WA), implying that the structure of Scotch broom-rhizobia mutualisms may not be driven entirely by symbiotic efficiency—assuming the observed strains are not equally efficient—but by which rhizobia are available. Subsequently, Scotch broom hosts may

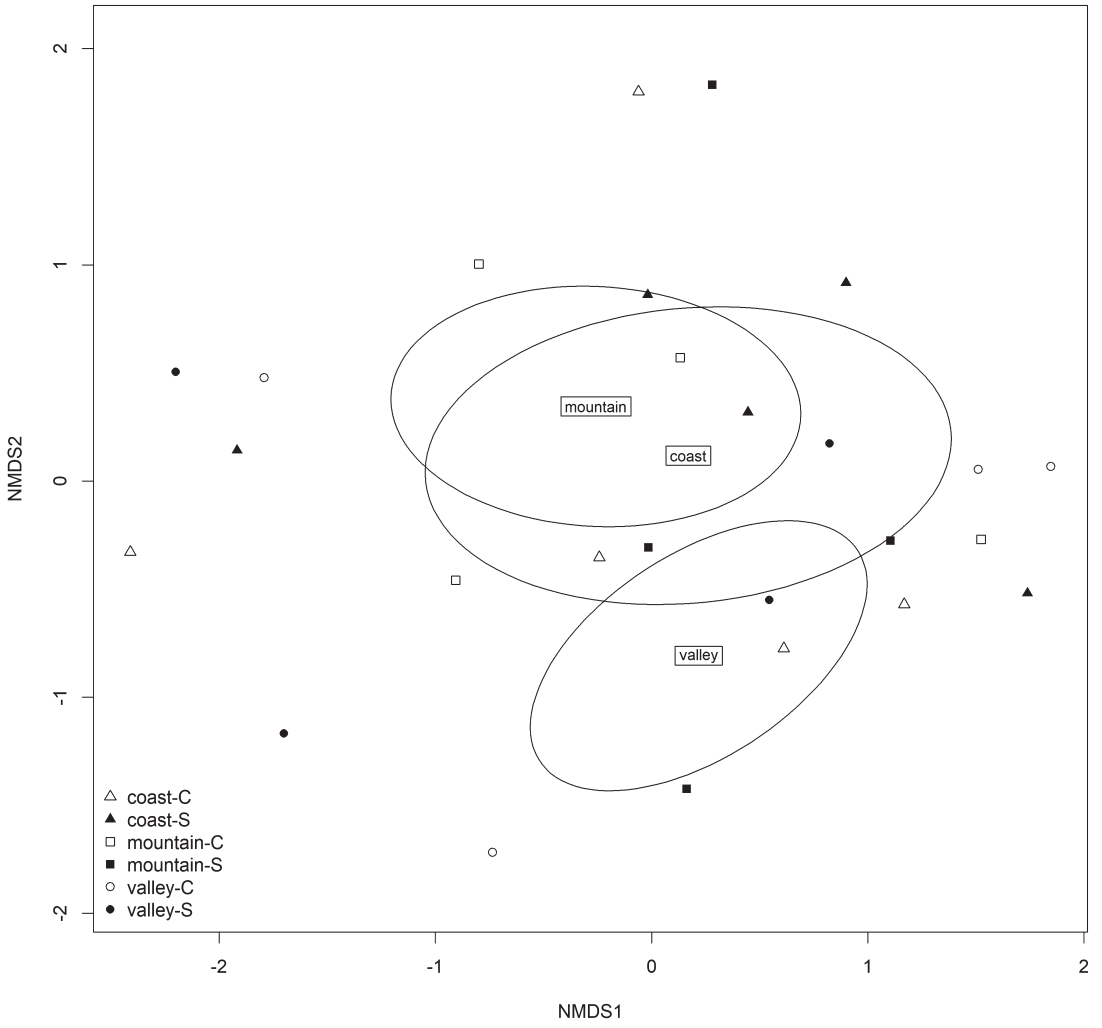

Figure 5. Non-metric multidimensional scaling ordination of rhizobial communities of Scotch broom (*Cytisus scoparius*) and sympatric legumes among three ecoregions (coast, mountain, and valley) in the western Pacific Northwest; ellipses represent 95% confidence intervals based on the standard error; *C. scoparius* (C) and sympatric (S) legumes are plotted as black/white forms of the same shapes for each ecoregion.

select less efficient rhizobial genotypes when the preferred ones are not present in the immediate rhizosphere.

In our study, sampled plants were, at minimum, 10 m apart. The mature seed pods of Scotch broom are spirally dehiscent and can eject seeds up to 1 to 2 m distance from the parent plant (Hulting et al. 2008). Thus, it is possible that the host plants that associated to less common OTUs were genetically different from those that associated to OTUs 1 and 2 at any given site. Successful nodulation of a particular rhizobial strain has been shown to be strongly influenced by the host plant's genotype (Wilkinson et al. 1996, Depret and Laguerre 2008, Godschalx et al. 2017, Nelson et al. 2017). Furthermore, Van Cauwenberghe et al. (2016) and Vuong et al. (2017) demonstrated that the structure of rhizobial communities is not only affected by host plant genotype but by an

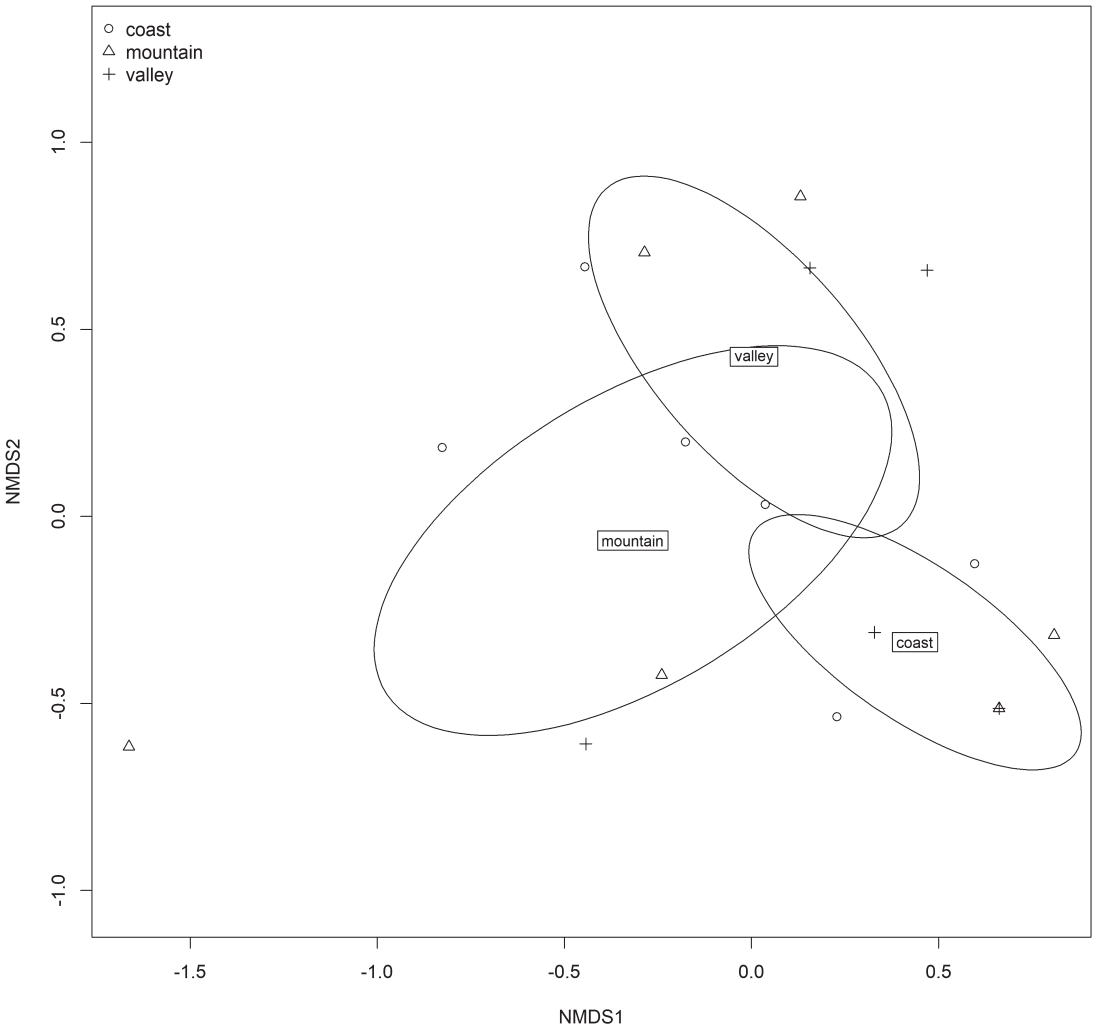

Figure 6. Non-metric multidimensional scaling ordination of nodule-forming rhizobial communities of Scotch broom among three ecoregions (coast, mountain, and valley) in the western Pacific Northwest; ellipses represent 95% confidence intervals based on the standard error.

interaction between genotype and environmental factors such as soil nitrogen, phosphorous, and water content. However, in the Scotch broom-rhizobia system, the exact reason for variation and diversity in rhizobial communities remains to be uncovered.

Our study revealed that Scotch broom was able to form associations with the same rhizobial genotypes as sympatric legumes, but usually did not. Many of the sympatric legumes collected in this study associated to rhizobia in the genus *Rhizobium*. Across the PNW, rhizobial communities of Scotch broom and other legumes were not different from each other, but at any given site it was rare to find them sharing symbionts of the same genotype. Contrary to findings by Parker et al. (2006), but in line with more recent studies conducted by Rodriguez-Echeverria et al. (2012) and La Pierre et al. (2017), our findings suggest that competition for rhizobia between Scotch broom and sympatric legumes is minimal, and

that Scotch broom is not reliant on leguminous neighbors to provide a source of suitable rhizobia mutualists. While the origins and invasive status of the Scotch broom-associated OTUs found in this study are unknown, this finding might provide support for the theory that exotic invasive legumes rely primarily on the co-introduction of compatible exotic rhizobia for successful nodulation and invasive persistence.

Rhizobial community composition, both within and between Scotch broom and neighboring legumes, was significantly affected by ecoregion. To our knowledge, this has not been seen before in the Scotch broom-rhizobia system, but it is not surprising since large-scale spatial patterns are expected to emerge from both abiotic and biotic variation among regions (Bryant et al. 2008, Neilsen et al. 2012, Richter et al. 2018, Wang et al. 2018, Regar et al. 2019), as well as from co-evolution in geographically distinct legume-rhizobia populations (Parker 1999; Rodriguez-Echiverria 2009, 2012). Such ecoregion effects could be further compounded by genetic similarity of host plants in a given region.

In this study, rhizobia communities in the valley ecoregion appear distinct from those in the mountain or coast ecoregions, which strongly overlap. This distinction could arise from greater differences in soil type and pH between the valley and the other two ecoregions, due in part to the coast and mountain regions having largely volcanic geologic histories, while the valley was formed by repeated Pleistocene flooding and sedimentation (Madin 2009). Soil type and pH are known to vary across ecoregions in the PNW, with major influencers being geologic history, local plant communities, elevation, and precipitation (Franklin and Dyrness 1973). This reasoning is consistent with findings by Cao et al. (2014), who demonstrated that rhizobia communities of *Phaseolus vulgaris* differed significantly across two ecoregions in China, with differential soil type and pH.

Conclusion

Our results suggest that PNW populations of Scotch broom—while able to promiscuously associate with *Bradyrhizobium* spp.—are not reliant on the provision of suitable mutualists by sympatric legumes to persist in novel geographic locations. Further, the observed structure of Scotch broom-rhizobia mutualisms in the PNW indicates that environmental variation and host plant characteristics (and possibly an interaction between the two) are drivers of successful nodulation by specific rhizobial genotypes. Future research will need to tease apart the ecoregional factors that contribute to the formation of spatial patterns. With a rapidly growing human population, anthropogenic pressure on ecological invasions will continue to increase, and understanding plant-microbe mutualisms will be a crucial factor in mitigating the spread of invasive species and creating sound management practices.

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