Taxonomy and Distribution of Phenazine-Producing *Pseudomonas* spp. in the Dryland Agroecosystem of the Inland Pacific Northwest, United States

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We investigated the taxonomic placement of phenazine-producing fluorescent *Pseudomonas* spp. in the Inland Pacific Northwest region of the United States. Five distinct species were identified, two of which were provisionally considered to be new. Agroclimatic zone and soil silt content affected the species diversity across the region.

Phenazines are functionally diverse heterocyclic nitrogen-containing compounds produced by bacteria containing *phz* genes (1, 2). Indigenous phenazine-producing (Phz⁺) pseudomonads are abundant in certain agricultural soils (1, 3, 4), and such strains inhibit soilborne plant pathogens (3, 4). In the Inland Pacific Northwest region of the United States (IPNW), Phz⁺ pseudomonads are genetically and phenotypically diverse (5) and particularly abundant in the low-precipitation zone, where they occur at densities of 10^5 to 10^7 CFU g root^{-1} (4, 6). However, the taxonomy of these Phz⁺ populations and the extent to which Phz⁺ rhizobacterial community composition is impacted by abiotic factors remains unclear. Here, we addressed this knowledge gap by using multilocus sequence analysis (MLSA) (7) of Phz⁺ isolates and sequence analysis of *phzF* alleles cloned from IPNW commercial wheat fields. Our results provide new insights into the prevalence of Phz⁺ pseudomonads in the environment and serve as a foundation for characterizing such species globally.

During this study, we sampled several wheat-growing areas throughout the IPNW (Fig. 1; see also Fig. S1 in the supplemental material) and isolated indigenous Phz⁺ strains (see Table S1 and Table S2 in the supplemental material) and extracted DNA from the rhizosphere of field-grown wheat (Table 1). Indigenous populations of Phz⁺ *Pseudomonas* were quantified using the terminal endpoint dilution assay (1, 8). Phz⁺ strains were isolated from rhizosphere suspensions and subjected to BOX-PCR profiling (5, 9). The newly isolated Phz⁺ strains, along with those isolated in previous surveys (1, 5), were subjected to profiling by BOX-PCR (5, 10). Isolates of the same banding profile from each location were grouped, and one representative isolate was selected for multilocus sequence analysis (MLSA) (7). Phylogeny was defined with MLSA using partial DNA sequences for 16S rRNA (1,384 nucleotides [nt]), *gyrB* (794 nt), *rpoB* (903 nt), and *rpoD* (698 nt) that were amplified, respectively, with the primer sets 8F/1492R (11), Up-1G-/Up-2G- (1), rpoBup1/rpoBlow1 (1) and LAPS/LAPS27 (12), and PsEG30F and PsEG790R (13) (see Table S4 in the supplemental material). Genomic DNA was extracted from strains using a GenElute bacterial genomic DNA kit (Sigma-Aldrich Co., St. Louis, MO). PCR amplifications were performed using GoTaq DNA polymerase (Promega, Inc., Madison, WI) on a PTC-200 thermal cycler (Bio-Rad, Hercules, CA), and the amplicons were sequenced by Elim Biopharm (Hayward, CA). DNA sequences were processed using CLC bio Main Workbench v.6.6.2 (CLC Bio, Aarhus, Denmark) and aligned in MEGA5 (14) using MUSCLE

(15). Phylogenetic distances were estimated using the Jukes-Cantor substitution model and bootstrapped with 1,000 replications. Simultaneous Alignment and Tree Estimation v.2.2.2 (SATé-II) (16) was used for maximum-likelihood MLSA phylogeny estimation with the suggested settings and GTR+G20 as the model. We also carried out detailed sequence analysis of *phzF* alleles amplified directly from rhizosphere DNA samples isolated from rhizosphere suspensions with a PowerSoil DNA isolation kit (Mo Bio, Carlsbad, CA). *phzF* alleles were amplified with the primers Ps_up1/ Ps_low1 (1), and the amplicons were cloned into the pGEM-T Easy vector (Promega, Madison, WI). The resultant recombinant plasmids were purified using a MinElute 96 UF kit (Qiagen, Valencia, CA) and sequenced by Elim Biopharm (Hayward, CA). The nucleotide sequences were analyzed in SATé-II (16), and the resultant phylogeny was visualized in Dendroscope v.3.2.2 (17). The *phzF*-based taxonomy classification was performed in Mothur v.1.26.0 (18) using "classify.seqs" and a taxonomy database constructed from the reference alleles. An alignment without reference *phzF* alleles was used to calculate the inverse Simpson diversity ($1/D$) estimate (19). All calculations in Mothur were carried out using a phylogenetic distance cutoff of 0.04 nucleotide substitution per site.

The largest clade found using BOX-PCR profiling and MLSA was provisionally named "*Pseudomonas aridus*" (*n* = 177) (Fig. 2), as it occurred only in fields that receive the least annual precipitation. The most closely related described species had between 4.1% and 4.5% sequence dissimilarity (Table 2). The second largest clade, provisionally named "*Pseudomonas cerealis*," included a subgroup with ca. 2% MLSA dissimilarity (*n* = 156) (Fig. 2) and was more cosmopolitan throughout the IPNW. "*Pseudomonas cerealis*" was most closely related to strains of *P. orientalis* (ca. 3.7% dissimilarity) and "*P. aridus*." "*P. cerealis*" and "*P. aridus*" were found at the majority of sites throughout the IPNW (Table 2) and
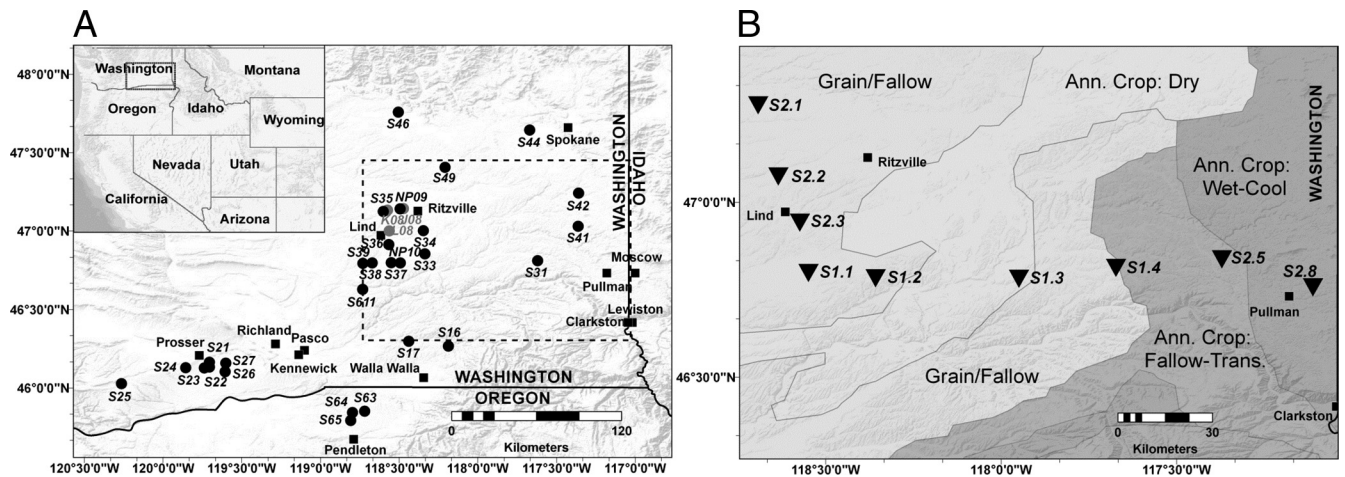
FIG 1 Distribution of sites sampled for Phz$^+$ populations throughout the Inland Pacific Northwest. All sites labeled with an "S" in panel A were dryland winter wheat fields sampled for Phz$^+$ *Pseudomonas* populations in a previous study (4) and used to isolate new Phz$^+$ strains to establish MLSA-based phylogenetic placement. Sampled sites in panel B were used to isolate DNA from the rhizosphere of winter wheat for subsequent amplification of *phzF* for cloning and sequencing. The map in panel B also indicates agroclimatic zones (22). Three sites (K08, J08, and L08) sampled previously (5) were also included and are shaded in gray. Spatial data visualization was performed in ArcGIS v.9.3 (Esri, Inc.). The map was created with ArcGIS (base map from USGS, Esri, Tele Atlas, and Automotive Navigation Data [AND]).

comprised over 67% of our Phz$^+$ isolates. The DNA sequence similarities of these groups were below 97%, the MLSA-defined cutoff for defining species within the *Pseudomonas* genus (7). Phenotypic differences in PCA and biosurfactant production *in vitro* also were observed between these two species (5), in addition to differential carbon substrate utilization profiles (5) (see Table S3 in the supplemental material).

The *P. orientalis* clade contained the type strain *P. orientalis* CFML-170 (20) and 140 IPNW Phz$^+$ strains (Fig. 2) as well as a small subgroup at 2.8% dissimilarity (see Table S2 in the supplemental material). The *P. synxantha* clade included the type strain *P. synxantha* LMG 2335 and the model strain 2-79 (21) ($n = 22$) (Fig. 2) and clearly diverged from other species (Table 2). This report provides the first evidence for phenazine production by either *P. orientalis* or *P. synxantha* (20). Finally, *Pseudomonas* sp. strain 1209 was genetically distinct from other pseudomonads, as it was only 95.2% similar by MLSA to any previously described species (Fig. 2; also, see Table S2 in the supplemental material).

We also found that three different dryland plant hosts (spring wheat, common yarrow, and needle-and-thread grass) supported largely different communities of Phz$^+$ species, although spring wheat and common yarrow shared some closely related "*P. cerealis*" strains (Fig. 2; also, see Table S1 in the supplemental material).

Among 454 sequenced *phzF* amplicons from different locations, the majority of alleles belonged to one of the four dominant Phz$^+$ species (Fig. 3). The largest percentage of these (45%) were classified as "*P. cerealis*." Another 10% (45 of 454) were classified as *Pseudomonas* sp. 1209-like, and 44 of these originated from sample S2.8 in the wet-cool annual crop agroclimatic zone (22), suggesting that this group favors higher-precipitation areas. No *phzF* alleles were assigned to *P. synxantha*, and a small group of sequences from samples S1.3 and S2.8 did not match any of the known Phz$^+$ species (Fig. 3). Rarefaction analysis of *phzF* revealed that the Phz$^+$ communities were nearly completely sampled at seven of the eight sites and S2.8 had the greatest OTU richness (see Fig. S2 in the supplemental material). Taken together with popu-

TABLE 1 Characteristics of sites sampled for total rhizosphere DNA

Sample	Geographic coordinates	Total no. of *phzF* clones	Phz$^+$ population density (log CFU g root^{-1} [fresh wt])[a]	Phz$^+$ plant colonization frequency	Agroclimate zone[b]	Mean annual precipitation (mm)[c]
S1.1	46°47′49″N, 118°32′57″W	51	5.4 ± 0.6	1.0	Grain/fallow	259
S1.2	46°46′53″N, 118°21′28″W	54	5.6 ± 1.0	0.7	Grain/fallow	297
S1.3	46°46′50″N, 117°56′58″W	52	5.3 ± 0.8	0.8	Annual crop; dry	371
S1.4	46°48′40″N, 117°40′22″W	55	6.3 ± 0.6	0.6	Grain/fallow	430
S2.1	47°16′33″N, 118°41′34″W	52	5.4 ± 1.3	0.6	Grain/fallow	273
S2.2	47°4′20″N, 118°38′7″W	52	5.7 ± 0.9	0.6	Grain/fallow	265
S2.3	46°56′26″N, 118°34′26″W	46	5.3 ± 0.8	0.8	Grain/fallow	258
S2.5	46°50′9″N, 117°22′18″W	44	4.8 ± 0.5	0.2	Annual crop; fallow-transition	512
S2.8	46°45′20″N, 117°6′41″W	48	5.0 ± 0.6	0.3	Annual crop; wet-cool	581

[a] At each location, 16 plants were randomly sampled and root suspensions were used to determine population density and plant colonization by the terminal endpoint dilution assay (1, 8). Values are means ± standard deviations.
[b] Agroclimatic zones defined by Douglas et al. (22).
[c] Mean annual precipitation data for 30 years (1971–2000) from the PRISM Climate Group (www.prism.oregonstate.edu/).
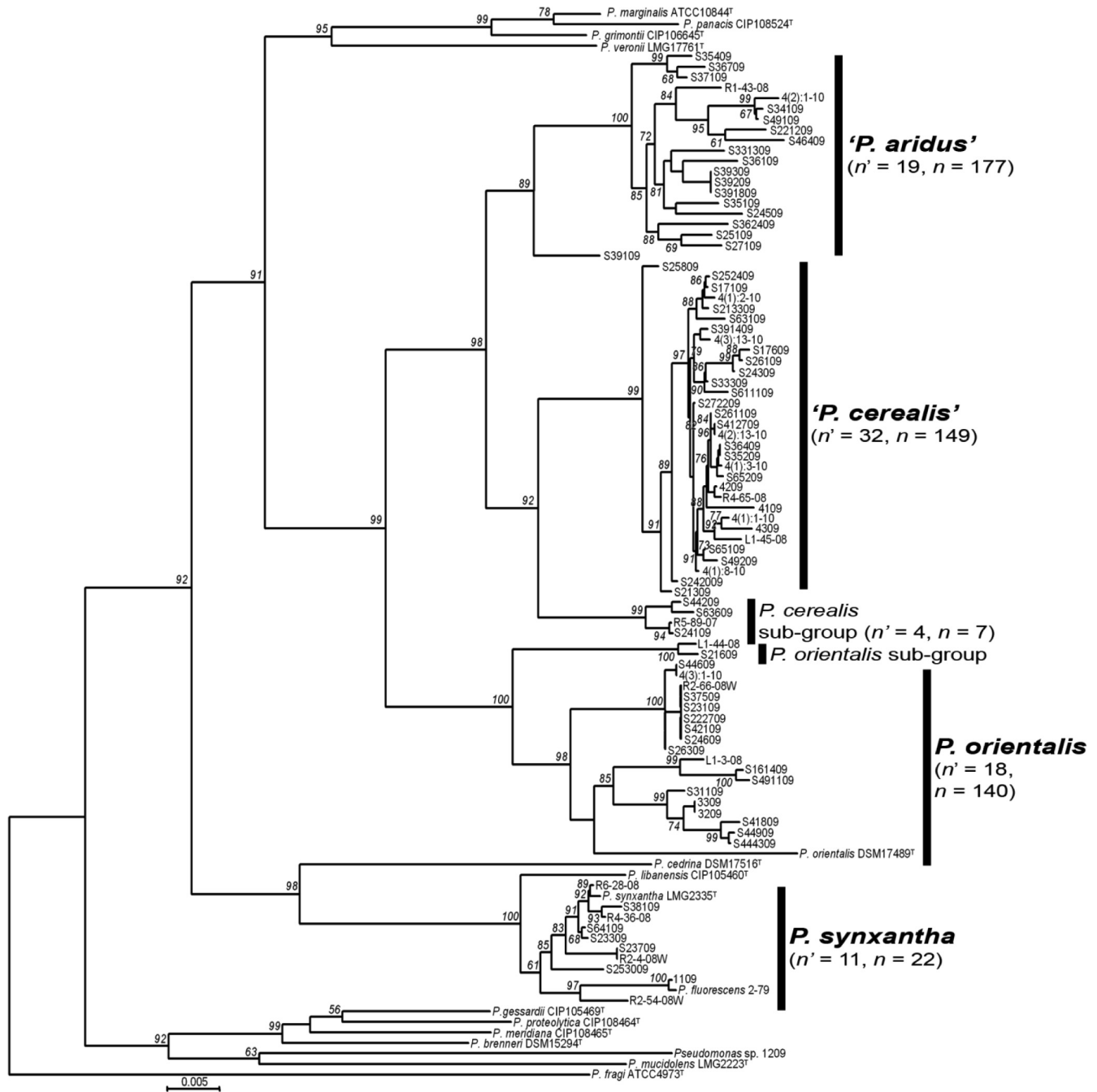
FIG 2 Phylogenetic placement of Phz⁺ *Pseudomonas* spp. isolates in the "*P. fluorescens*" and "*P. gessardii*" subgroups as defined by Mulet et al. ([7]). Eighty-eight representative Phz⁺ isolates (n') were analyzed alongside 14 *Pseudomonas* type strains, including the outgroup *P. fragi* ATCC 4973[T]. The total number of strains defined by BOX-PCR in this study (n) is also indicated. The scale bar indicates the number of nucleotide substitutions per site.

lation densities of 10^5 to 10^6 Phz⁺ CFU per gram of root, these results indicate very low overall diversity in the Phz⁺ community (Table 1). We also estimated the diversity of *phzF* by calculating the inverse Simpson's index ($1/D$) ([19]) and found that S2.5 had the highest diversity, at 2.47. Sites S2.8 and S1.4 had the next highest, at 2.01 and 1.58, respectively, whereas sites S1.1 and S2.1 had the lowest diversity, at 1.00. Mean annual precipitation had only a slight effect on diversity (t-test P value = 0.0560; analysis of variance [ANOVA] F-test P value = 0.0593); however, soil per-

cent silt (t-test P value = 0.0220; ANOVA F-test P value = 0.0233) and agroclimatic zone (t-test P value = 0.0188; ANOVA F-test P value = 0.0217) showed a much greater effect where overall diversity was lowest under "grain/fallow" management (see Fig. S3 in the supplemental material). Future studies on the effect of soil type, crop management, and water availability on the Phz⁺ community composition under controlled conditions are needed to determine the specific influence of these factors on the ecology of Phz⁺ rhizobacteria.

TABLE 2 MLSA-based sequence dissimilarity comparison of Phz$^+$ strain groups to species of the *P. fluorescens* lineage

Strain	Dissimilarity value (%)[a]				
	P. aridus group	*P. cerealis* group	*P. orientalis* group	*P. synxantha* group	*Pseudomonas* sp. strain 1209
P. brenneri DSM15294[T]	5.3 ± 0.3	5.4 ± 0.3	5.8 ± 0.4	5.0 ± 0.3	5.2 ± 0.3
P. cedrina DSM17516[T]	5.2 ± 0.3	5.0 ± 0.3	4.6 ± 0.3	4.5 ± 0.3	6.3 ± 0.4
P. gessardi CIP105469[T]	5.5 ± 0.3	5.4 ± 0.3	5.7 ± 0.4	5.0 ± 0.3	5.0 ± 0.3
P. grimontii CIP106645[T]	4.1 ± 0.3	4.2 ± 0.3	4.5 ± 0.3	4.6 ± 0.3	5.6 ± 0.4
P. libanensis CIP105460[T]	4.8 ± 0.3	4.9 ± 0.3	5.0 ± 0.3	1.5 ± 0.2	6.2 ± 0.4
P. marginalis ATCC10844[T]	4.3 ± 0.3	4.3 ± 0.3	4.6 ± 0.3	4.7 ± 0.3	5.7 ± 0.4
P. meridiana CIP108465[T]	4.8 ± 0.3	4.8 ± 0.3	5.2 ± 0.3	5.3 ± 0.3	4.8 ± 0.3
P. mucidolens LMG2223[T]	6.2 ± 0.4	6.0 ± 0.4	6.8 ± 0.4	5.8 ± 0.4	5.5 ± 0.3
P. orientalis DSM17489[T]	4.1 ± 0.3	3.7 ± 0.3	2.4 ± 0.2	4.8 ± 0.3	6.6 ± 0.3
P. panacis CIP108524[T]	4.5 ± 0.3	4.5 ± 0.3	4.5 ± 0.3	4.8 ± 0.3	5.6 ± 0.4
P. proteolytica CIP108464[T]	5.0 ± 0.3	5.0 ± 0.3	5.4 ± 0.3	4.9 ± 0.3	4.9 ± 0.3
P. synxantha LMG2335[T]	4.5 ± 0.3	4.5 ± 0.3	4.6 ± 0.3	0.6 ± 0.1	6.2 ± 0.4
P. veronii LMG1776[T]	4.5 ± 0.3	4.5 ± 0.3	4.5 ± 0.3	4.8 ± 0.4	5.2 ± 0.3

[a] The sequence dissimilarity values (means ± standard errors) were calculated as *p* distance in MEGA5 using 1,000 bootstrap replications.

This study provides new insights into the evolutionary ecology and global distribution of beneficial plant-associated Phz$^+$ bacteria. While it previously appeared that the capacity to produce phenazines was limited to just a few *Pseudomonas* species (1), we have now demonstrated the presence of an extensive complex of Phz$^+$ species belonging to the *P. fluorescens* lineage in the IPNW.

The provisional "*P. aridus*" and "*P. cerealis*" species are particularly abundant in this semiarid region and may have unique traits allowing them to thrive under dryland conditions. We also demonstrated that, in addition to mean annual precipitation, the agroclimatic zone and soil silt content significantly affect the diversity of indigenous Phz$^+$ communities. These results contribute to the rational exploitation of beneficial microbial communities for the purpose of improving plant health and managing soilborne pathogens in agroecosystems.

Nucleotide sequence accession numbers. MLSA and *phzF* DNA sequences were deposited in the NCBI GenBank database with accession numbers KC834074 to KC834377 and KC842530 to KC843059, respectively.
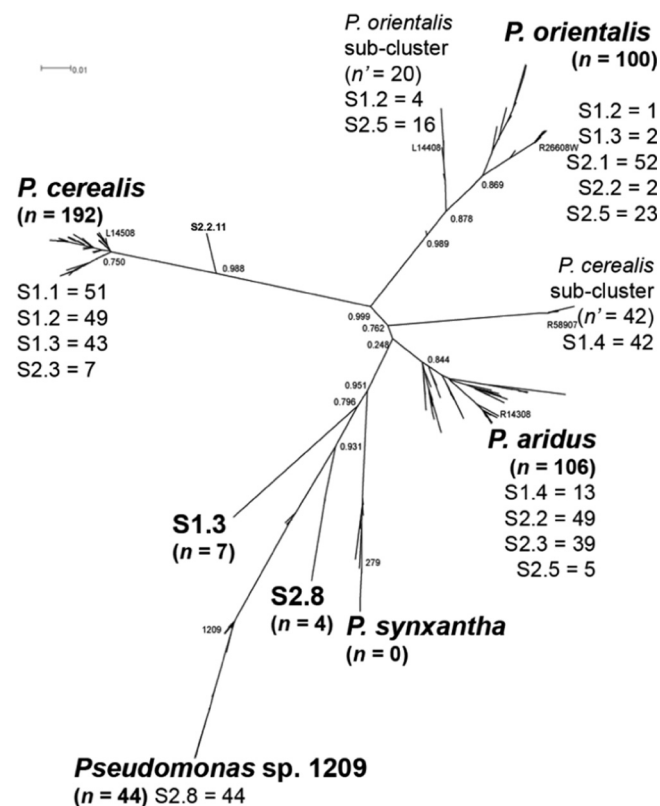
FIG 3 Unrooted maximum-likelihood phylogenetic tree of partial (389 nt) *phzF* DNA sequences obtained from representative strains featured in this (*n* = 76) and previous (*n* = 37) (1, 5) studies, as well as from samples of rhizosphere DNA (*n* = 454). The total number of clones within the cluster (*n*) or subcluster (*n'*) specific to each geographic site is indicated. A representative strain from the MLSA analysis is also shown for each cluster.

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