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Genome-Derived Criteria for Assigning Environmental *narG* and *nosZ* Sequences to Operational Taxonomic Units of Nitrate Reducers▽†

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Ninety percent of cultured bacterial nitrate reducers with a 16S rRNA gene similarity of ≥97% had a *narG* or *nosZ* similarity of ≥67% or ≥80%, respectively, suggesting that 67% and 80% could be used as standardized, conservative threshold similarity values for *narG* and *nosZ*, respectively (i.e., any two sequences that are less similar than the threshold similarity value have a very high probability of belonging to different species), for estimating species-level operational taxonomic units. Genus-level tree topologies of *narG* and *nosZ* were generally similar to those of the corresponding 16S rRNA genes. Although some genomes contained multiple copies of *narG*, recent horizontal gene transfer of *narG* was not apparent.

Nitrate reducers (i.e., both dissimilatory nitrate reducers and denitrifiers) reduce nitrate to nitrite, which can then be reduced to ammonium by dissimilatory nitrate reducers or sequentially reduced to nitric oxide, nitrous oxide, and dinitrogen by denitrifiers (29). *narG* codes for the alpha subunit of the dissimilatory nitrate reductase, which reduces nitrate to nitrite and is thus common to both dissimilatory nitrate reducers and denitrifiers (29). *nosZ* codes for nitrous oxide reductase, which reduces nitrous oxide to dinitrogen and is common to denitrifiers but not dissimilatory nitrate reducers (29). Both *narG* and *nosZ* are commonly used as gene markers for community level analysis of nitrate reducers (2, 8, 9, 16, 18, 19, 20, 25). However, standardized criteria for assigning environmental *narG* and *nosZ* sequences to operational taxonomic units (OTUs) are required so that diverse data sets on nitrate-reducing communities can be normalized. The widespread ability of bacteria and archaea to denitrify (29) complicates the development of such criteria for genes involved in denitrification. Some closely related *narG* and closely related *nosZ* genes occur in distantly related taxa, and *narG* or *nosZ* phylogenies do not always reflect 16S rRNA phylogenies (17). However, *nosZ*-based phylogenies in general have a high degree of congruency with 16S rRNA gene-based phylogenies (3, 10, 30), and recent horizontal gene transfer of *nosZ* seems unlikely (10), indicating that denitrifier structural genes might be used for estimating the species-level novelty, as well as species-level diversity, of denitrifiers in environmental samples. The limited amount of data on horizontal gene transfer of *narG* (4, 24) identifies a need to extend such an approach to this gene. The limited number of studies that have compared 16S rRNA with *narG* or *nosZ* phylogenies accentuates the need for a more thorough analysis of the phylogenetic relatedness of these three genes (3, 4, 7). Thus, the main objectives of this study were to (i) resolve criteria for standardizing OTU assignment of environmental *narG* and *nosZ* sequences, (ii) determine whether those criteria can be used as indicators of novel species, and (iii) investigate the impact of horizontal gene transfer on *narG*.

Analysis of *narG* and *nosZ* sequence data. One hundred fourteen *narG* and 85 *nosZ* sequences from pure cultures were retrieved from GenBank along with the associated 16S rRNA gene sequences (see Table S1 in the supplemental material). The fragments of *narG*, *nosZ*, and the 16S rRNA gene that were analyzed correspond to regions amplified by the commonly used primers narG1960f/-2650r (18), nosZF/-R (19), and 27F/1492R (14), respectively. Alignments of 16S rRNA gene fragments and in silico-translated *narG* and *nosZ* fragments were performed with ClustalW implemented in MEGA 4.0 (13) and manually refined. The number of base or amino acid differences per site (D) was calculated for pairwise comparisons of all *narG*, *nosZ*, and 16S rRNA gene fragments. The similarity (S) was expressed as $S = 1 - D$. The percent similarity of *narG* or *nosZ* from nucleic acid- and amino acid-based comparisons was plotted against the percent similarity of the corresponding 16S rRNA gene. This approach is similar to that used for derivation of 16S rRNA gene-based criteria for species delineation, which is based on DNA/DNA similarities (as a valid criterion to distinguish two species) (28).

Phylogenetic trees based on nucleotide sequences of 16S rRNA gene, *narG*, and *nosZ* fragments were calculated in MEGA 4.0 (13) using the neighbor-joining algorithm (21) and 500 bootstrap replications (5). Tree topologies were compared to evaluate the use of *narG* and *nosZ* as indicators of new species.

The impact of horizontal gene transfer on *narG* diversity was assessed by locating putatively horizontally transferred genes in whole-genome sequences of species using the score-based identification of genomic islands–hidden Markov model algorithm (SIGI-HMM) as implemented in Colombo (http://www.tcs.informatik.uni-goettingen.de/colombo-sigihmm), which identifies putatively alien genes based on differences in codon usage (27), and using SeqWord, which can be used to identify putatively alien genes based on oligonucleotide biases (6).

Correlations between structural gene sequences, species diversity, and horizontal gene transfer. A 16S rRNA gene similarity of ≥97% (a conservative threshold similarity for assign-

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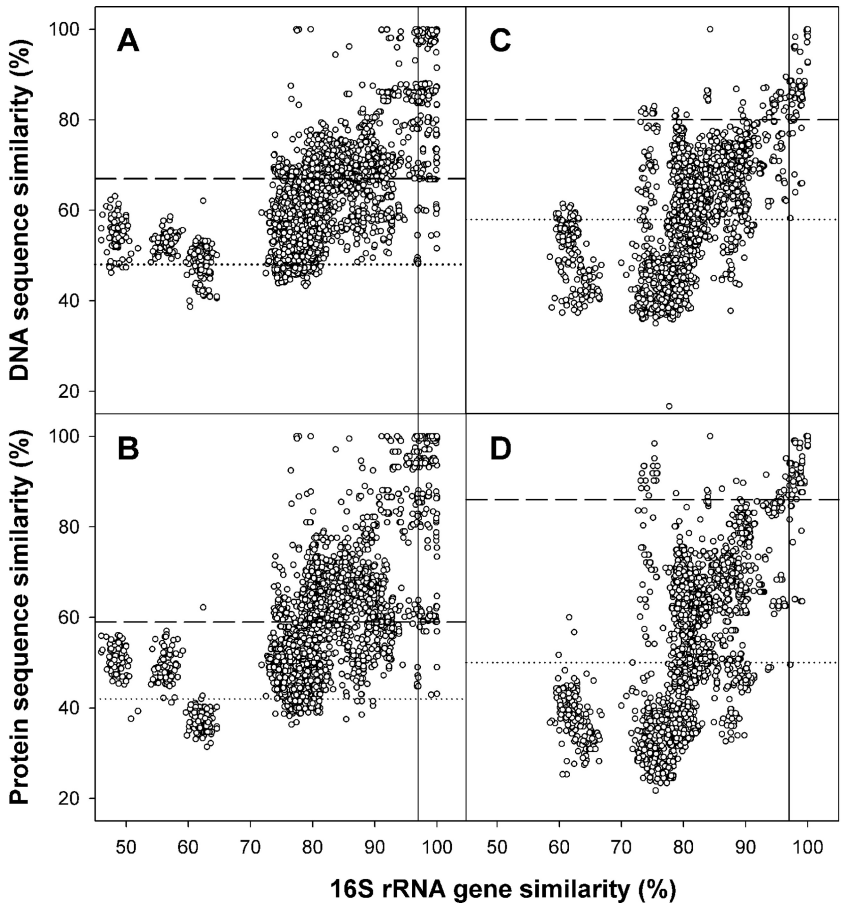
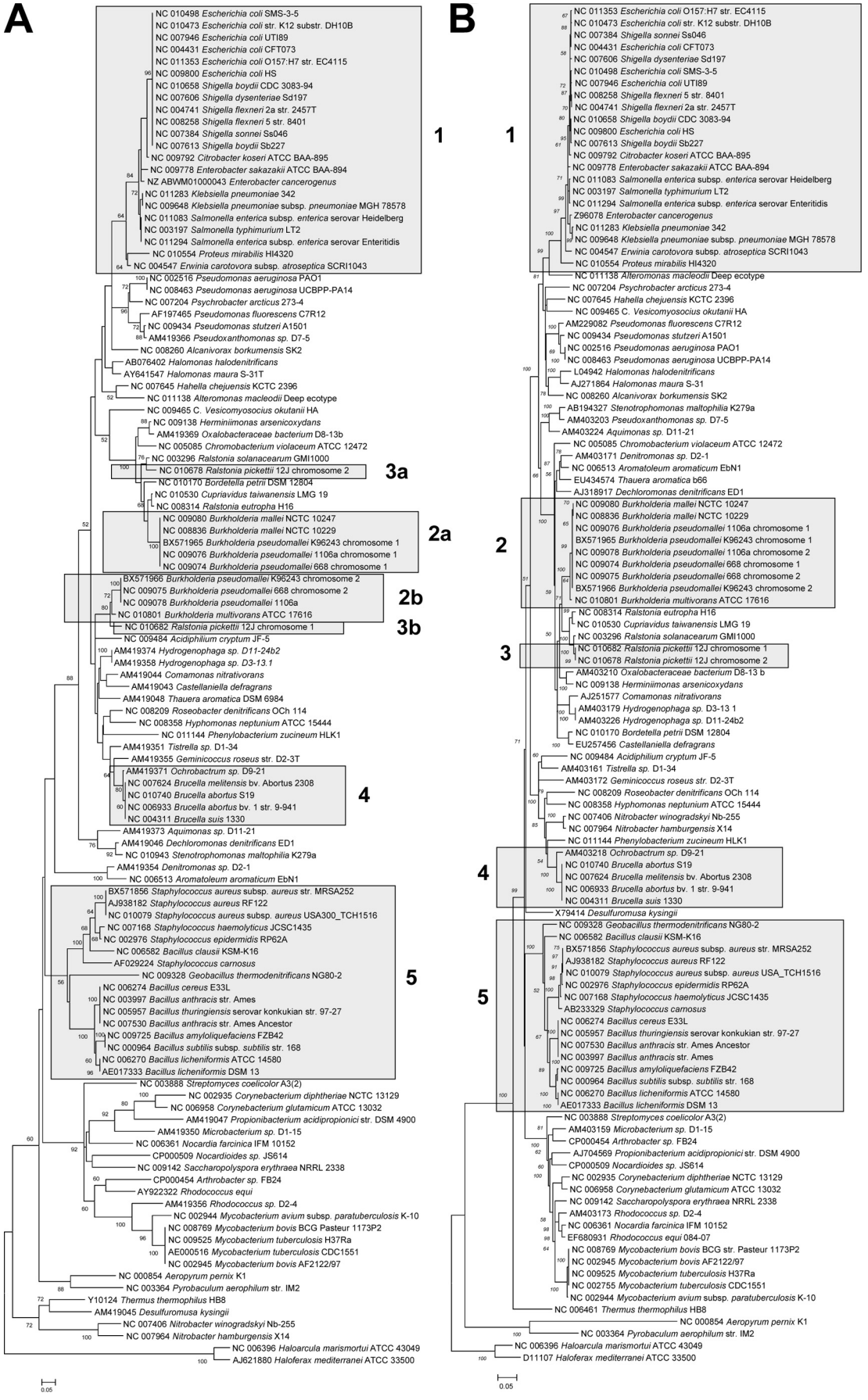


FIG. 1. Correlation of DNA and in silico-translated amino acid sequence similarities of *narG* (A, B) and *nosZ* (C, D) versus 16S rRNA gene similarity. Dotted lines represent the similarity values, below which two sequences always had less than 97% 16S rRNA gene sequence similarity. The dashed lines represent the 90% quantile of pairwise sequence comparisons with a 16S rRNA gene sequence similarity of 97% (i.e., threshold similarity). The solid lines mark the 97% 16S rRNA gene similarities.

ing two organisms to different species [22, 23]) corresponded to an average *narG* sequence similarity of 85% (86% for translated amino acids). Nitrate reducers that had a <48% *narG* sequence similarity (or <42% for translated amino acids) always had a 16S rRNA gene similarity of <97% (Fig. 1) and therefore belonged to different species. Ninety percent of the nitrate reducers with a 16S rRNA gene similarity of ≥97% had a *narG* sequence similarity of ≥67% and a *narG* translated amino acid similarity of ≥59%. The same gene sequence and translated amino acid similarities were obtained for 90% of nitrate reducers with a 16S rRNA gene similarity of ≥98.7%, which has recently been suggested as a threshold similarity for species differentiation (22). The correlation analysis suggests that a *narG* sequence similarity of 67% and a *narG* amino acid similarity of 59% could be used as threshold similarities (i.e., any two sequences that are less similar than the threshold similarity value have a very high probability of belonging to different species) for estimating *narG*-derived species-level OTUs. These relationships are indicative of different evolutionary rates for *narG* and the 16S rRNA gene. Tree topologies of *narG* and 16S rRNA phylogenies were generally similar at the genus level, since species of the same genus mostly clustered together in both trees (Fig. 2). However, multiple copies of *narG* were present in some species. In such cases, the multiple *narG* copies clustered separately (Fig. 2), while the multiple copies of the 16S rRNA genes from the organisms analyzed clustered closely together (data not shown).

The percentage of putatively alien genes found in whole-genome sequences ranged from 0% to 20.6% based on codon usage analysis, and the Nar operon was predicted to be putatively alien only in *Pseudomonas stutzeri* A1501 and was estimated to be transferred from the *Gammaproteobacteria* (see Table S1 in the supplemental material). In *Nitrobacter winogradskyi* Nb-255, *narI* and *narJ* were identified as putatively alien genes transferred from the *Gammaproteobacteria* (see Table S1 in the supplemental material). It appeared to be unlikely, based on oligonucleotide bias, that the Nar operon was horizontally transferred in any species (e.g., see Fig. S1 to S3 in the supplemental material).

A 16S rRNA gene similarity of ≥97% (a conservative threshold similarity for assigning two organisms to different species [22, 23]) corresponded to an average *nosZ* sequence similarity of 89% (92% for translated amino acids). Denitrifiers with a <58% *nosZ* similarity (or <50% similarity of translated amino acids) always had a 16S rRNA gene similarity of <97% (Fig. 1) and therefore belonged to different species.


A
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NC 010473 Escherichia coli str. K12 substr. DH10B
NC 007946 Escherichia coli UTI89
NC 004431 Escherichia coli CFT073
NC 011353 Escherichia coli O157:H7 str. EC4115
NC 009800 Escherichia coli HS
NC 010658 Shigella boydii CDC 3083-94
NC 007606 Shigella dysenteriae Sd197
NC 004741 Shigella flexneri 2a str. 2457T
NC 008258 Shigella flexneri 5 str. 8401
NC 007384 Shigella sonnei Ss046
NC 007613 Shigella boydii Sb227
NC 009792 Citrobacter koseri ATCC BAA-895
NC 009778 Enterobacter sakazakii ATCC BAA-894
NZ ABWM01000043 Enterobacter cancerogenus
NC 011283 Klebsiella pneumoniae 342
NC 009648 Klebsiella pneumoniae subsp. pneumoniae MGH 78578
NC 011083 Salmonella enterica subsp. enterica serovar Heidelberg
NC 003197 Salmonella typhimurium LT2
NC 011294 Salmonella enterica subsp. enterica serovar Enteritidis
NC 010554 Proteus mirabilis HI4320
NC 004547 Erwinia carotovora subsp. atroseptica SCRI1043
1
NC 002516 Pseudomonas aeruginosa PAO1
NC 008463 Pseudomonas aeruginosa UCBPP-PA14
NC 007204 Psychrobacter arcticus 273-4
AF197465 Pseudomonas fluorescens C7R12
NC 009434 Pseudomonas stutzeri A1501
AM419366 Pseudoxanthomonas sp. D7-5
NC 008260 Alcanivorax borkumensis SK2
AB076402 Halomonas halodenitrificans
AY641547 Halomonas maura S-31T
NC 007645 Hahella chejuensis KCTC 2396
NC 011138 Alteromonas macleodii Deep ecotype
NC 009465 C. Vesicomyosocius okutanii HA
NC 009138 Herminiimonas arsenicoxydans
AM419369 Oxalobacteraceae bacterium D8-13b
NC 005085 Chromobacterium violaceum ATCC 12472
NC 003296 Ralstonia solanacearum GMI1000
NC 010678 Ralstonia pickettii 12J chromosome 2
3a
NC 010170 Bordetella petrii DSM 12804
NC 010530 Cupriavidus taiwanensis LMG 19
NC 008314 Ralstonia eutropha H16
NC 009080 Burkholderia mallei NCTC 10247
NC 008836 Burkholderia mallei NCTC 10229
BX571965 Burkholderia pseudomallei K96243 chromosome 1
NC 009076 Burkholderia pseudomallei 1106a chromosome 1
NC 009074 Burkholderia pseudomallei 668 chromosome 1
2a
BX571966 Burkholderia pseudomallei K96243 chromosome 2
NC 009075 Burkholderia pseudomallei 668 chromosome 2
NC 009078 Burkholderia pseudomallei 1106a
NC 010801 Burkholderia multivorans ATCC 17616
2b
NC 010682 Ralstonia pickettii 12J chromosome 1
3b
NC 009484 Acidiphilium cryptum JF-5
AM419374 Hydrogenophaga sp. D11-24b2
AM419358 Hydrogenophaga sp. D3-13.1
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AM419043 Castellaniella defragrans
AM419048 Thauera aromatica DSM 6984
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NC 008358 Hyphomonas neptunium ATCC 15444
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AM419351 Tistrella sp. D1-34
AM419355 Geminicoccus roseus str. D2-3T
AM419371 Ochrobactrum sp. D9-21
NC 007624 Brucella melitensis bv. Abortus 2308
NC 010740 Brucella abortus S19
NC 006933 Brucella abortus bv. 1 str. 9-941
NC 004311 Brucella suis 1330
4
AM419373 Aquimonas sp. D11-21
AM419046 Dechloromonas denitrificans ED1
NC 010943 Stenotrophomonas maltophilia K279a
AM419354 Denitromonas sp. D2-1
NC 006513 Aromatoleum aromaticum EbN1
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NC 010079 Staphylococcus aureus subsp. aureus USA300_TCH1516
NC 007168 Staphylococcus haemolyticus JCSC1435
NC 002976 Staphylococcus epidermidis RP62A
NC 006582 Bacillus clausii KSM-K16
AF029224 Staphylococcus carnosus
NC 009328 Geobacillus thermodenitrificans NG80-2
NC 006274 Bacillus cereus E33L
NC 003997 Bacillus anthracis str. Ames
NC 005957 Bacillus thuringiensis serovar konkukian str. 97-27
NC 007530 Bacillus anthracis str. Ames Ancestor
NC 009725 Bacillus amyloliquefaciens FZB42
NC 000964 Bacillus subtilis subsp. subtilis str. 168
NC 006270 Bacillus licheniformis ATCC 14580
AE017333 Bacillus licheniformis DSM 13
5
NC 003888 Streptomyces coelicolor A3(2)
NC 002935 Corynebacterium diphtheriae NCTC 13129
NC 006958 Corynebacterium glutamicum ATCC 13032
AM419047 Propionibacterium acidipropionici str. DSM 4900
AM419350 Microbacterium sp. D1-15
NC 006361 Nocardia farcinica IFM 10152
CP000509 Nocardioides sp. JS614
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CP000454 Arthrobacter sp. FB24
AY922322 Rhodococcus equi
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NC 002944 Mycobacterium avium subsp. paratuberculosis K-10
NC 008769 Mycobacterium bovis BCG Pasteur 1173P2
NC 009525 Mycobacterium tuberculosis H37Ra
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NC 007406 Nitrobacter winogradskyi Nb-255
NC 007964 Nitrobacter hamburgensis X14
NC 006396 Haloarcula marismortui ATCC 43049
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B
NC 011353 Escherichia coli O157:H7 str. EC4115
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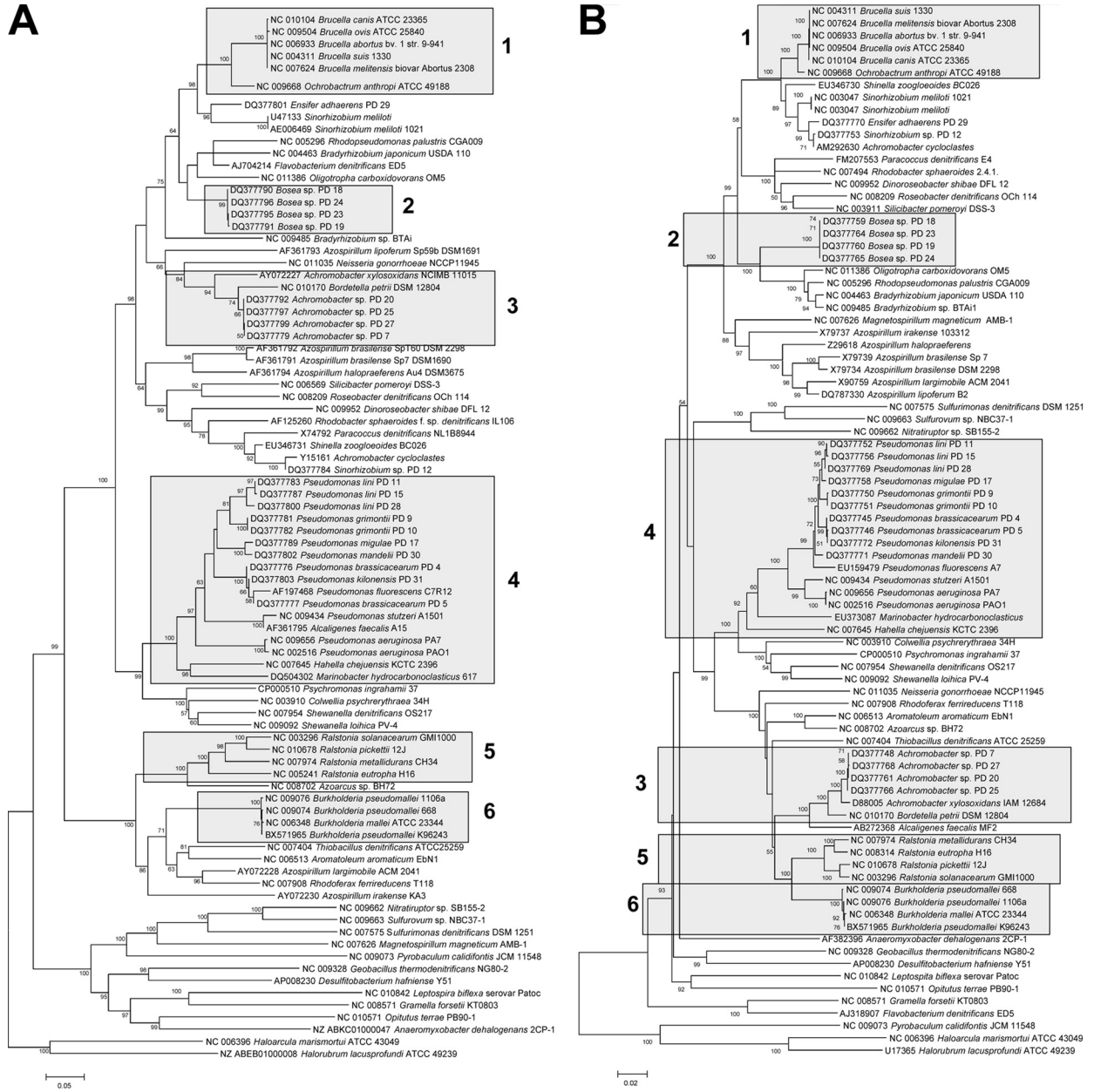

FIG. 3. Comparison of *nosZ* (A) and 16S rRNA (B) phylogenies. Boxes 1 to 6 display examples of closely related species clustering together in the *nosZ* tree. Not all sequences of *Azospirillum* sp. cluster together in the *nosZ* tree. Neighbor-joining trees were constructed from nucleotide sequences of *nosZ* and 16S fragments with approximate lengths of 700 and 1,400 bp, respectively. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. Bootstrap supports below 50% are not shown. The bars represent estimated sequence dissimilarities of 5% (A) and 2% (B).

Ninety percent of denitrifiers with a 16S rRNA gene similarity of ≥97% had a *nosZ* sequence similarity of ≥80% and a translated amino acid similarity of ≥86%. Ninety percent of denitrifiers with a 16S similarity of ≥98.7% had a *nosZ* sequence similarity of ≥85% and a translated amino acid similarity of ≥90%. The correlation analysis suggests that a *nosZ* sequence similarity of 80% and a *nosZ* amino acid similarity of 86% could be used as threshold similarities for estimating

FIG. 2. Comparison of *narG* (A) and 16S rRNA (B) phylogenies. Boxes 1, 4, and 5 display examples of closely related species clustering together in the *narG* tree. Boxes 2 and 3 illustrate that multiple copies of *narG* from the same organism cluster separately. Neighbor-joining trees were constructed from nucleotide sequences of *narG* and 16S fragments with approximate lengths of 650 and 1,400 bp, respectively. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. Bootstrap supports below 50% are not shown. The bars represent estimated sequence dissimilarities of 5% (A) and 0.1% (B).

nosZ-derived species-level OTUs with a very high probability of estimating correctly. The calculated threshold similarities are in good agreement with those calculated for the *nosZ* fragment amplified by the primers nosZ661F/-1773R (9). Phylogenetic trees of *nosZ* and 16S rRNA genes had similar clustering at the genus level (Fig. 3). Multiple copies of *nosZ* per organism were not found. The different threshold similarities of *nosZ* and *narG* suggest that *nosZ* is more conserved than *narG*, indicating that species-level OTU assignment of *nosZ* might be more reliable than that of *narG*.

Conclusions. The above considerations indicate that *narG* or *nosZ* analyses can be used to estimate species-level diversity of the associated bacteria on the basis of sequence similarities. Such analysis is considered to yield a minimum number of species in a sample (i.e., the true species-level diversity might be much higher). Analyses of gene markers of different functional groups revealed similar threshold similarities indicating species-level OTUs (e.g., for *dsrAB* and *amoA* gene fragments, they were 80 to 90%) (11, 12). Since *narG* or *nosZ* sequences from organisms of the same genus generally form coherent clusters in phylogenetic trees (Fig. 2 and 3), distinct clusters of environmental sequences could provide evidence for new genus-level diversity. Recent horizontal gene transfer does not appear to have occurred for either *narG* (as indicated by the results in this study) or *nosZ* (as indicated by the results in reference 10). The adaptation of genomic features of an alien gene to those of a host genome takes several hundred million years (15), and genes that were horizontally transferred 50 million years ago can be reliably detected by sequence analyses (26). Sequence differences might also be caused by gene duplication and diversification (e.g., in species in which multiple gene copies are present) rather than by horizontal gene transfer (1). However, analysis of codon usage and oligonucleotide bias does not always reveal the transfer of genes from closely related organisms with similar genomic features (27).

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