Escape from bacterial diversity: potential enemy release in invading yellow starthistle (*Centaurea solstitialis*) microbiomes

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Total word count (excluding summary, references and legends):	5753	No. of figures:	7
Summary:	195	No. of Tables:	0
Introduction:	1147	No of Supporting Information files:	1
Materials and Methods:	1908		
Results:	803		
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Acknowledgements:	133		

SUMMARY

- Invasive species may benefit from introduction to new regions where they can escape their natural enemies. Here we examined whether geographic patterns of microbial community composition support a role for enemy escape in the invasion of California, USA by yellow starthistle, a highly invasive plant in western North America.
- We used high-throughput sequencing of the 16S V4 region to characterize bacterial community composition in the phyllosphere, rhizosphere, leaves, and roots of plants from seven populations in California and eight populations in the native European range. We compared bacterial diversity between the native and invaded ranges, and with previously published estimates of plant genetic diversity within each population.
- Bacterial communities differed significantly among plant compartments, and between native and invaded ranges within compartments, with consistently lower diversity in the invaded range. Plant genetic diversity did not explain this pattern in bacterial diversity, but a positive relationship was found within ranges between bacterial diversity in roots and plant genetic diversity within populations.
- Our observation of lower bacterial diversity in the invaded relative to the native range of yellow starthistle is consistent with potential enemy escape, providing some of the first evidence for this scenario in plant microbiomes.

KEYWORDS

bacteria, *Centaurea solstitialis*, endophyte, genetic diversity, invasive species, microbiome, phyllosphere, rhizosphere

INTRODUCTION

Humans continue to transport plant species around the globe, and increasing numbers of these translocations result in the invasive expansion of non-native species into recipient communities (Lonsdale, 1999; Butchart *et al.*, 2010; Essl *et*

al., 2011; Ellis *et al.*, 2012). There is a longstanding hypothesis that many species become invasive after escaping from enemies that reduce invader fitness and limit their populations in their native ranges (Darwin, 1859; Williamson, 1996). Known as the 'Enemy Release' hypothesis, this idea is highly intuitive and forms a basis for the biological control of invasive species (Keane & Crawley, 2002). Initial tests of enemy release focused on quantifying visible changes in above-ground herbivore damage (Keane & Crawley, 2002), but there has been increasing recognition that microbial enemies above- and below-ground can have large effects on plant fitness, and could thus determine whether invasive plants benefit from escaping negative species interactions (Callaway *et al.*, 2004; Colautti *et al.*, 2004; Torchin & Mitchell, 2004; Agrawal *et al.*, 2005; Mitchell *et al.*, 2006; Kulmatiski *et al.*, 2008; van der Putten *et al.*, 2013; Dawson & Schrama, 2016; Faillace *et al.*, 2017).

In recent years, microbial communities have emerged as particularly likely candidates for generating enemy release. Although many interactions between plants and microbes can be beneficial, microbial communities often appear to have negative net effects on plant fitness which may become more negative over time, e.g., via plant-soil feedbacks (Bever, 2003; Reinhart & Callaway, 2006; Kulmatiski *et al.*, 2008; Petermann *et al.*, 2008). It is now apparent that interactions between plants and their microbiomes can vary over space and environment (Nemergut *et al.*, 2013; van der Putten *et al.*, 2013; terHorst & Zee, 2016), creating opportunities for introduced plants to escape the microbial communities that characterize their native ranges. Moreover, evidence is building that reductions in microbial diversity are occurring in response to environmental change and human disturbances, and these reductions in diversity may reduce the resistance of ecosystems to invasion (Schnitzer *et al.*, 2011; Wagg *et al.*, 2014; Dawson & Schrama, 2016; van der Putten *et al.*, 2016).

Invasive plant species have provided some of the best evidence to date that microbial interactions can be locally evolved, and can vary considerably over

geographic regions (Rout & Callaway, 2012). Invaders have been shown to vary in their response to soil communities from their native and invaded ranges, and there are now many examples of more favorable interactions between plants and soil from the invaded range, consistent with escape from enemies (Reinhart *et al.*, 2003; Callaway *et al.*, 2004; Mitchell *et al.*, 2006; Engelkes *et al.*, 2008; Kulmatiski *et al.*, 2008; Maron *et al.*, 2014; van der Putten *et al.*, 2016). Plant-microbe interactions which provide net benefits to invasive species can be explained by reduced negative effects of key microbial pathogens, increased direct beneficial effects of mutualistic taxa, or increased indirect benefits from taxa that affect competitors more negatively than they do the invader (Dawson & Schrama, 2016). These mechanisms should manifest as differences in the microbial communities associated with invading vs. native plants, specifically as divergence in taxonomic composition, reduction in diversity, and/or the loss or gain of groups known to have pathogenic or mutualistic effects, where taxonomic resolution permits inference of function (Herrera Paredes & Lebeis, 2016).

Release from enemies is expected to be beneficial in and of itself, but it may further promote invasion by changing the pattern of natural selection on resource allocation by the invader (Sakai *et al.*, 2001). Plants that require reduced defenses against negative enemy interactions have the potential to adapt to invest a larger proportion of resources in traits that increase competitiveness, reproduction, and/or spread. This idea, known as the Evolution of Increased Competitive Ability (EICA) hypothesis (Blossey & Notzold, 1995) has received a great deal of attention but mixed empirical support (Maron *et al.*, 2004; Bossdorf *et al.*, 2005; Mitchell *et al.*, 2006; Felker-Quinn *et al.*, 2013). Potential contributors to evolutionary responses to enemy release are likely to become better resolved as our understanding of microbial community interactions increases, particularly since adaptive responses to microbial disease are known to be among the most rapid evolutionary changes that occur in any organism (Tiffin & Moeller, 2006; Bomblies *et al.*, 2007; Salvaudon *et al.*, 2008).

Here we conduct one of the first comparisons of plant microbiomes between invading populations and their native source region, explicitly testing for patterns consistent with enemy release (see also Gundale *et al.*, 2016). We ask whether changes in plant-associated microbial communities have the potential to generate enemy escape in the highly invasive plant yellow starthistle (*Centaurea solstitialis*). Yellow starthistle is native to a wide region of Eurasia and was introduced to South America in the 1600's and North America in the 1800's as a contaminant of alfalfa seed (Gerlach, 1997). This herbaceous annual is a colonizer of grassland ecosystems, and is often called one of the '10 Worst Weeds of the West' in North America (DiTomaso & Healy, 2007). Its extensive invasion of California in the USA (>14 million acres; Pitcairn *et al.*, 2006) is well-studied, and invading genotypes in this region have been shown to grow larger and produce more flowers than plants in the native range, suggesting an adaptive shift in resource allocation and an increase in invasiveness (Widmer *et al.*, 2007; Eriksen *et al.*, 2012; Dlugosch *et al.*, 2015). Previous research has demonstrated that yellow starthistle throughout all of its native and invaded ranges experiences net fitness reductions when grown with its local soil communities (Andonian *et al.*, 2011, 2012; Andonian & Hierro, 2011). However, these studies have also indicated that this negative interaction is weaker (more favorable) in California, raising the possibility that escape from microbial enemies has promoted this aggressive invasion.

We sample microbial communities associated with leaves (phyllosphere and endosphere) and roots (rhizosphere and endosphere) of yellow starthistle plants in both the California invasion and its source region in Europe. Previous experiments with fungicide treatments have shown that plant-soil interactions between yellow starthistle and fungi in California are *more* negative (less favorable) than those in the native range, inconsistent with a role for fungi in escape from microbial enemies (Hierro *et al.*, 2016). Here, we focus on bacterial communities as candidates for a potential role in enemy escape in this system. We use high-throughput sequencing of prokaryotic ribosomal 16S sequences to

142 quantify diversity and relative abundance of taxa in yellow starthistle
143 microbiomes, designing a novel modified peptide nucleic acid clamp (Lundberg
144 *et al.*, 2013) to reduce non-target sequencing of host plastids. We ask whether
145 there are patterns of reduced taxonomic diversity and/or potential loss of
146 pathogens in the invaded range, and whether patterns of diversity in plant-
147 associated bacteria can be explained by geographic patterns of plant genetic
148 diversity. Loss of potential pathogens would be consistent with opportunities for
149 enemy escape that could contribute to the evolution of increased invasiveness in
150 yellow starthistle's highly successful invasion of California.
151

152 **MATERIALS AND METHODS**

153 **Study species**

154 Yellow starthistle (*Centaurea solstitialis* L., Asteraceae) is an obligately
155 outcrossing annual plant, diploid throughout its range (Heiser & Whitaker, 1948;
156 Widmer *et al.*, 2007; Öztürk *et al.*, 2009). Plants form a taproot and grow as a
157 rosette through mild winter and/or spring conditions, bolting and producing
158 flowering heads (capitula) throughout the summer. The species is native to
159 Eurasia, where distinct genetic subpopulations have been identified in
160 Mediterranean western Europe, central-eastern Europe, Asia (including the
161 Middle East), and the Balkan-Apennine peninsulas (Barker *et al.*, 2017). The
162 invasion of California as well as those in South America appear to be derived
163 almost entirely from western European genotypes (Fig. 1; Barker *et al.*, 2017).
164

165 **Sample collection**

166 Fifteen populations of yellow starthistle were sampled for microbial communities:
167 seven populations across the invasion of California, six in western Europe, and
168 two in eastern Europe (Fig. 1; Supporting Information Table S1). At each
169 population, plants were sampled every meter (or to the nearest meter mark)
170 along a 25 meter transect, to yield 25 individuals per population. Individuals in
171 rosette or early bolting stages were preferentially selected. In one population
172 (HU29), low plant density yielded 20 individuals along the 25 meter transect.

173 Using sterile technique, plants were manually pulled and each individual sampled
174 for phyllosphere, rhizosphere, leaves, and roots using modified versions of
175 protocols by Lundberg *et al.* (2013) and Lebeis *et al.* (2015) as described below.
176 A control (blank) sample was collected for each population. Plants were pressed
177 and dried after sampling, and submitted to the University of Arizona Herbarium
178 (ARIZ; Supporting Information Table S1).
179
180 *Phyllosphere and rhizosphere* — one to three basal, non-senescent leaves were
181 collected from each plant, as well as the upper 2-5 cm of the taproot, together
182 with accompanying lateral roots (excess soil was brushed or shaken off). Leaf
183 and root samples were placed in individual 50 ml tubes containing 25 ml of sterile
184 wash solution (45.9 mM NaH_2PO_4, 61.6 mM Na_2HPO_4, 0.1% Tween 20). Tubes
185 were shaken by hand for one minute (timed). Leaf and root samples were then
186 removed and stored on ice in separate tubes (leaves in empty tubes, roots in
187 tubes containing 10 ml of wash solution) until further processing. Wash samples
188 were stored on ice during transport, then refrigerated at 4°C. Phyllosphere and
189 rhizosphere washes were pooled per population, then centrifuged at 2,200 g at
190 4°C for 15 minutes. Supernatants were discarded, and pellets were air-dried and
191 stored at -20°C until DNA extraction.
192
193 *Leaf endosphere* — leaves were surface sterilized by submerging in bleach
194 solution (10% commercial bleach, 0.1% tween 20) for two minutes. Leaves were
195 then rinsed in distilled water, patted dry using clean kimwipe, and sealed in
196 individual sterile surgical envelopes (Fisherbrand #01-812-50). Envelopes were
197 kept in silica gel desiccant until leaf tissue was completely dry, then stored at
198 room temperature until DNA extraction.
199
200 *Root surface and endosphere (hereafter 'whole root')* — roots were further
201 washed by shaking in 10 ml of wash solution until visible residual soil was
202 removed. Washed roots were stored and dried as described above for leaves.
203

204 *Controls* — at each collection site, a tube of sterile wash solution was left
205 uncapped while sampling plants. Disinfected tools were periodically swished in
206 the blank wash tube before sterilization and use for the next sample collection.
207 For each population, rinse water and wipes used to process tissue samples were
208 represented in controls by rinsing and wiping flame-sterilized forceps, then
209 swishing the forceps in the blank wash tube. Controls were stored and processed
210 in the same manner as phyllosphere and rhizosphere samples.
211
212 **DNA extraction**
213 Extractions were carried out using sterile technique in a laminar flow hood. Leaf
214 and root DNA was extracted as bulk samples from tissue pooled by population
215 (15 total populations), and as individual samples from 8 plants from each of 10
216 populations (80 total individuals). For pooled tissue extractions, equal sections of
217 leaf tissue (50 mm^2) and root tissue (12.5 mm^3 plus 10 mm of lateral roots) were
218 collected from each individual sample per population and pooled prior to
219 extraction. Control (blank) samples were collected for each batch of extractions
220 by swabbing tools and surfaces, then extracting DNA from the swab head.
221
222 All DNA samples were extracted using the MO BIO PowerSoil kit (MO BIO
223 Laboratories, Inc.). Phyllosphere and rhizosphere DNA was extracted from up to
224 0.25 g of wash pellets following the standard kit protocol. Leaf and root tissues
225 were ground to powder or sawdust consistency in liquid nitrogen using sterile
226 mortars and pestles. Leaf and root DNA was extracted from 20 mg (leaf) or 100
227 mg (root) of ground tissue with the following modification to the standard
228 protocol: tissue was incubated at 65°C for 10 minutes in extraction buffer, then
229 vortexed for 1 minute, followed by a second 10 minute incubation (as described
230 under “alternative lysis methods” in the kit protocol). Control DNA was extracted
231 by placing whole swab heads directly into extraction tubes. Extracted DNA was
232 eluted in PCR-grade water and stored at -20°C pending library preparation.
233
234 **Library preparation and sequencing**

To remove secondary compounds inhibiting PCR, DNA extracted from root and leaf tissue (together with corresponding blanks) was purified using a ZR-96 genomic DNA clean-up kit (Zymo Research). All DNA concentrations were quantified using a Qubit fluorometer high-sensitivity assay for double-stranded DNA (Invitrogen), and standardized to equimolar amounts.

Library preparation followed a dual barcoded two-step PCR protocol. In the first step (target-specific PCR), the V4 region of the 16S rRNA gene was amplified using target specific primers (515F and 806R) appended with common sequence (CS) tags through a linker sequence which varied from two to five nucleotides in length. Target-specific PCR was carried out using Phusion Flash master mix (Thermo Scientific) in 25 µl reaction volume in a Mastercycler pro thermocycler (Eppendorf) under the following conditions: 25 cycles of 1 s at 98°C, 5 s at 78°C, 5 s at 57°C, 15 s at 72°C. Products were visualized on an agarose gel and diluted by up to 1:15 (depending on yield); 1 µl of diluted product was then used as template in the second step (barcode-adapter attachment PCR). Using reagents and equipment as described above, barcoded primer pairs incorporating Illumina P5 and P7 adapters were used to amplify products from target-specific PCR in 25 µl reaction volumes under the following conditions: 10 cycles of 1 s at 98°C, 5 s at 78°C, 5 s at 51°C, 15 s at 72°C. Barcoded amplicons were quantified by fluorometry, pooled in equimolar amounts, cleaned, and submitted to the University of Idaho's IBEST Genomic Resources Core for QC and sequencing. Amplicons were multiplexed to use half the capacity of one 2 × 300 bp run on an Illumina MiSeq platform. Raw sequence data are deposited in the NCBI Short Read Archive under accession number XXXXXX [pending submission].

Peptide nucleic acid clamps (PNAs) were included in both PCR steps of library preparation to block amplification of plant chloroplast and mitochondrial 16S as recommended by Lundberg *et al.* (2013). Clamp sequences published by Lundberg *et al.* (2013) were compared with chloroplast and mitochondrial 16S sequences from yellow starthistle and three other species of Asteraceae with

266 published organellar genomes (*Centaurea diffusa*, *Helianthus annuus*, *Lactuca*
267 *sativa*). We found a single nucleotide mismatch between the Asteraceae
268 chloroplast 16S and the plastid PNA sequences, and designed an alternative
269 plastid PNA specific to the Asteraceae sequence (5'—
270 GGCTCAACTCTGGACAG—3'). All samples for this study were amplified using
271 the plastid PNA of our design, together with the mitochondrial PNA published by
272 Lundberg *et al.* (2013). To gauge the effectiveness of our alternative PNA, two
273 duplicate samples were processed using both PNAs published by Lundberg *et al.*
274 (2013).
275
276 **Identification of operational taxa**
277 Demultiplexed paired reads were merged and quality filtered using tools from the
278 USEARCH package version 9.0.2132 (Edgar & Flyvbjerg, 2015). Merged reads
279 were truncated to uniform length and primer sequences were removed using a
280 combination of the seqtk toolkit version 1.2 (github.com/lh3/seqtk) and a custom
281 script. The UPARSE pipeline (Edgar, 2013) implemented in the USEARCH
282 package was used for further data processing and analysis: unique sequences
283 were identified, and those represented only once or twice in the processed read
284 set were discarded as likely PCR or sequencing errors. Remaining sequences
285 were clustered into operational taxonomic units (OTUs) at a 97% threshold,
286 chimeras were filtered out, and per-sample OTU read counts were tabulated
287 using the UPARSE-OTU algorithm. Assignment of OTUs to nearest taxonomic
288 match in the Greengenes database (McDonald *et al.*, 2012) was carried out
289 using the UCLUST algorithm implemented in QIIME version 1.9.1 (Caporaso *et*
290 *al.*, 2010; Edgar, 2010). Data were further processed using tools from the QIIME
291 package: reads mapping to chloroplast and mitochondrial OTUs were removed,
292 and samples were rarefied by plant compartment. Rarefaction levels were
293 chosen to reflect the distribution of read counts per sample within plant
294 compartments, subsampling to the minimum number of reads necessary to
295 include all samples except those that were outliers for low read count.
296

297 **Microbial community analyses**

298 All statistical analyses were performed in R (R Core Team, 2015). We evaluated
299 overall differences in bacterial community composition between plant
300 compartments, and between native and invaded ranges within plant
301 compartments, by performing non-metric multidimensional scaling (NMDS) using
302 the R packages vegan (Oksanen *et al.*, 2016) and MASS (Venables & Ripley,
303 2002). Ordinations were based on Bray-Curtis distances, and were performed
304 using a two-dimensional configuration to minimize stress, using Wisconsin
305 double standardized and square root transformed data, with expanded weighted
306 averages of species scores added to the final NMDS solution. Significant
307 differences among plant compartments and between native and invaded samples
308 were assessed using the envfit function in vegan. Ellipses were drawn on NMDS
309 plots using the vegan function ordiellipse, representing 95% confidence limits of
310 the standard error of the weighted average of scores.

311

312 We further explored the underlying correlates of bacterial community variation
313 using Principal Components Analysis (PCA; using R function prcomp) for
314 samples from native and invaded ranges within each plant compartment. We
315 identified the OTUs with the highest loading on the dominant PC axis of variation
316 by examining the matrix of variable loadings produced by prcomp. The OTU
317 composition of samples pooled by population (phyllosphere, rhizosphere, and
318 bulk root samples; hereafter 'bulk samples') was visualized using a heatmap
319 generated in ggplot2 (Wickham, 2009). Bulk samples were hierarchically
320 clustered by Bray-Curtis dissimilarity (hclust function in R) using McQuitty's
321 method (McQuitty, 1966).

322

323 We compared the diversity of OTUs between the native and invaded range for
324 each plant compartment using both richness (R) and the Shannon diversity index
325 (H'; Shannon, 1948), which reflects the contributions of both taxonomic richness
326 and evenness to diversity. These values were calculated using the vegan
327 package, and compared between native and invaded ranges using a

328 nonparametric Kruskal-Wallis rank sum test on rarefied read counts. For plant
329 tissue samples that included multiple individuals per site, we compared the
330 diversity among sites using a Kruskal-Wallis test within regions.
331
332 Finally, we asked whether the geographic distribution of plant-associated
333 bacterial diversity could be explained by the geographic distribution of genetic
334 diversity in the plants. Measurements of plant genetic diversity at each of our
335 sampling sites were obtained from previously published genome-wide marker
336 analyses by Barker and colleagues (Barker *et al.*, 2017), calculated as the
337 average proportion of pairwise nucleotide differences between alleles (π) at
338 variable sites across the yellow starthistle genome. Diversity estimates (H') for
339 each plant compartment were predicted using linear models that included fixed
340 effects of plant genetic diversity, region (native vs. invaded), and the interaction
341 between these two effects.
342
343 **RESULTS**
344 **Sequencing and data processing**
345 Sequencing yielded 9,672,898 read pairs, of which 6,217,852 remained after
346 merging and quality control; these were 253 bp in length after removing artificial
347 and primer sequences. The number of raw read counts per sample ranged from
348 16 to 306,200 with a median of 21,964.
349
350 Analysis of the merged and processed reads resulted in 4,014 OTUs, of which 60
351 were identified as plastid or mitochondrial. Sequences representing yellow
352 starthistle chloroplast and mitochondrial 16S accounted for 40% and 1% of all
353 reads, respectively. Amplification of host chloroplast in samples using the
354 Asteraceae-specific plastid PNA was reduced by up to 51% compared with the
355 Lundberg *et al.* (2013) PNA (Supporting Information Table S2). This is consistent
356 with results from a broader comparison of the two PNAs, using five Asteraceae
357 species, which also found that blocking of host chloroplast amplification was
358 improved by using the Asteraceae-specific PNA (FitzPatrick *et al.*, unpublished).

359 Despite PNA blocking activity, 83% of the total reads from leaf endosphere
360 samples were yellow starthistle chloroplast sequences. This might potentially be
361 attributable to high chloroplast DNA concentrations relative to endophyte DNA in
362 leaf tissue total DNA extracts. After removal of chloroplast and mitochondrial
363 reads, remaining read counts for most leaf endosphere samples were low (Fig.
364 2), so no further analysis of leaf endosphere bacterial communities was
365 performed.
366
367 Rarefaction levels (chosen to reflect the minimum number of reads per sample
368 by compartment, not including outliers) were 18,000 reads per sample for
369 phyllosphere, 17,000 for rhizosphere, and 5,000 for whole root samples. These
370 levels resulted in the exclusion of six samples which were outliers for low read
371 count: one phyllosphere (DIA), one rhizosphere (SAZ), one bulk root (CAN), and
372 three individual root samples (two from SAZ; one from SIE).
373
374 **Microbial community analyses**
375 Results from NMDS ordinations indicated that bacterial communities differed
376 overall among the phyllosphere, rhizosphere, and whole root compartments (Fig.
377 3a; $P = 0.001$). Within compartments, NMDS further revealed significant
378 differences between native and invaded range whole root samples (Fig. 3b; $P =$
379 0.001) and rhizosphere samples ($P = 0.001$). Native and invaded range
380 phyllosphere samples differed with marginal significance ($P = 0.05$). The
381 dominant phyla among bacterial communities were Proteobacteria,
382 Actinobacteria, Bacteroidetes, and Firmicutes (Fig. 4), which is consistent with
383 the findings of previous characterizations of plant-associated bacterial
384 communities (reviewed by Bulgarelli *et al.*, 2013). Principal component analyses
385 suggested that the strongest contributions to changes in bacterial community
386 composition between the native and invaded ranges were made by shifts in the
387 representation of *Pseudomonas*, *Erwinia*, *Chryseobacterium*,
388 Xanthomonadaceae, and *Bacillus* taxa (Supporting Information Fig. S1; Table
389 S3). Clustering analyses within the phyllosphere and rhizosphere compartments

390 consistently grouped invaded range samples together, as well as samples from
391 the source region in western Europe (Supporting Information Fig. S2). Native
392 range samples from eastern Europe (HU01 and HU29) clustered together in
393 these compartments but were variable in their relationship to the other regions.
394 Bulk root samples showed less consistent clustering by range.
395
396 Bacterial OTU diversity (H': Fig. 5) was significantly lower in the invaded range in
397 the phyllosphere (X^2_1 = 5.36, P = 0.02) and rhizosphere (X^2_1 = 6.21, P = 0.01),
398 and marginally lower in bulk whole root samples (X^2_1 = 3.01, P = 0.08). Diversity
399 in whole root individual samples (Fig. 5d) did not vary significantly among
400 populations within the native range (X^2_4 = 1.82, P = 0.77), but did vary
401 significantly within the invaded range (X^2_4= 15.30, P = 0.004), such that the two
402 most extreme populations (TRI, SIE) were significantly different from one another
403 but not the remaining sites. Bacterial OTU richness (R) values showed patterns
404 similar to H' in general, but differences between native and invading regions were
405 much weaker overall (Supporting Information Fig. S3), indicating that both
406 richness and evenness of OTU representation contributed to differences in
407 diversity between the ranges. For whole roots, our most extensively sampled
408 plant compartment, an analysis of OTUs observed across all bulk and individual
409 samples combined indicated that the native and invaded range shared 51% of
410 observed OTUs, with 31% fewer unique OTUs observed in the invaded relative
411 to the native range (Fig. 6a). These patterns were reflected across both major
412 groups of Proteobacteria and Actinobacteria (Fig. 6b,c).
413
414 A linear model predicting microbial diversity (H') from plant diversity was
415 significant for bulk whole root samples (Fig. 7; $F_{(2,12)}$ = 4.99; P = 0.02; r^2_{adj} =
416 0.36), with significant main effects of both plant genetic diversity (P = 0.04) and
417 region (native vs. invaded; P = 0.009). The interaction between these two effects
418 was not significant (P = 0.69) and was removed from the final model. Similar
419 linear models did not identify significant effects of plant diversity when predicting

420 phyllosphere (*P* = 0.42) or rhizosphere (*P* = 0.11) diversity, nor the median
421 diversity of individual whole root samples at a site (*P* = 0.95).
422
423 **DISCUSSION**
424 Our study revealed strikingly lower diversity of bacterial communities in yellow
425 starthistle's invasion of California (USA), relative to native European populations
426 within and beyond the source region for the invasion. Reduced bacterial diversity
427 was apparent across phyllosphere, rhizosphere, and whole root communities,
428 and across dominant bacterial phyla. These patterns are consistent with
429 opportunities for enemy escape in this invasion and could explain why soils have
430 more favorable (less negative) effects on yellow starthistle fitness in California
431 relative to other parts of its range (Andonian *et al.*, 2011, 2012; Andonian &
432 Hierro, 2011).
433
434 In line with other surveys of plant microbiomes, we found that differences in
435 bacterial communities were greatest among plant compartments (i.e.,
436 phyllosphere, rhizosphere, and roots; (Bulgarelli *et al.*, 2013; Vandenkoornhuyse
437 *et al.*, 2015). The numbers and diversity of taxa (OTUs) that we recovered in
438 samples from each compartment were generally similar in magnitude to those
439 reported in other studies of prokaryotic 16S sequences, from angiosperm groups
440 as diverse as e.g. Agavaceae (Coleman-Derr *et al.*, 2016), Brassicaceae
441 (Bodenhausen *et al.*, 2013), Cactaceae (Fonseca-García *et al.*, 2016), and other
442 Asteraceae (Leff *et al.*, 2016). Notably, we found that diversity was approximately
443 twice as high in the roots than the rhizosphere. Higher root endosphere diversity
444 relative to the rhizosphere is also reported in some other recent studies
445 (Fonseca-García *et al.*, 2016; Leff *et al.*, 2016), but previous reviews have
446 concluded that root endosphere communities are typically less diverse than
447 those in the rhizosphere (Bulgarelli *et al.*, 2013; Vandenkoornhuyse *et al.*, 2015).
448 Our root collections were not surface sterilized and may represent some of the
449 rhizoplane/rhizosphere in addition to the endosphere, elevating our estimates of
450 diversity, though it is also possible that yellow starthistle deviates from previous

trends. In contrast to phyllosphere, rhizosphere, and root compartments, we recovered few microbial sequences for leaf bacterial endophytes, even when compared to control (blank) samples, suggesting low sequence coverage due to persistent chloroplast contamination, and potentially low overall bacterial loads within yellow starthistle leaves.

Within compartments, differences in community composition between ranges were substantial and dominated by differences in OTU diversity, which ranged from 18-40% lower in the invaded range. This variation in diversity is similar in scale to other studies that have sampled distant geographic locations (e.g. across regions of North America: (Bodenhausen *et al.*, 2013; Peiffer *et al.*, 2013). A variety of factors may explain this pattern, including environmental differences across sites (Fierer & Jackson, 2006; Bulgarelli *et al.*, 2013; Nemergut *et al.*, 2013; Vandenkoornhuyse *et al.*, 2015). Soil type appears to have a particularly strong influence on microbial communities (e.g. Bulgarelli *et al.*, 2012; Lundberg *et al.*, 2012), and is known to differ broadly across yellow starthistle's range (Hierro *et al.*, 2016). In addition, populations in California include temperature and precipitation environments that are on the warm and dry extreme of yellow starthistle's climatic niche (Dlugosch *et al.*, 2015), and our sampling was conducted at the end of a period of severe drought in California (Griffin & Anchukaitis, 2014; Diffenbaugh *et al.*, 2015), which could have amplified microbial differences related to climate (Schrama & Bardgett, 2016). Interestingly, a recent study of grassland plants found that microbial diversity increased under drought, whereas we found reduced diversity in our drought-affected invaded range (terHorst *et al.*, 2014).

We also observed an effect of plant genotypic diversity on microbial diversity in bulk samples of roots. We note that bacterial diversity at the level of the individual plant did not covary with plant population genetic diversity, indicating that it was only when samples from different plants were combined that an effect of plant genotype variation was apparent. Such within-species plant genotype

482 effects have been observed in other studies and may interact with the effect of
483 environment to shape microbial communities (Peiffer *et al.*, 2013; terHorst & Zee,
484 2016). In many cases, it appears that genotype effects are minor relative to site
485 effects (e.g. (Lundberg *et al.*, 2012; Peiffer *et al.*, 2013; Bodenhausen *et al.*,
486 2014; Bulgarelli *et al.*, 2015), and we also found that genotypic effects were not
487 stronger than between-region variation in yellow starthistle. Moreover, plant
488 genotype effects were only significant in the roots, the only endophytic
489 compartment analyzed: consistent with plant genotype having the strongest
490 influence on microbial taxa colonizing within the plant itself (Bulgarelli *et al.*,
491 2012; Lundberg *et al.*, 2012; Lebeis *et al.*, 2015). The ability of plants to shape
492 the composition and quantity of endophytic microbial taxa in particular has been
493 suggested to contribute to a 'core' microbiome shared across environments
494 (Lundberg *et al.*, 2012; Shade & Handelsman, 2012; Reisberg *et al.*, 2013), and
495 indeed we found that the majority of OTUs observed in yellow starthistle roots
496 were shared across invading and native populations on different continents.
497
498 Importantly, yellow starthistle's invasion to high density could be a cause rather
499 than an effect of low microbial diversity. Species invasions and range expansions
500 have been shown to change microbial composition over short timescales (Collins
501 *et al.*, 2016; Gibbons *et al.*, 2017). Yellow starthistle invasions are denser than
502 populations surveyed in the native range by an order of magnitude or more
503 (Uygur *et al.*, 2004; Andonian *et al.*, 2011). Invaded communities that are
504 dominated by yellow starthistle include lower diversity of plant species overall
505 (Seabloom *et al.*, 2003; Zavaleta & Hulvey, 2004; D'Antonio *et al.*, 2007), and low
506 plant diversity may depress the diversity of plant-associated microbes in the
507 environment (Garbeva *et al.*, 2004; Schnitzer *et al.*, 2011; Coleman-Derr *et al.*,
508 2016). Such an effect of plant density could provide an explanation for a general
509 pattern of weaker plant-soil interactions for invasive species in their introduced
510 ranges (Kulmatiski *et al.*, 2008; Dawson & Schrama, 2016). These results
511 reinforce a growing need for explicit observational and experimental tests of the
512 association between microbial diversity and the potential influences of plant

density, plant community diversity, and environmental gradients (Dawson & Schrama, 2016).

This study is among the first to examine differences in microbial taxa between the native and introduced ranges of an invasive species. Gundale and colleagues (Gundale *et al.*, 2014) also identified more favorable soil interactions in invasions of lodgepole pine (*Pinus contorta*), and explored potential enemy escape in its fungal endophyte community (Gundale *et al.*, 2016). For lodgepole pine, microbial communities differed among regions, but there was no consistent pattern of loss of potential fungal pathogens or gain of mutualists in the invaded range, and it remains unclear what part of the soil community is responsible for observed differences in interactions across ranges (Gundale *et al.*, 2016). Finkel and colleagues (Finkel *et al.*, 2011; 2016) similarly explored the phyllosphere community of multiple species of *Tamarix* in native and introduced parts of their range, finding that communities are most strongly structured by geographic region. Our study reveals that this type of comparative microbiome approach can be fruitful for identifying changes in species interactions that might be contributing to invasion success.

One of the central challenges in testing the hypothesis that invaders are benefitting from enemy release is quantifying the impact of all types of enemies, with the microbial community being historically the hardest to observe (Keane & Crawley, 2002; Beckstead & Parker, 2003; Dawson & Schrama, 2016; Müller *et al.*, 2016; van der Putten *et al.*, 2016; Crawford & Knight, 2017). For yellow starthistle, a great deal of effort has gone into the identification of potential native herbivores/seed predators that could be used as biocontrol in California. Six specialist biocontrol insect species and one fungal foliar pathogen have been released into this area without resulting in effective control (DiTomaso *et al.*, 2006; Swope & Parker, 2012), suggesting that escape from these species has not facilitated the invasion. Our finding that invaded populations have not only

unique, but also less diverse bacterial communities, suggests particularly strong opportunities for pathogen escape in this system.

We have previously argued that yellow starthistle has invaded into a low competition environment in California, benefitting from the historical loss of plant competitors for water in this system (Dlugosch *et al.*, 2015). Disturbance of the native community is critical for yellow starthistle establishment, and functionally similar native species compete well against it in experiments; however, key competitors have been lost from the ecosystem due to a variety of perturbations prior to the yellow starthistle invasion (Zavaleta & Hulvey, 2004; Hooper & Dukes, 2010; Hierro *et al.*, 2011, 2016; Hulvey & Zavaleta, 2012). Any benefits to yellow starthistle of reduced bacterial diversity could be independent of these interactions with native plant species, but there are clear opportunities for these factors to be related. If a lack of competition allowed yellow starthistle to increase in density, then this could have reduced plant-associated microbial diversity in the environment, as noted above. However, while this scenario could explain lower diversity among bacteria, it appears that density is unlikely to explain reduced negative interactions with the soil community in California. Yellow starthistle experiences some of its strongest negative plant-soil feedbacks across generations in California soils (Andonian *et al.*, 2011), suggesting that the build up of high plant densities is unlikely to explain patterns of enemy release. Alternatively, the historical loss of native species diversity in California (D'Antonio *et al.*, 2007) could have resulted in the loss of associated microbial diversity, generating particularly strong opportunities for invasion into a system with both reduced competition and reduced pathogen diversity. Microbial surveys of remnant native communities, as well as across densities of yellow starthistle would help to clarify alternative interacting effects of plant and microbial diversity, and it may be particularly enlightening to explore microbial communities preserved on native plant specimens pre-dating the extensive invasion of yellow starthistle into this region.

Conclusions

To our knowledge, our study is the first to find evidence consistent with opportunities for release from microbial enemies during invasion. We find lower overall bacterial diversity in invading plant populations, similar in scale to geographic variation in bacterial diversity that has been observed in other studies. These patterns suggest that yellow starthistle may have benefitted from introduction into disturbed plant communities with relatively low microbial diversity. Microbial interactions appear to be important for plant fitness in this system, but may interact with other factors shaping invasiveness, including disturbance and lack of effective competition from native plant species. In particular, escape from both microbial enemies and plant competitors might have created an opportunity for adaptive allocation of resources away from defensive functions and towards reproduction and the evolution of increased invasiveness in yellow starthistle. Comparative surveys of the microbiome in invading and native populations, as presented here, can reveal important variation in the species interactions that are shaping patterns of invasion.

ACKNOWLEDGMENTS

We thank D. Lundberg and S. Lebeis for helpful discussions regarding sampling design; G. Reardon and C. Chandeyson for assistance with field collections; E. Arnold, J. Aspinwall, J. Braasch, E. Carlson, K. Hockett, T. O'Connor, M. Schneider, J. U'Ren, and N. Zimmerman for 16S library preparation assistance and discussion; A. Gerritsen, D. New and staff at iBEST for assistance with sequencing; K. Andonian, J. Hierro, and # reviewers for helpful feedback on the manuscript. We are particularly indebted to C.E. Morris for hosting the European sample processing at INRA Station de Pathologie Végétale, Montfavet, France. Data collection and analyses performed by the IBEST Genomics Resources Core at the University of Idaho were supported in part by NIH COBRE grant P30GM103324. This study was supported by USDA grant 2015-67013-23000 to K.M.D., D.A.B., and S.M.S.

605 **AUTHOR CONTRIBUTIONS**

606 P.L-I., D.A.B., and K.M.D. designed the study. P.L-I. and J.H. collected the
607 samples with assistance from H.S., S.M.S., and S.R.W. P.L-I conducted the
608 microbial sequencing and bioinformatics. P.L-I, S.R.W., and K.M.D. analyzed the
609 data. P.L-I and K.M.D. wrote the manuscript, which was edited by all authors.

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Fig. 1. The distribution (gray) of yellow starthistle and sampling sites for this study. Maps detail the native range in Eurasia (a) and the invasion of western North America (b). Previous work has indicated that western Europe is the source for the severe invasion of California, USA (both in dark shading; Barker et al. 2017). Sampling included seven locations in California (b, filled circles), six locations in western Europe and an additional two locations in eastern Europe (a, open circles).

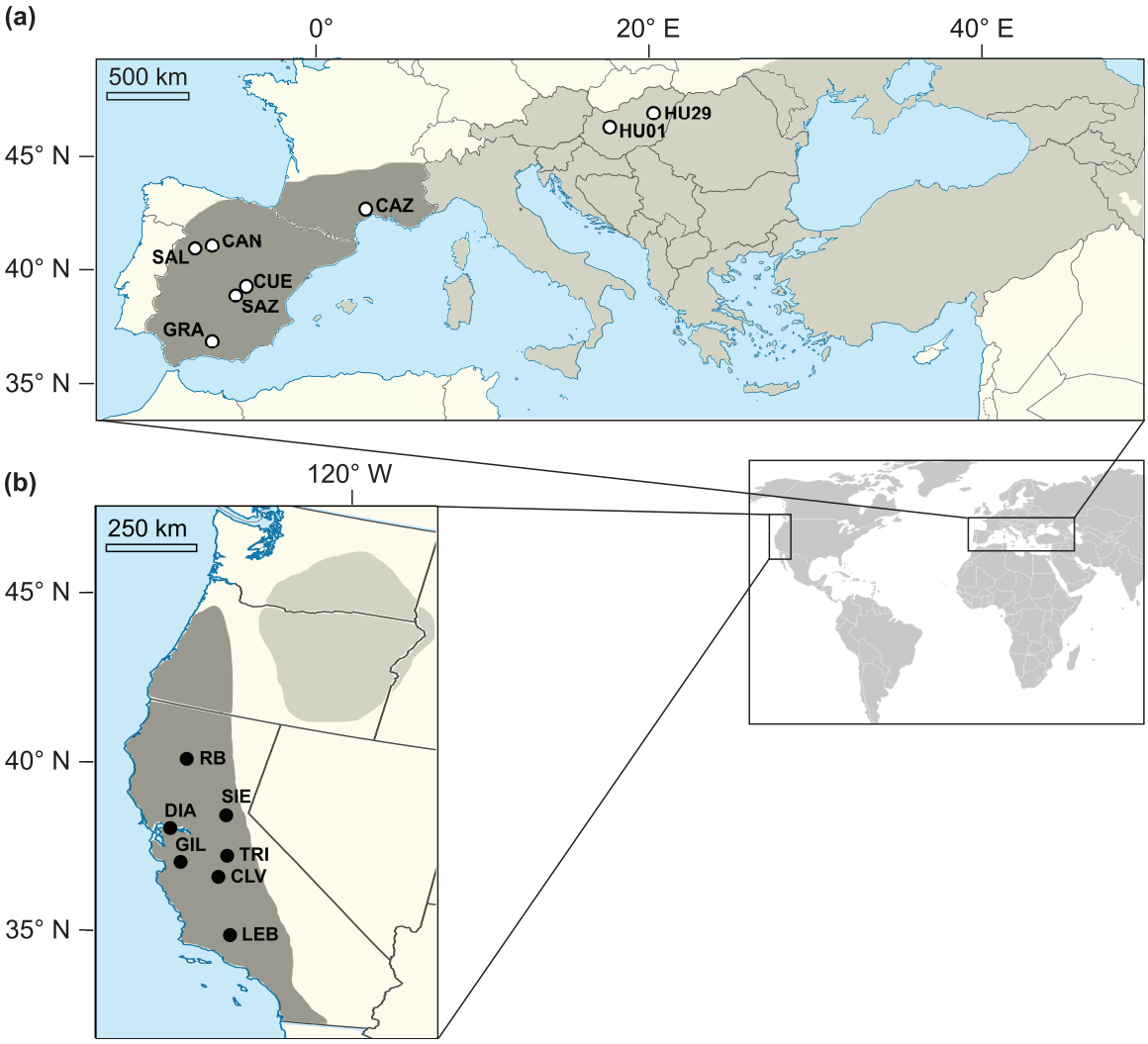

Fig. 2. Distribution of read counts for bulk samples from all four compartments (native and invading population samples combined), as well as control (blank) samples.

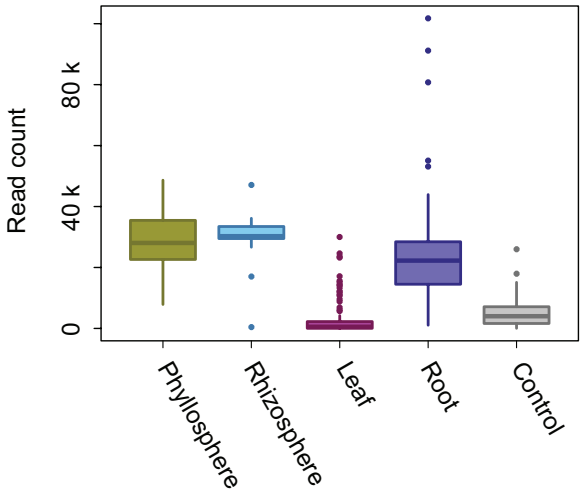

Fig. 3. NMDS plots of bacterial OTU composition in phyllosphere (green), rhizosphere (light blue), and whole root (dark blue) samples for native (open symbols) and invading (closed symbols) populations. Plotted are a) bulk samples for each population, showing overall separation by compartment and by range within compartment, and b) individual whole root samples within native and invading populations. Ellipses indicate 95% confidence intervals for samples grouped by range (native range: dashed line; invaded range: solid line).

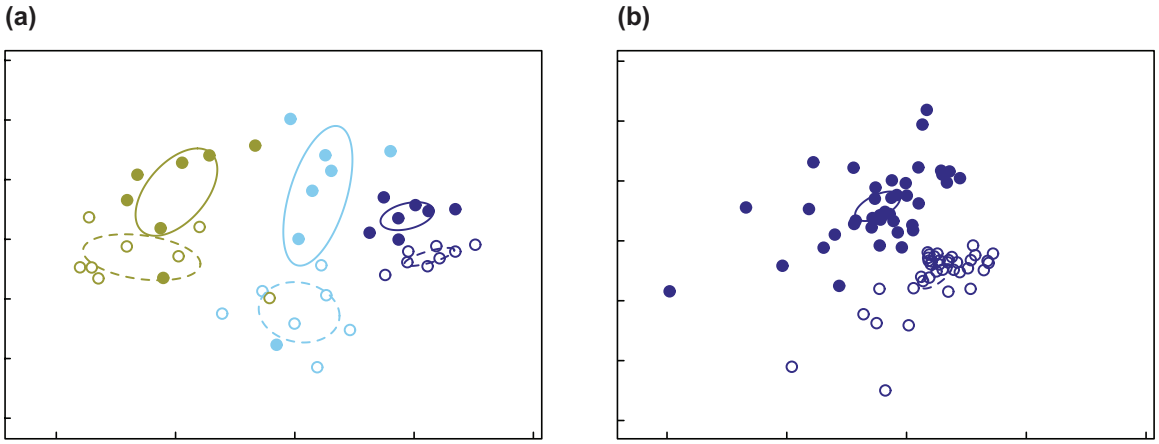

Fig 4. Relative abundance of (proportion of reads mapping to) dominant phyla in (a) phyllosphere, (b) rhizosphere, and (c) whole root bulk samples from native and invaded ranges.

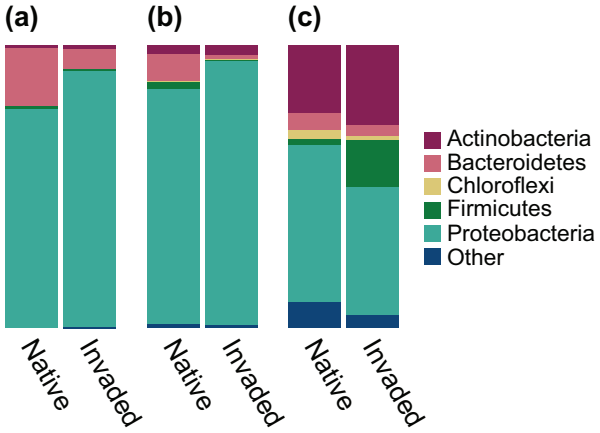

Fig. 5. Comparison of diversity (H') between samples from native and invaded ranges for (a) phyllosphere, (b) rhizosphere, (c) bulk whole roots by population, (d) individual whole roots. Significance levels from Kruskal-Wallis tests indicated with asterisks: *P < 0.05, (*)P < 0.1.

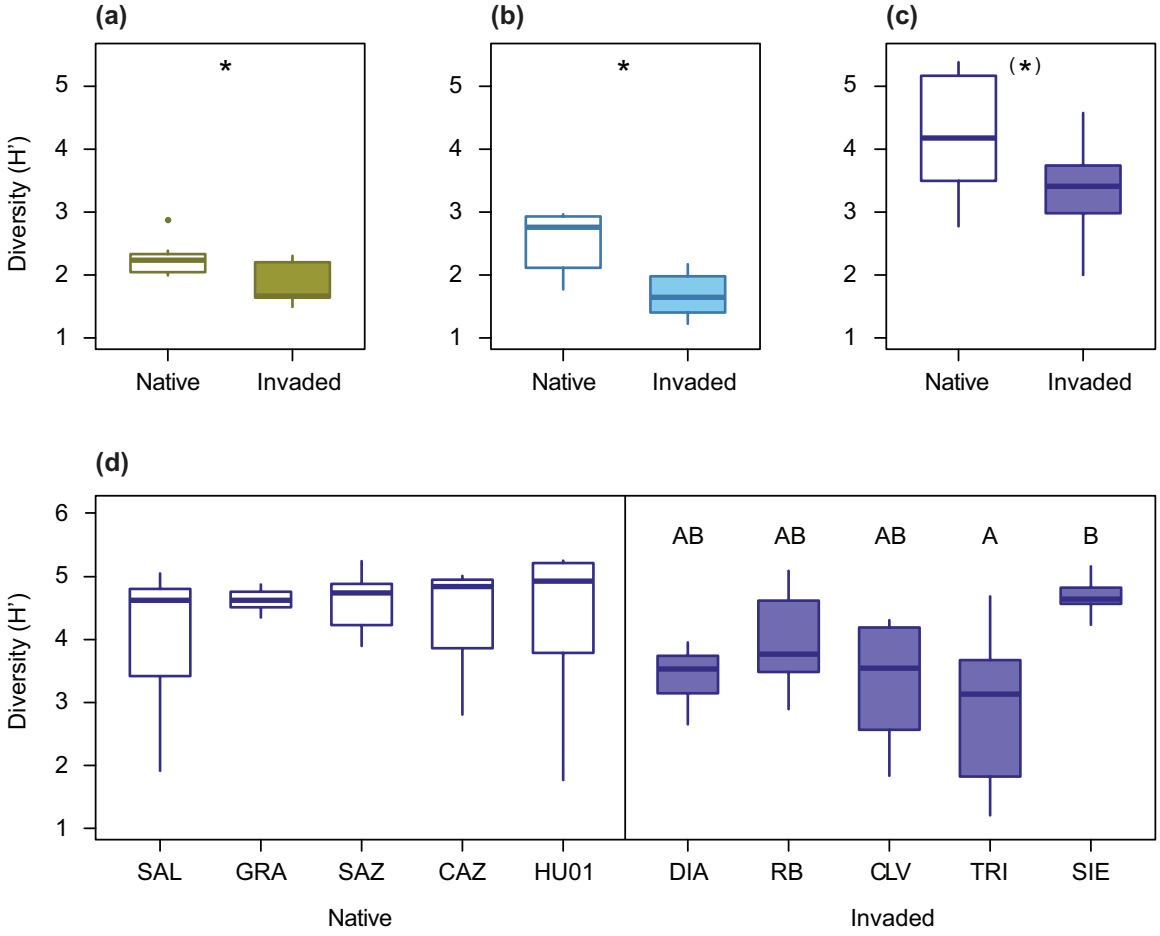

Fig 6. Venn diagrams indicating the number of OTUs shared between native and invaded ranges, and unique to each range, for whole root samples. Shown are OTUs from bulk and individual samples combined, for (a) all OTUs, and for the dominant phyla Proteobacteria (b) and Actinobacteria (c).

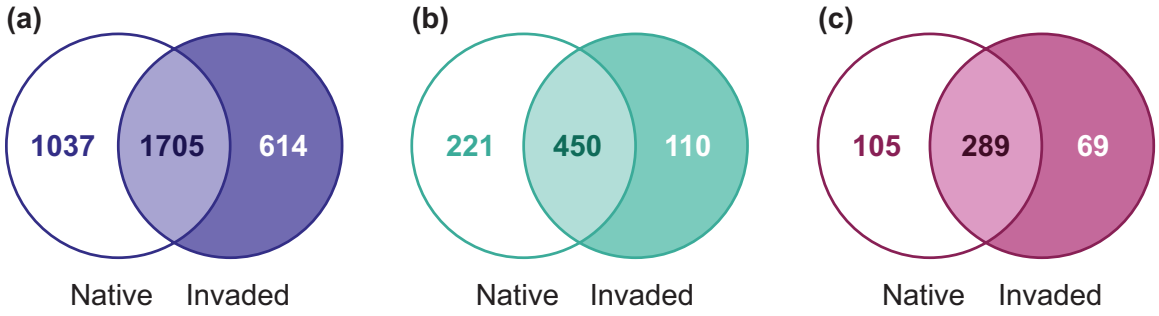
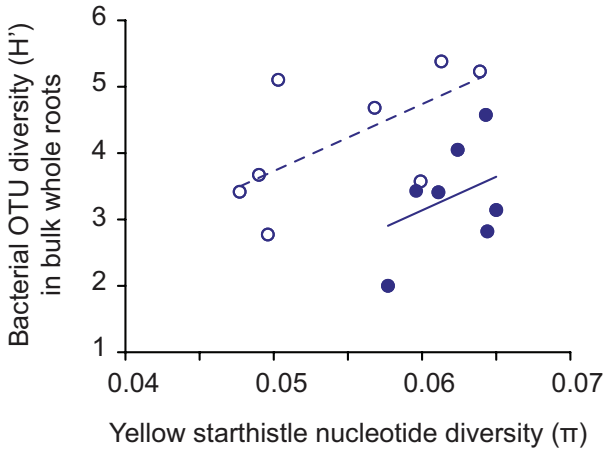
Fig 7. Bacterial diversity (H') in bulk whole root samples for each population as a function of the genetic diversity among plants in those populations (calculated as the average proportion of pairwise nucleotide differences between alleles (**π**) at variable sites across the genome; from Barker *et al.*, 2017). Lines show significant positive relationships (linear model: $P < 0.02$) between microbial and plant diversity in both the native range (open symbols, dashed line) and invaded range (closed symbols, solid line).