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Reassessing PCR primers targeting *nirS*, *nirK* and *nosZ* genes for community surveys of denitrifying bacteria with DGGE

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Abstract

We re-evaluated PCR primers targeting *nirS*, *nirK* and *nosZ* genes for denaturing gradient gel electrophoresis as a tool to survey denitrifying community composition in environmental samples. New primers for both *nirS* and *nosZ* were combined with existing primers, while for *nirK* the previously published F1aCu:R3Cu set was chosen for denaturing electrophoresis. All three sets yielded amplicons smaller than 500 bp and amplified the correct fragment in all environmental samples. The denaturing gradient gel electrophoresis worked satisfactorily for *nirK* and *nosZ*, but not for *nirS*. This was probably due to the multiple melting domains in this particular *nirS* fragment. From the excised and sequenced bands, only sequences related to the target genes were detected and tree analysis showed that the selected primers acted as broad range primers for each of the three genes. By use of the new *nirS* primers it was demonstrated that agricultural soil harbours a substantial diversity of *nirS* denitrifiers.
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Keywords: Diversity; Denitrification; Soil; Peat; Activated sludge; Functional genes; Environmental clones; Nitrite reductase; Nitrous oxide reductase; Uncultured bacteria

1. Introduction

Community and diversity surveys of bacteria have for nearly 10 years focused on the phylogenetic diversity of bacteria in the environment. However, species composition based on 16S rRNA analysis only provides marginal information on communities and certain metabolic groups. An exciting direction in molecular ecology is the analysis of genes encoding important functions for the ecosystems. The analysis of functional diversity and its physiological dynamics is essential for improved understanding of the microbial ecology and biogeochemistry of different environments. Generally, functional genes have more sequence variation than the relatively conserved 16S rRNA genes. They can therefore be exploited as biomarkers to discriminate between closely related but ecologically different populations [1]. Moreover, some important functions are not associated with a specific taxonomic group. One example is denitrification, which is a functional trait found within more than 50 genera [2].

Denitrification is the stepwise reduction of nitrate (NO_3^-) to dinitrogen (N_2), associated with oxidative phosphorylation and release of nitric oxide (NO) and nitrous oxide (N_2O) gases. This process is of global concern since it leads to nitrogen losses from agricultural soils and because nitrous oxide contributes to ozone depletion in the stratosphere and is a potent greenhouse gas [3]. Denitrifying bacteria can also be used to remove excess nitrogen in wastewater treatment plants and to degrade organic pollutants. Most bacteria with this functional trait belong to a wide range of various subclasses of *Proteobacteria*. However, the ability to denitrify has also been found in some *Archaea*, in the halophilic and hyperthermophilic branches, and in mitochondria of certain fungi [2]. Lateral gene transfer is the most likely explanation for this widespread ability to denitrify [4].

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Functional genes that encode for the enzymes involved in the denitrification pathway, such as nitrite, nitric oxide and nitrous oxide reductases, can be exploited by targeting conserved regions. The reduction of nitrite (NO_2^-) to nitric oxide distinguishes denitrifiers from other nitrate-respiring bacteria [2]. This reaction is central to denitrification and is catalysed by two different types of nitrite reductases (Nir), either a cytochrome cd_1 enzyme encoded by *nirS* or a Cu-containing enzyme encoded by *nirK*. The reduction of nitrous oxide is the last step in the denitrification pathway and is catalysed by nitrous oxide reductase encoded by the *nosZ* gene. However, some denitrifiers lack this enzyme. The *nosZ* gene can be used as a target for the different populations of the denitrifying bacteria capable of nitrous oxide reduction. Most work on molecular ecology of denitrifying bacteria has been based on *nirK* and *nirS*, as well as *nosZ*, e.g. [5,6]. Recently, the gene, *norB*, encoding nitric oxide reductase was used as a marker for denitrifying bacteria in freshwater and marine sediments [7].

Surveys of bacterial community composition in environmental samples are often based on the polymerase chain reaction (PCR). Reliable PCR primers are a prerequisite for microbial community surveys since they ultimately determine what is detected in the environmental sample. Ward [8] made the first attempt to design PCR primers targeting the *nirS* gene and they were based on only three sequences from two different species. A better attempt to amplify *nirK* and *nirS* was published in 1998 [9], and alternatives or modifications followed [10–15]. Primers for detection of the *nosZ* gene were first published in 1998 [16] and these were later made more degenerate [17]. Kloos et al. [18] developed more pertinent broad range primers for *nosZ*. The different *nir* and *nosZ* primers have mainly been used to study community composition of denitrifying bacteria in marine sediments [5,6,16,19–22] but also in estuarine sediments [17], cyanobacterial bloom [23], soil [13,24–29], wastewater treatment reactors [30,31] and groundwater [15]. The oldest and most frequently used primers to detect denitrifying bacteria target *nirS* and *nirK* [9] and were designed based on a limited number of sequences, mainly from laboratory strains. Since then the number of partial *nir* and *nosZ* sequences deposited in the GenBank have increased almost a 100-fold and it has become apparent that the primer sites are more variable than previously shown.

The aim of our study was to re-evaluate primers for amplification of *nirS*, *nirK* and *nosZ* gene fragments in silico and in vitro, and to introduce denaturing gradient gel electrophoresis (DGGE) [32] as a tool to survey the denitrifying community composition in environmental samples. A limiting factor when designing primers for DGGE is that the fragments should not be much longer than 500 bp for successful analysis [33]. Nevertheless, the most commonly used primers for the denitrifying genes are not suitable for DGGE since they amplify fragments that are approximately 600–1100 bp. DGGE of partial 16S rDNA has been successfully employed for analysis of community DNA even in such complex environments as soil (e.g. [34]). However, the use of DGGE with functional genes is still in its beginnings.

2. Materials and methods

2.1. Bacterial strains, growth conditions and environmental samples

Twenty one of the 28 denitrifying strains used in this study (Table 1) were cultivated aerobically in nutrient broth (Oxoid, Basingstoke, UK) at 30 °C. *Bradyrhizobium japonicum* 526 and *Rhizobium meliloti* 50 were grown at 30 °C in yeast extract mannitol medium [35]. *Blastobacter denitrificans* DSM 1113 was grown in peptone yeast extract glucose medium (DSM medium 621) at 40 °C and *Hyphomicrobium denitrificans* DSM 1869 in methylamine hydrochloride medium (DSM medium 166) at 30 °C. *Rhodobacter sphaeroides* DSM 158 was grown anaerobically in the presence of light in a medium containing yeast extract and ethanol (DSM medium 27) at 25 °C. Both *Pseudomonas aeruginosa* CCUG 241 and *P. aeruginosa* Mi11 were cultivated in nutrient broth at 37 °C. The cells were harvested by centrifugation (14,000 rpm, 10 min, 22 °C), washed twice in sodium phosphate (4.5 mM K_2HPO_4 and 1.2 mM KH_2PO_4, pH 7.3) buffer and stored in 15% glycerol at −70 °C. As controls, DNA from the three non-denitrifying strains *Escherichia coli* TG1 [36], *Lactobacillus reuteri* DSM 20016 and *Staphylococcus aureus* 8325-4 [37], was kindly provided by colleagues at the Department of Microbiology, SLU, Sweden.

Six environmental samples were used to evaluate the primers and the DGGE analysis. Samples of arable soil were collected from the upper 20 cm layer at three different sites (Alunda [A], Brunnby, [B] and Ulleråker [U]) in the Mälar region in Sweden (Table 2). Each soil was sieved (<4 mm) and thoroughly mixed. One peat sample (P) was collected from the true peat layer of a small fen (ca. 1 ha) in a forested area close to Uppsala, Sweden. The vegetation was dominated by *Sphagnum* mosses, and *Vaccinium oxycoccus*, *Eriophorum vaginatum* and *Potentilla palustris* were the most common vascular plants. The sample was collected from just below the level of the water table at a depth of 20–30 cm below the moss surface. Activated sludge samples were collected from full-scale nitrogen removal processes at the Kungsängen (K) and Henriksdal (H) municipal wastewater treatment plants in Uppsala and Stockholm, respectively. One-millilitre portions of the fresh sludge samples were centrifuged at 14,000 rpm for 10 min and the su-

Table 1
Bacterial strains used in this study, and results of PCR amplification of denitrifying genes (*nirS*, *nirK* and *nosZ*) with different sets of primers

Bacterial strains	*nirS*[e]															
	F1acd: R4bcd	F1dcd: R3cd	F1dcd: nirS4R	F1dcd: nirS6R	nirS1F: R4bcd	nirS1F: R3cd	nirS1F: nirS4R	nirS1F: nirS6R	nirS3F: R4cd	nirS3F: nirS6R	nirS3Fa: R3bcd	nirS3Fa: nirS4R	nirS4F: R4bcd	nirS4F: nirS6R	cd3aF: R4cd	cd3aF: R3cd
Denitrifying strains with *nirS*																
Alcaligenes denitrificans CCUG 407T	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	+
Comamonas testosteroni CCUG 1426T	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+
Paracoccus denitrificans CCUG 13798	+	+	+	+	+	–	–	+	+	M	+	+	+	+	+	+
Paracoccus denitrificans CCUG 30144	–	+	–	–	–	–	–	–	–	–	–	–	+	+	+	+
Paracoccus denitrificans Pd1222[a]	+	+	+	+	–	–	+	–	–	–	+	–	–	+	+	+
Pseudomonas aeruginosa CCUG 241	+	+	+	+	+	+	+	+	+	M	–	+	+	+	+	+
Pseudomonas aeruginosa Mi11[b]	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+	+
Pseudomonas fluorescens ATCC 33512	+	–	–	–	+	–	–	–	–	M	+	+	–	–	+	+
Pseudomonas fluorescens Mi32[b]	+	+	+	+	+	+	–	+	+	–	–	–	+	+	+	+
Pseudomonas putida CCUG 2479	–	+	+	–	M	–	+	–	+	M	+	–	–	–	–	–
Pseudomonas stutzeri ATCC 14405	+	+	+	+	+	+	+	M	+	M	+	+	+	+	+	+
Pseudomonas stutzeri CCUG 29240	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+	+
Ralstonia eutropha ATCC 17699	–	+	+	–	–	–	–	+	+	–	–	–	–	–	–	+
Ralstonia eutropha CCUG 13724	+	+	+	+	+	+	M	M	–	–	+	+	+	–	+	+
Denitrifying strains with *nirK*																
Alcaligenes sp. DSM 30128																
Alcaligenes faecalis ATCC 8750	+	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–
Alcaligenes faecalis CCUG 2348																
Achromobacter cycloclastes ATCC 21921																
Blastobacter denitrificans DSM 1113																
Bradyrhizobium japonicum 526[b]																
Hyphomicrobium denitrificans DSM 1869																
Nitrosomonas europaea NCIMB 11850																
Nitrosospira multiformis NCIMB 11849																
Pseudomonas denitrificans CCUG 1783																
Pseudomonas denitrificans CCUG 2519																
Rhizobium meliloti 50[b]																
Rhodobacter sphaeroides DSM 158																
Strain R51g[c]																
Environmental samples																
Alunda soil	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+
Brunnby soil	–	–	–	–	+	+	+	+	–	–	–	–	+	–	+	+
Ulleråker soil	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+
Sludge from Henriksdals WWTP[d]	–	+	+	+	+	+	–	+	–	–	+	–	+	+	–	+
Sludge from Kungsängens WWTP[d]	–	+	+	+	+	–	–	+	–	+	+	–	+	+	–	+
Peat	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	+

Table 1 (*continued*)

	nirS[e]				*nirK*[e]								
	cd3aF: nirS4R	cd3bF: nirS6R	F3nirS: R4bcd	F3nirS: NirS6R	F1aCu: R3Cu	F1aCu: nirK3R	F1aCu: nirK5R	Cunir3: R3Cu	Cunir3: nirK3R	Cunir3: nirK5R	nirK1F: R3Cu	nirK1F: nirK3R	nirK1F: nirK5R
Denitrifying strains with *nirS*													
Alcaligenes denitrificans CCUG 407T	–	–	–	+									
Comamonas testosteroni CCUG 1426T	–	–	–	–									
Paracoccus denitrificans CCUG 13798	+	+	+	+									
Paracoccus denitrificans CCUG 30144	+	+	–	–									
Paracoccus denitrificans Pd1222[a]	+	–	–	–									
Pseudomonas aeruginosa CCUG 241	+	+	+	+									
Pseudomonas aeruginosa Mi11[b]	+	+	+	+									
Pseudomonas fluorescens ATCC 33512	+	–	–	–									
Pseudomonas fluorescens Mi32[b]	+	+	+	–									
Pseudomonas putida CCUG 2479	–	–	–	–									
Pseudomonas stutzeri ATCC 14405	+	+	+	+	–	–	–	–	–	–	–	–	–
Pseudomonas stutzeri CCUG 29240	+	+	+	+									
Ralstonia eutropha ATCC 17699	+	–	–	–									
Ralstonia eutropha CCUG 13724	+	+	+	+									
Denitrifying strains with *nirK*													
Alcaligenes sp. DSM 30128					+	–	+	–	–	–	+	+	+
Alcaligenes faecalis ATCC 8750	–	–	–	+	+	+	+	M	M	–	–	–	–
Alcaligenes faecalis CCUG 2348					–	–	+	–	–	–	–	–	–
Achromobacter cycloclastes ATCC 21921					+	–	+	–	+	M	+	+	+
Blastobacter denitrificans DSM 1113					+	+	+	M	+	M	+	+	+
Bradyrhizobium japonicum 526[b]					–	–	–	–	–	–	–	–	–
Hyphomicrobium denitrificans DSM 1869					+	+	M	–	–	M	+	+	+
Nitrosomonas europaea NCIMB 11850					–	–	–	–	–	–	–	–	–
Nitrosospira multiformis NCIMB 11849					+	+	–	M	M	–	–	–	–
Pseudomonas denitrificans CCUG 1783					+	+	–	M	–	M	+	M	+
Pseudomonas denitrificans CCUG 2519					+	+	M	–	–	M	M	–	M
Rhizobium meliloti 50[b]					+	–	–	–	–	–	+	+	+
Rhodobacter sphaeroides DSM 158					–	–	–	–	–	–	–	–	–
Strain R51g[c]					+	–	–	M	+	–	–	–	–
Environmental samples													
Alunda soil	–	–	–	–	+	–	–	–	–	–	–	–	M
Brunnby soil	+	–	+	–	+	–	+	–	–	M	+	–	M
Ulleråker soil	–	–	–	–	+	–	–	+	–	M	+	–	M
Sludge from Henriksdals WWTP[d]	+	–	+	–	+	+	+	+	–	–	+	+	M
Sludge from Kungsängens WWTP[d]	+	–	+	–	+	+	+	+	+	M	+	+	M
Peat	–	–	–	–	+	+	+	+	+	–	+	–	M

	nosZ[e]						
	nosZF: nosZR	nosZF: Nos1773R	nosZF: nosZ1622R	Nos661F: Nos1527R	Nos661F: Nos1773R	Nos1527F: Nos1773R	nosLb: nosRb
Denitrifying strains with *nirS*							
Alcaligenes denitrificans CCUG 407T	+	+	+	+	+	+	–
Comamonas testosteroni CCUG 1426T	–	–	+	–	–	–	–
Paracoccus denitrificans CCUG 13798	+	+	+	+	+	+	+
Paracoccus denitrificans CCUG 30144	–	+	+	–	–	–	+
Paracoccus denitrificans Pd1222[a]	+	+	+	+	+	+	–
Pseudomonas aeruginosa CCUG 241	+	+	+	–	–	+	+
Pseudomonas aeruginosa Mi11[b]	+	+	+	+	+	+	+
Pseudomonas fluorescens ATCC 33512	M	+	+	+	–	+	+
Pseudomonas fluorescens Mi32[b]	–	+	+	+	–	–	+
Pseudomonas putida CCUG 2479	–	+	–	–	–	–	–
Pseudomonas stutzeri ATCC 14405	+	+	+	+	+	+	+
Pseudomonas stutzeri CCUG 29240	+	+	+	+	+	+	+
Ralstonia eutropha ATCC 17699	–	+	+	+	–	+	–
Ralstonia eutropha CCUG 13724	+	+	+	+	+	+	–
Denitrifying strains with *nirK*							
Alcaligenes sp. DSM 30128	+	+	+	–	–	–	–
Alcaligenes faecalis ATCC 8750	+	+	+	+	–	+	–
Alcaligenes faecalis CCUG 2348	+	+	+	–	–	+	–
Achromobacter cycloclastes ATCC 21921	+	–	+	–	–	+	–
Blastobacter denitrificans DSM 1113	+	+	+	–	–	–	–
Bradyrhizobium japonicum 526[b]	–	–	–	–	–	–	–
Hyphomicrobium denitrificans DSM 1869	+	+	+	–	–	–	–
Nitrosomonas europaea NCIMB 11850	–	–	+	–	–	–	–
Nitrosospira multiformis NCIMB 11849	–	–	+	–	–	–	–
Pseudomonas denitrificans CCUG 1783	+	+	+	+	+	+	–
Pseudomonas denitrificans CCUG 2519	–	+	+	–	–	–	–
Rhizobium meliloti 50[b]	+	–	+	+	–	–	–
Rhodobacter sphaeroides DSM 158	–	–	–	–	–	–	–
Strain R51g[c]	+	+	+	–	–	+	+
Environmental samples							
Alunda soil	+	+	+	M	+	+	–
Brunnby soil	+	+	+	M	+	+	–
Ulleråker soil	+	+	+	M	+	+	–
Sludge from Henriksdals WWTP[d]	M	+	+	M	M	+	–
Sludge from Kungsängens WWTP[d]	M	+	+	M	M	+	–
Peat	M	+	+	M	M	+	M

[a] A.H. Stouthamer, Vrije University, Amsterdam, The Netherlands.

[b] Swedish University of Agricultural Sciences.

[c] β-proteobacteria, rubrivivax group. G. Dalhammar, Royal Institute of Technology, Stockholm, Sweden.

[d] Wastewater treatment plant.

[e] +, visible band of the expected size; M, multiple bands, including band of expected size; –, no visible band.

Table 2
General soil characteristics

Site	Soil type	pH	Clay content (%)	Organic C (%)	Total N (%)
Alunda (A)	Silty clay loam	7.8	39	4.95	0.49
Brunnby (B)	Clay loam	5.6	41	1.23	0.13
Ulleråker (U)	Sandy loam	5.8	14	1.82	0.13

pernatant was discarded prior to storage. All the environmental samples were stored at −20 °C.

2.2. DNA extraction

Chromosomal DNA was isolated from the pure cultures using the DNeasy Tissue Kit™ (Qiagen, Valencia, CA, USA) as specified by the manufacturer. Total DNA from the environmental samples was extracted using the FastDNA® Spin Kit for soil (Qbiogene, Carlsbad, CA, USA) according to the manufacturer's protocol with a few modifications in the pre-treatment step. The soil and peat samples (300 mg) were mixed with 978 μl of the phosphate buffer in the kit using a blender and transferred to a multimix-tube, and 122 μl MT buffer from the kit was then added. The sludge pellets were dissolved in 978 μl phosphate buffer and transferred to multimix-tubes to which 122 μl MT buffer was added. The isolated DNA was stored at −20 °C.

2.3. PCR amplification of nirS, nirK and nosZ genes

The *nirS*, *nirK* and *nosZ* sequences presently available from the GenBank database were aligned, and conserved regions that could provide suitable primer target sites were re-assessed. For the *nirS* gene, 11 forward and five reverse primers were tested. For the *nirK* gene, three forward and three reverse primers were evaluated for PCR amplification of gene fragments, while five forward and six reverse primers were tested for *nosZ* (Table 3, Fig. 1). All possible primer combinations for *nirK* and *nosZ*, except those including nosLb and nosRb, were evaluated against the denitrifying strains, the non-denitrifying strains and the environmental samples. For *nirS* merely combinations yielding amplicons >400 bp were tested. The reason for this was to reduce the number of combinations and to only work with primers that amplify fragments with enough information to be useful in environmental studies. In the case of minor variations in the primer sequence, e.g., modifications of F1acd (Table 3), only the result from the best combination is shown in Table 1. The oligonucleotides were purchased from Invitrogen (Carlsbad, CA, USA).

The criteria to evaluate the different primer sets for *nirK*, *nirS* and *nosZ* were: (i) the number of strains that the gene was amplified from, (ii) the number of genera that the gene was amplified from, (iii) the number of environmental samples that the gene was amplified from and (iv) the size of the amplicon. In the case of criteria (i)–(iii), the higher the better. For community surveys, larger amplicons is beneficial and in order to be successful also in DGGE analysis the size should not exceed 500 bp.

All primer pairs amplifying gene fragments of *nirS* and *nirK* were run with an initial denaturation of the DNA at 94 °C for 2 min, followed by 35 cycles of 30 s at 94 °C, 1 min at 51 °C and 1 min at 72 °C. The reaction was completed after 10 min at 72 °C. The reaction mixtures were placed in a minicycler (MJ Research, Waltham, M, USA). Due to inadequate amplification, primer pair Cunir3:nirk5R (also named Cunir4) was also run according to Casciotti and Ward [12]. The PCR for primer pairs, cd3aF:R3cd and F1aCu:R3Cu, was further optimised and the annealing temperature was increased to 57 °C for both primer pairs. The annealing temperature for the primers encoding *nosZ* differed depending on the specific pair, but the PCR program was otherwise as described above. Primer combinations Nos661F:Nos1527R, Nos661F:Nos1773R, Nos1527F:-Nos1773R, nosZ-F:Nos1773R and nosZ661b:-nosZ1773b were run at an annealing temperature of 55 °C, combination nosZ-F:nosZ-R at 50 °C and combination nosZ-F:nosZ1622R at 53 °C. Primers nosLb and nosRb were run according to Chèneby et al. [24] but also with a lower annealing temperature (56 °C) due to inadequate amplification. The detection and the size of the amplicons were determined by agarose (0.9%) gel electrophoresis and UV translumination after ethidium bromide staining.

PCR amplification was performed in a total volume of 25 μl containing 2.5 μl of 10× PCR buffer (500 mM KCl, 15 mM $MgCl_2$ and 100 mM Tris–HCl, pH 9.0, at room temperature), 200 μM of each deoxynucleotide triphosphate, 1.25 U of Taq polymerase (Amersham Biosciences, NY, USA), 1.0 mM of each primer and 10–100 ng DNA. In accordance with Braker et al. [9], an additional 1.0 mM $MgCl_2$, 400 ng μl^{-1} BSA and 0.1% Triton X-100 were added for amplification with primers nirK1F, nirK3R, nirK5R, nirS1F, nirS3F, nirS3Fa, nirS4R and nirS6R. An additional 1.0 mM $MgCl_2$ was added for the primer pair Cunir3:nirK5R [12]. For amplification of the environmental samples, BSA was added in the PCRs with all other primer combinations. For *nirS* reactions, 1000 ng μl^{-1} was added, for *nirK* 400 and for *nosZ* 600.

The relative amounts of PCR products obtained for *nirS*, *nirK* and *nosZ* in the environmental samples were estimated by comparing band intensities on agarose gels.

Table 3
Primer sequences and positions used to amplify fragments from *nirS*, *nirK* and *nosZ* genes in the denitrification pathway

Primer[a]	Position[b]	Primer sequence (5′-3′)	Reference
nirS1F	763–780	CCT A(C/T)T GGC CGC C(A/G)C A(A/G)T	[9]
F1acd	856–871	TA(C/T) CAC CC(C/G) GA(A/G) CCG C	[10]
F1bcd	856–872	TA(C/T)CAC CC(C/G) GA(A/G) CCG CG	This study
Heme 832F	856–875	TA(C/T) CAC CC(C/G) GA(A/G) CCG CGC GT	[15]
F1dcd	859–875	CAC CC(C/G) GA(A/G) CCG CGC GT	This study
cd3aF	916–935	GT(C/G) AAC GT(C/G) AAG GA(A/G) AC(C/G) GG	[11]
cd3bF	916–935	GTG AAC GT(C/G) AAG GA(A/G) AC(C/G) GG	[11]
nirS3F	1002–1019	TTC CT(T/C/G) CA(C/T) GAC GGC GGC	[9]
nirS3Fa	1002–1018	TTC CT(T/C/G) CA(C/T) GAC GG(C/T) GG	This study
F3nirS	1132–1148	CCG CAC CCG GG(T/C/G) CG(C/T) GG	This study
nirS4F	1317–1336	TTC (A/G)TC AAG AC(C/G) CA(C/T) CCG AA	[9]
nirS4R	1317–1336	TTC GG(G/A) TG(C/G) GTC TTG A(T/C)G AA	[9]
R3cd	1322–1341	GA(C/G) TTC GG(A/G) TG(C/G) GTC TTG A	This study
R4cd	1636–1654	CGT TGA ACT T(G/A)C CGG T(C/G)G G	[10]
R4bcd	1636–1654	CGT TGA A(C/T)T T(G/A)C CGG T(C/G)G G	This study
nirS6R	1638–1653	CGT TGA ACT T(A/G)C CGG T	[9]
Cunir3	504–521	CGT CTA (C/T)CA (C/T)TG CGC (A/C/G)CC	[12]
nirK1F	526–542	GG(A/C) ATG GT(G/T) CC(C/G) TGG CA	[9]
F1aCu	568–584	ATC ATG GT(C/G) CTG CCG CG	[10]
nirK3R	898–918	GAA CTT GCC GGT (A/C/G)G(C/T) CCA GAC	[9]
R3Cu	1021–1040	GCC TCG ATC AG(A/G) TTG TGG TT	[10]
nirK5R	1023–1040	GCC TCG ATC AG(A/G) TT(A/G) TGG	[9]
Nos661F	303–320	CGG CTG GGG GCT GAC CAA	[16]
nosZ661b	303–320	CGG (C/T)TG GGG (C/G)(A/C)(A/T) (T/G)AC CAA	[17]
nosLb	1124–1144	CCC GCT GCA CAC C(A/G)C CTT CGA	[24]
Nos1527F	1169–1187	CGC TGT TC(A/C/T) TCG ACA G(C/T)C A	[16]
nosZ-F	1169–1188	CG(C/T) TGT TC(A/C) TCG ACA GCC AG	[18]
Nos1527R	1171–1188	CTG TTC (A/C/T)TC GAC AG(T/C) CAG	[16]
Nos1773R	1396–1415	AAC GA(A/C/G) CAG (T/C)TG ATC GA(T/C) AT	[16]
nosZ1773b	1396–1415	AA(C/T) GA(A/G/C/T) CA(G/A) (T/C)TG ATC CG(T/C) AT	[17]
nosRb	1405–1425	CGT CGC C(C/G)G AGA TGT CGA TCA	[24]
nosZ1622R	1603–1622	CGC (G/A)A(C/G) GGC AA(G/C) AAG GT(G/C) CG	This study
nosZ-R	1849–1869	CAT GTG CAG (A/C/G/T)GC (A/G)TG GCA GAA	[18]

[a] Primers are indicated Cu or nirK for the *nirK* gene, cd or nirS for the *nirS* gene and nosZ or nos for *nosZ*.
[b] Positions in the *nirS* gene of *Pseudomonas stutzeri* ZoBell ATCC 14405 (X56813), in the *nirK* gene of *Alcaligenes faecalis* S-6 (D13155), and in the *nosZ* gene of *Pseudomonas aeruginosa* DSM 50071 (X65277).

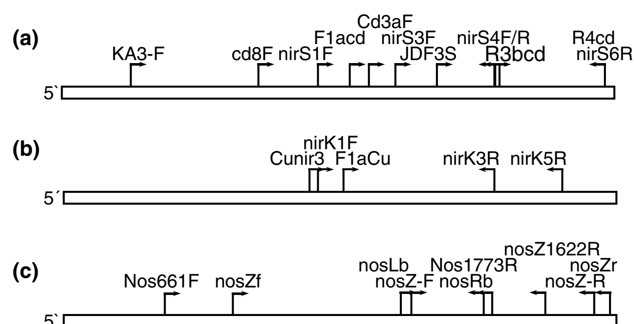

Fig. 1. PCR-primers for: (a) *nirS* relative to the 1683 bp *nirS* sequence of *Pseudomonas stutzeri* ZoBell (Accession No. X56813), (b) *nirK* relative to the 1131 bp *nirK* sequence of *Alcaligenes faecalis* S-6 (Accession No. D13155) and (c) *nosZ* relative to the 1905 bp *nosZ* sequence of *Pseudomonas aeruginosa* (Accession No. X65277).

In order to get approximately the same amount from each sample, the amplicons were concentrated through freeze-drying (3×10^{-1} mbar and −40 to −50 °C; Edwards Modulyo Freeze Dryer, BOC Edwards, Crawley, UK) to approximately 1/5 of the original volume before DGGE analysis.

2.4. DGGE

The melting profiles of the *nirS*, *nirK* and *nosZ* fragments from the best primer combinations were analysed with the computer program WinMelt (Bio-Rad Laboratories Inc., Hercules, CA). To modify the melting profile and avoid complete denaturing of the amplified fragments, a 33-bp GC-clamp (5′ GGC GGC GCG CCG CCC GCC CCG CCC CCG TCG CCC 3′) was attached to the 5′ end of the R3cd, R3Cu and nosZ1622R primer, respectively. The PCR conditions for reactions with the GC-clamped primers were the same as those for the ordinary primers, as described above. Following PCR of four independent reactions, the amplified products were pooled and resolved on DGGE gels using a Dcode sys-

tem (Bio-Rad Laboratories Inc.). PCR products from three denitrifying strains were applied two times to each DGGE gel as markers to check the electrophoresis run and to compare fragment migration between gels. For *nirS* the strains were (in the order of increasing migration distance): *Pseudomonas fluorescens* Mi32, *Paracoccus denitrificans* CCUG 13798 and *P. denitrificans* Pd1222; for *nirK*: *Alcaligenes faecalis* ATCC 8750, *Achromobacter cycloclastes* ATCC 21921 and *Alcaligenes* sp. DSM 30128; and for *nosZ*: *Ralstonia eutropha* CCUG 13724, *B. denitrificans* DSM 1113 and *P. denitrificans* Pd 1222.

One-mm thick, 16 by 16 cm polyacrylamide gels (7.0% [v/v] acrylamide-bisacrylamide [37.5:1]; denaturant [urea and formamide]) were poured using a gradient maker (Bio-Rad Laboratories Inc.). A mixture of 7 M urea and 40% formamide was defined as 100% denaturant [32]. Fifteen microlitres of the PCR products that had been amplified with the cd3aF:R3cd-GC, F1aCu:R3Cu-GC and nosZ-F:nosZ1622R-GC primer pairs were run on denaturing gradients of 60–80% for 17 h, 50–70% for 13 h and 40–70% for 17 h, respectively. The gels were run in 1× TAE (40 mM Tris-acetate and 1 mM EDTA) at 130 V and 60 °C. Migration patterns were visualised by staining with 1:10 000 (v/v) SYBR Gold (Molecular probes, Eugene, Canada) for 30 min followed by UV translumination. Images were documented with the Gel Doc 2000 System from Bio-Rad (Bio-Rad Laboratories Inc.), and digital pictures were analysed with the Quantity One software from Bio-Rad (Bio-Rad Laboratories Inc.).

2.5. Cloning, sequencing and computer analysis

The middle portion of each selected DGGE band was excised for sequence analysis and placed in 160 μl distilled H_2O. The DNA was eluted through freezing and thawing (12 h at −70 °C, 1 h at room temperature and 1 h at −70 °C) followed by thawing at 8 °C overnight. The eluted fragments were PCR-amplified with primers without a GC-clamp using 4 μl DNA in a total reaction volume of 50 μl but otherwise as described above. Prior to sequencing, 45 μl of each PCR product was purified with Microspin S-400 HR columns (Amersham Biosciences, NY, USA). To test the resolution on the DGGE gels, the bands were further cloned using the TOPO TA Cloning® Kit for Sequencing (Invitrogen, Carlsbad, CA). Cells from randomly picked clones were collected with a toothpick and re-suspended in 25 μl pre-prepared PCR mixtures, and the inserts were amplified as described above. From each excised DGGE band, 10 clones with the correct insert were chosen for sequencing. The plasmids containing *nirS*, *nirK* or *nosZ* gene fragments were isolated using the QIAprep Spin Miniprep Kit Protocol from Qiagen (Valencia, CA). Both strands of the insert were sequenced with the DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Biosciences) with 4 μl of the eluted plasmids using the plasmid-specific primers M13F and M13R. Twelve bands from each gel were randomly chosen for direct sequencing. The original PCR primers (cd3aF and R3cd; F1aCu and R3Cu; and nosZ-F and nosZ1622R) were then used as sequencing primers. An ABI PRISM 377 (Perkin–Elmer, Wellesley, MA) automated DNA sequencer was used for sequencing.

Derived nucleotide sequences of *nirS*, *nirK* and *nosZ* were aligned with nucleotide sequences of equivalent length from the GenBank (NCBI) database using the CLUSTAL W software (http://www.ebi.ac.uk/clustalw/). A selection of environmental *nir* clones from marine sediment samples [5,22], estuarine sediments [17], cyanobacterial bloom [23], forested upland and wetland soils [28], agricultural soil [25], temperate forest soil [13] and contaminated groundwater [15] were included as well as *nosZ* clones from marine sediments [6], arable soil [26,27] meadow soil [29], coastal seawater (unpublished, Accession Nos. AB089825, AB089829 and AB089832) and forest soils [13]. The environmental clones from each study were chosen to cover the greatest possible sequence variation. The tree analysis was performed with the software TREECON [38]. Distance matrix analyses were performed with the Jukes and Cantor correction [39]. The trees were reconstructed using the neighbor-joining method by Saitou and Nei [40] and tree topology was evaluated by bootstrap analysis using 100 replicates.

2.6. Nucleotide sequence accession numbers

The partial *nirS* gene sequences that were generated in this study have been deposited in the GenBank database under Accession Nos. AY4245134–AY425145 and AY583405–AY583455. The *nirK* sequences are available under AY425146–AY425157 and AY583380–AY583404, while the *nosZ* sequences are found under AY425158, AY425160–AY425169 and AY577558–AY577577.

3. Results and discussion

3.1. Evaluation of PCR-primers for nirS *detection*

Several new options for possible primers were found when the *nirS* sequences were aligned (Table 3; Fig. 1a). They are all located in the second half of the gene, where the majority of the sequences have been derived. Unfortunately, there are only nine complete *nirS* gene sequences in the databases. For several *nirS* primers, small modifications were tested, but in Table 1 only the results from the best primer targeting each primer site is shown. The best result was obtained with the modified version of primer cd3F [11] cd3aF in combination with the *nirS*

primer R3cd, which partly overlaps with primer nirS4R [9]. The pair managed to amplify the correct fragment in 13 out of 14 strains as well as in all environmental samples. No other bands were detected, which eliminates the need to purify the correct fragment from agarose gels before downstream analysis. When applying this primer pair to a known *nirK*-denitrifier (*A. faecalis* ATCC 8750) or to non-denitrifiers, no PCR products were obtained. Primer combinations F1acd:R4bcd, nirS1F:R4bcd and F3nirS:nirS6R managed to amplify a fragment of the correct size in the *nirK* denitrifier *A. faecalis* ATCC 8750. Moreover, a fragment was also amplified from *L. reuteri* DSM 20016 with the nirS4F:R4bcd set. In all these cases, the region for the reverse primer is the same, which indicates that this region is less specific than previously believed.

Primer cd3F was originally designed for quantification of cd_1-denitrifiers in marine sediments [11]. Two additional primer sites were used in this earlier study. Forward primer cd8F is located approximately 200 bp upstream of the nirS1F site [9], but only seven sequences are known in this region. Moreover, only a few bases seem to be conserved. Primer cd4R corresponds to primers nirS6R [9] and R4cd [10]. The most commonly used primers, nirS1F and nirS6R, were less successful in our study and amplified the correct fragment in only eight organisms within four genera. Other potential primers have also been used to survey denitrifying communities. Yan et al. [15] modified the two *nirS* primers F1acd (renamed to Heme 832F) and R4cd (Heme 1606) published by Hallin and Lindgren [10] to determine diversity of denitrifying bacteria in groundwater and these were also used for studies of denitrification genes in sediments [22]. The forward primer (KA3-F) used by Rösch et al. [13] targets a region on the *nirS* gene corresponding to bases 183–206 in *Pseudomonas stutzeri* ATCC 14405. The primer was not used in our evaluation since there are only seven sequences available for this region and they do not appear to be very conserved. Their reverse primer is similar to the one developed by Braker et al. [9].

A number of primer combinations targeting *nirS* failed to amplify the gene in several of the environmental samples (Table 1). The *nirS* gene was readily amplified from the activated sludge samples with most of the primers, but we had severe problems with Alunda and Ulleråker soil samples and the peat. It was only from the new primer pair cd3aF:R3cd that we obtained an amplicon of the correct size from all samples. The commonly used nirS1F:nirS6R primers did not work well on soil samples and we only obtained a product from Brunnby soil. Others have also encountered problems in detecting *nirS* in soils with these primers and reported a low *nirS* abundance and diversity in soil [28]. This could likely be a consequence of the primers used, although it might also be a correct description of the samples. Based on the results from our evaluation, we conclude that the cd3aF:R3cd primer set would be the best for community analysis of *nirS* denitrifiers. The fragment size is also suitable for DGGE.

3.2. Evaluation of PCR-primers for nirK detection

For amplification of *nirK*, no better alternatives to the already published primers were found (Table 3; Fig. 1b). Amplification with most of the combinations resulted in fragments of the correct size in only about half of the strains that were tested. However, no PCR products were found when tested with *P. stutzeri* ATCC 14405 or the three non-denitrifying strains for any combination. The best results were obtained with the primers F1aCu and R3Cu [10] that amplified the correct fragment in 10 out of 14 strains without extra bands. In addition, fragments of the correct size were amplified from all six environmental samples.

The nirK1F and nirK5R primers [9] are the most widely used tools to survey diversity of the NirK denitrifying bacteria in environmental samples. The nirK1F primer binds to a region of the *nirK* gene that has an insert of three bases in some sequences of denitrifying bacteria (Accession. Nos. AJ002516, U62291, AF339044–AF339048 and AB076606). This insert may be the explanation for the poor amplification of this particular primer. Rösch et al. [13] used a forward primer that targets the same area as primer nirK1F, but is a few bases longer. This primer faces the same problem as nirK1F. Yan et al. [15] as well as Liu et al. [22] used a modified version of the F1aCu primer together with a modification of primer nirK3R [9]. The nirK3R primer was unsuccessful both in the original study and in the present re-evaluation, and the small number of conserved bases within the sequences may be the explanation. The Cunir3 primer designed by Casciotti and Ward [12] targets a highly conserved region encoding for a copper-binding site, but unfortunately there are only a few sequences available in this region. If they are not representative, this would explain the poor results from the amplification of denitrifying strains in the present study. There are only 12 complete *nirK* gene sequences in the databases.

As in the case of *nirS*, the *nirK* gene was amplified from activated sludge and peat with the majority of the primer combinations (Table 1). It was more difficult with the soil samples. Only primer combinations F1aCu:R3Cu and nirK1F:nirK5R amplified all environmental samples. Both of these sets generate amplicons of almost the right size for DGGE, but we chose to use the F1aCu:R3Cu set because it yields an amplicon that is ca. 40 bp shorter and less than 500 bp. Moreover, the results from PCR with the strains were better and the three base insert in the primer site of nirK1F is avoided.

3.3. Evaluation of PCR-primers for nosZ detection

Five forward and six reverse *nosZ* primer candidates were found (Table 3, Fig. 1c). For the *nosZ* primers, Nos661F and Nos1773R, small modifications were tested but in Table 1 only the best result, which was from the original primers, is shown. None of the *nosZ* primer sets resulted in fragments from the non-denitrifying strains. The best combination was shown to be primers nosZ-F [18] and nosZ1622R, which was designed in the present study. Together they amplified the correct fragment in 25 of the 28 denitrifying bacterial strains and in all environmental samples.

Kloos et al. [18] screened for *nosZ* in different *Azospirillum* strains and other plant growth-promoting rhizobacteria with the primers nosZ-F and nosZ-R. The same primers were later used to investigate *nosZ* in an acid forest soil [13] and meadow soil [29]. In our evaluation, the forward primer, nosZ-F, was combined with the reverse primers nosZ-R, Nos1773R [16] and nosZ1622R, with satisfactory results, but the combination nosZ-F:nosZ1622R gave even better results. Nos661F and Nos1773R were the first primers targeting *nosZ* [16], and were based on just a few sequences. They were primarily used to amplify *nosZ* from marine sediments, but were recently used to survey *nosZ* in native and cultivated soil [26]. Nogales et al. [17] modified these primers (nosZ661b and nosZ1773b) to be more degenerate. However, none of the sets were successful in the present re-evaluation. Chèneby et al. [24] constructed primers (nosLb and nosRb) for amplification of *nosZ* genes in two agricultural soils. The primers were not efficient in our study as shown by amplification of the correct gene fragment from only nine of the pure cultures. The two primers (nosZf and nosZr) designed by Delorme et al. [41] amplify a 1433-bp fragment and have been used to specifically study genetic diversity in fluorescent pseudomonads in soil. This fragment is too long for DGGE analysis but nosZf could be combined with the nos661F target site to generate a 250-bp fragment. However, since this re-evaluation has shown that Nos661F is not suitable, nosZf was excluded from the evaluation. Otherwise, the primer looks promising with many conserved bases within the sequences. The nosZr is located at the very end of the *nosZ* gene, from which about 15 sequences are available. These sequences do not appear to be very conserved and, therefore, it cannot serve as a general *nosZ* primer.

All *nosZ* primer combinations, except nosLb:nosRb, amplified a fragment of the correct size in the six environmental samples (Table 1) making all these sets promising for community surveys. If other criteria for evaluation of the primers are taken into account, such as number of strains and genera that can be amplified, the nosZ-F [18] in combination with nosZ1622R from this study were the best. This set is also the only one yielding amplicons of sufficient size both for community surveys and for DGGE.

3.4. Optimisation of DGGE analysis of partial nirS, nirK and nosZ genes

DGGE is simplified for a given fragment when the region of interest lies in a single domain. However, functional genes such as the *nirS*, *nirK* and *nosZ* genes have great sequence variation, and melting profiles obtained in WinMelt showed that all three denitrification genes had multiple melting domains. Some of the *nirS* fragments that were analysed had up to six different domains. To minimise the effects of these domains and avoid complete denaturing of the PCR-amplified fragments, a 33-bp GC-clamp was added to the three reverse primers, R3cd, R3Cu and nosZ1622R. The introduction of the GC-clamp did not affect the amplification efficiency of either the denitrifying strains or the environmental samples. After amplification, the three different DGGE methods in our study were optimised regarding gradient concentration and running time using amplicons from the denitrifying strains as controls. We initially experienced problems with multiple bands, but once the DGGE methods were optimised, only one band appeared on the gel from each of the pure cultures without any smear. After the DGGE optimisations, partial *nirK* and *nosZ* genes from denitrifying pure cultures were clearly separated from each other and covered the whole gradient, but the *nirS*-gene separation was not as good (e.g., Fig. 2).

3.5. DGGE analysis of partial nirS, nirK and nosZ genes

The DGGE analysis of partial *nirS* genes resulted in only a few bands in all the environmental samples with 2–3 dense bands dominating in the middle of the gel (Fig. 2a). This indicated that the resolution of the DGGE was insufficient for the environmental *nirS* amplicons. To test the resolution, as well as primer specificity, almost all visible bands were cloned and sequenced from the soil samples on the *nirS* gel as well as four bands from the peat, all three bands from the Kungsängen sludge sample and two of the three bands from Henriksdal. Comparison with the NCBI database using a BLAST search revealed that all 63 clones amplified with primers cd3aF:R3cd-GC showed homology with known *nirS* sequences. The formation of heteroduplex DNA fragments was not detected in any of the sequenced clones. All 31 bands except AS5, AS6, AS8 and US4 contained between two and six different clones, which proves that the fragments did not separate to the desired extent. The poor resolution could possibly be due to the multiple melting domains observed in the particular fragment. Multiple melting domains typically result in fuzzy bands in the migration direction, ham-

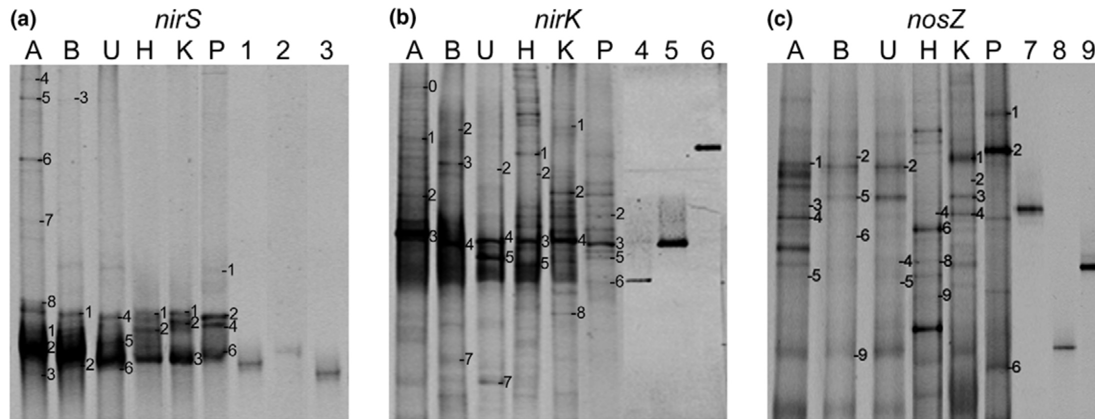

Fig. 2. DGGE fingerprints of dominating denitrifying communities in Alunda soil (A), Brunnby soil (B), Ulleråker soil (U), Henriksdal activated sludge (H), Kungsängen activated sludge (K) and Peat (P). (a) *nirS* gene fragments amplified with primers cd3aF and R3cd-GC. (1) *Paracoccus denitrificans* CCUG 13798, (2) *Pseudomonas fluorescens* Mi32, (3) *Paracoccus denitrificans* Pd1222; (b) *nirK* gene fragments amplified with primers F1aCu and R3Cu-GC. (4) *Alcaligenes* sp. DSM 30128, (5) *Achromobacter denitrificans* ATCC 21921, (6) *Alcaligenes faecalis* ATCC 8750 and (c) *nosZ* gene fragments amplified with primers nosZ-F and nosZ1622R-GC. (7) *Ralstonia eutropha* CCUG 13724, (8) *Paracoccus denitrificans* Pd1222, (9) *Blastobacter denitrificans* DSM 1113.

pering band resolution [42]. It has been shown that rDNA fragments, specific for methane oxidising bacteria, with substantial sequence variation could not be resolved by DGGE [43] although Nübel et al. [44] could separate fragments from different *rrN* operons differing in only one base pair. Different regions of a gene might result in different resolutions of separation and some fragments may be less useful for DGGE analysis.

In contrast to *nirS*, the DGGE analysis of *nirK* and *nosZ* genes worked satisfactorily. The analyses were reproducible. Also, there was no variation in the pattern between duplicate DNA extracts run with separate PCR amplification of *nirK* from samples of the same origin (Fig. 3). The DGGE banding pattern of *nirK* and *nosZ* genes was more composite in the soil samples than in the activated sludge and peat samples, indicating a higher number of denitrifying populations in soil (Fig. 2b and c). At least 25 visible bands were detected for each soil sample with equal intensity, although there also appeared to be some dominant *nirK* and *nosZ* populations within the denitrifying communities. The complexity of the soil DGGE analysis resembled what has been shown for 16S rDNA in soil [34]. The activated sludge samples and the peat were composed of a few dominant populations according to the DGGE banding pattern.

Comparison with the NCBI database using BLAST revealed that all 37 *nirK* and all 31 *nosZ* clones amplified with primers F1aCu:R3Cu-GC and nosZ-F:nosZ1622R-GC, respectively, showed homology with known *nirK* and *nosZ* sequences. No heteroduplex molecules were observed. The resolution of *nirK* and *nosZ* amplicons was satisfactory. For the 24 *nirK* bands that were excised, five bands contained two sequences and the bands PK6, UK4, BK3 and BK4 held three sequences. For *nosZ*, five of the 22 bands were composed of two different sequences and three sequences were found in bands BZ5 and HZ9. Sequences appearing in the same band were in most cases closely related and were therefore difficult to separate. However, a few bands contained distantly related *nirK* and *nosZ* clones, indicating co-migration of DNA. This is not unusual [45], and to resolve these sequences either cloning or running the fragment on new DGGE can be employed. The cloning approach is often used for sequencing

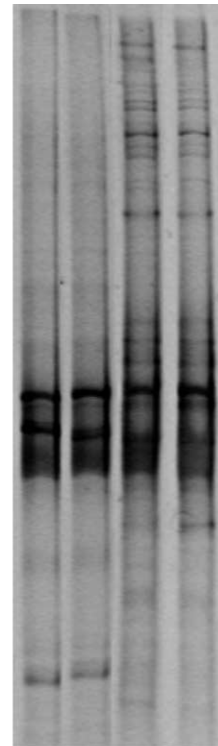

Fig. 3. DGGE analysis of *nirK* gene fragments amplified with the primer pair F1aCu:R3Cu-GC from duplicate DNA extracts from Henriksdals municipal wastewater treatment plant and Ulleråker soil.

bands from complex 16S rDNA profiles (e.g. [34,46] but has also been used to separate *amoA* fragments in less complex profiles [47]. Distances between different *nirK* or different *nosZ* sequences were not consistently correlated to distances between bands. However, banding patterns are the result of fragment mobility after partial

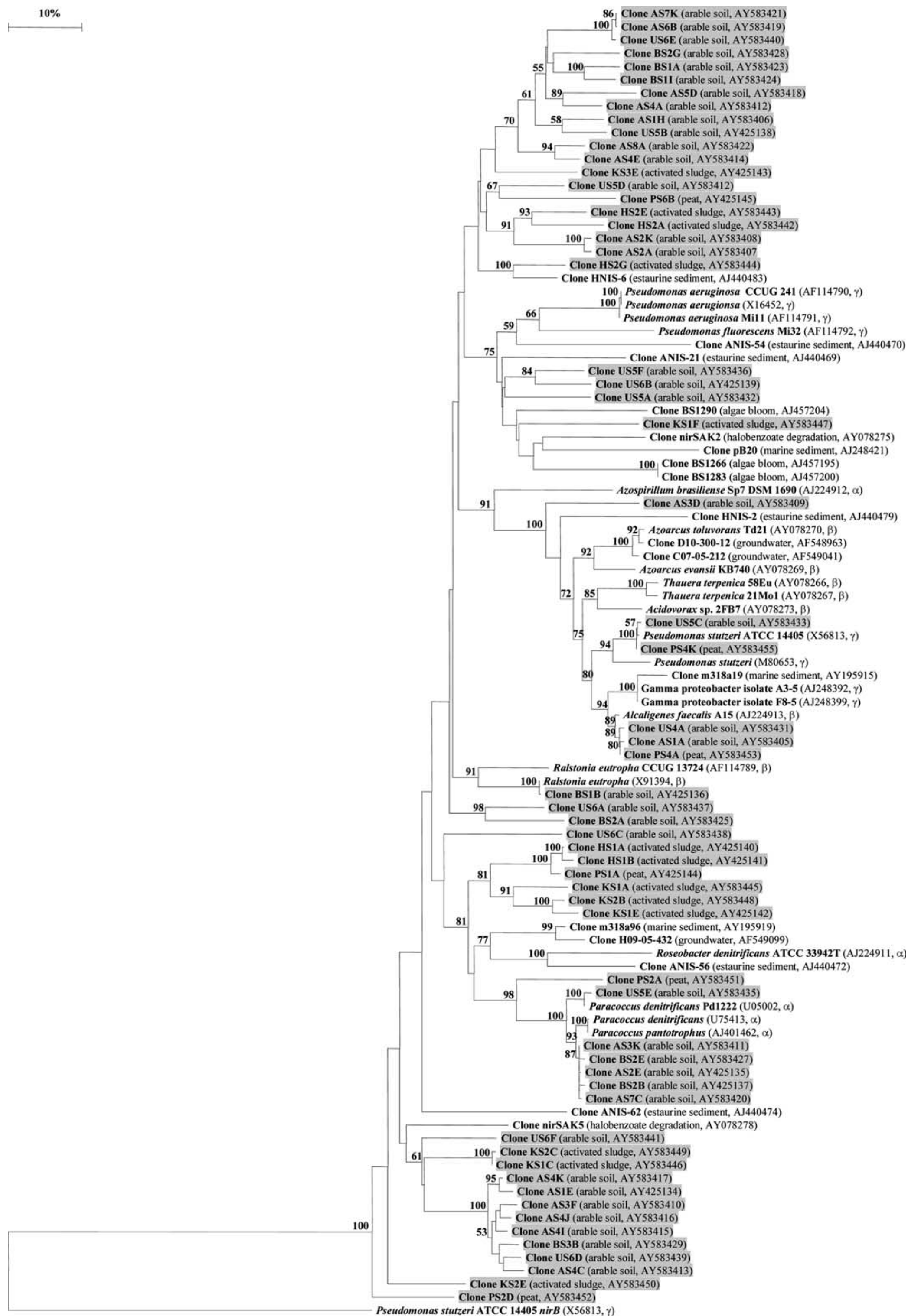

Fig. 4. Phylogram for *nirS* based on partial gene fragments (365 bp). The tree is based on distance matrix analysis and a neighbor-joining method. The scale bar indicates 10% nucleotide substitutions. Bootstrap values greater than 50 from 100 replicate trees are reported at the nodes. Phylogenetic positions of pure cultures based on 16S rDNA are indicated by α, β and γ for *Proteobacteria* affiliation. The clones from this study are framed and shaded in gray. The sequence of *nirB* from *Pseudomonas stutzeri* (Accession No. X56813) served as an outgroup to root the phylogram.

denaturing and not sequence per se. Molecules with different sequences may have a different melting behaviour, and stop migrating at different positions, but not necessarily. One should therefore be careful to draw conclusions on denitrifying bacterial diversity from the DGGE patterns alone. Moreover, DGGE only provides

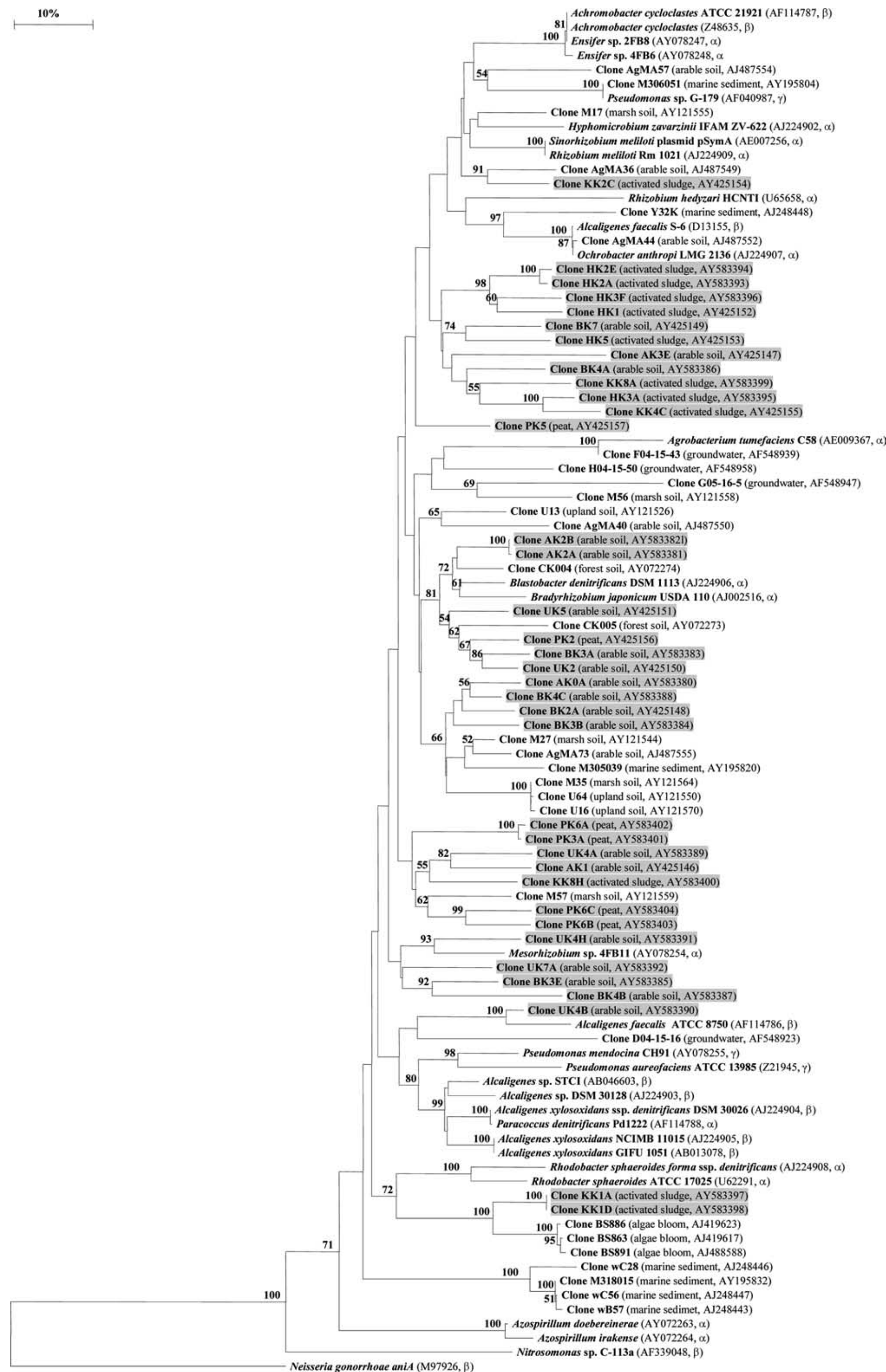

Fig. 5. Phylogram for *nirK* based on partial gene fragments (345 bp). The tree is based on distance matrix analysis and a neighbor-joining method. The scale bar indicates 10% nucleotide substitutions. Bootstrap values greater than 50 from 100 replicate trees are reported at the nodes. Phylogenetic positions of pure cultures-based 16S rDNA are indicated by α, β and γ for *Proteobacteria* affiliation. The clones from this study are framed and shaded in gray. The sequences of *aniA* from *Neisseria gonorrhoae* (Accession No. M97926) served as an outgroup to root the phylogram.

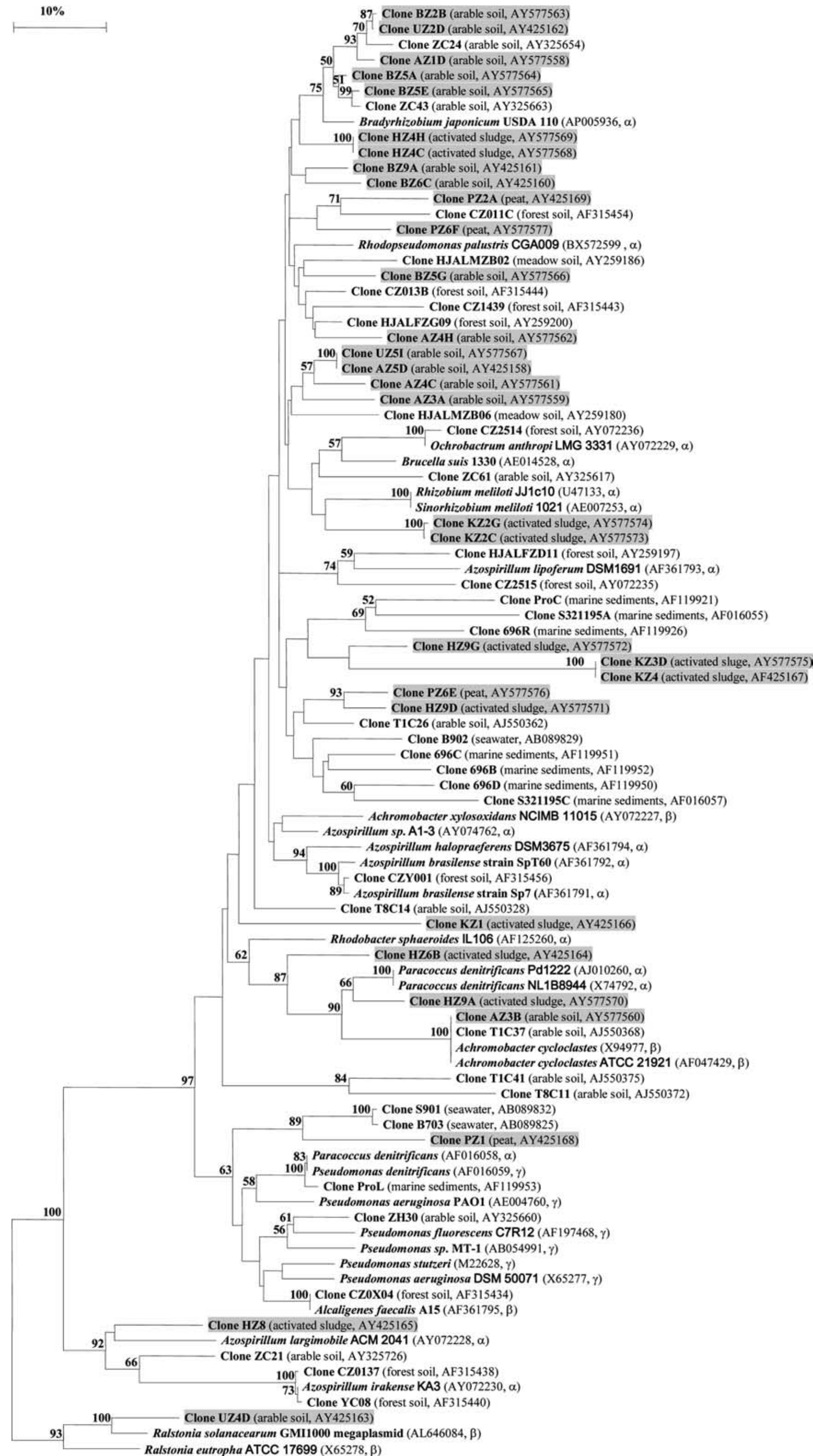

Fig. 6. Phylogram for *nosZ* based on partial gene fragments (190 bp). The tree is based on distance matrix analysis and a neighbor-joining method. The phylogram was an alignment of only 190 bp to allow a comparison with the *nosZ* sequences published by Scala and Kerkhof [20], Stres et al. [26] and the unpublished sequences from coastal seawater (Accession No. AB089825, AB089829 and AB089832). The scale bar indicates 10% nucleotide substitutions. Bootstrap values greater than 50 from 100 replicate trees are reported at the nodes. Phylogenetic positions of pure cultures based on 16S rDNA are indicated by α, β and γ for *Proteobacteria* affiliation. The clones from this study are framed and shaded in gray. The *nosZ* sequences of different Ralstonias served as an outgroup.

information of the predominant populations in the community.

Exploration of diversity is one issue but the study of population changes is another. For the latter the traditional cloning approach is not well suited. Fingerprinting methods like DGGE are useful for multiple sample analysis of complex dynamics such as successional population changes due to seasonal variations or environmental perturbations. Another fingerprinting method, terminal restriction fragment length polymorphism (T-RFLP) analysis of *nirS* [19], *nirK* [25] and *nosZ* [20], has been used in several studies to compare denitrifying community patterns in different environments. In contrast to T-RFLP, with DGGE it is possible to detect not only the major differences but also the sequences of the predominant denitrifying populations without the need of large clone libraries. With the possibility to directly sequence differences or shifts in the denitrifying communities, redundant sequencing or screening of hundreds of clones can be avoided.

3.6. *Comparison of* nirS*,* nirK *and* nosZ *genes from DGGE with environmental clones*

The *nirS* clones were compared to clones in the database from different environments as well as pure cultures in a tree based on distance matrix analysis and neighbor-joining (Fig. 4). There appeared to be three major clusters within the *nirS* tree, but they were not statistically supported by the bootstrap analysis. Tree topology differed only slightly from that reported by Priemé et al. [28]. This was despite the use of *nirB* instead of *nirF* as outgroup, the introduction of new environmental clones derived in this study and the exclusion of their clones, which covered another region. Other *nirS* trees have different topologies, but these were reconstructed with either *nirS* from *Roseobacter denitrificans* as an outgroup or lacked outgroup [5,17,22]. The environmental clones in our study were scattered all over the tree showing that the primers covered a broad range of *nirS* denitrifiers. There appeared to be no habitat-specificity among the environmental clones. Most of the *nirS* sequences were not related to known denitrifying strains, although clone BS1 showed more than 99% identity to *R. eutropha* and several soil clones from Brunnby and Alunda were similar to *Paracoccus pantotrophus*. In the literature and the databases *nirS* clones derived from soil are rare. As can be seen in this study, the *nirS* denitrifiers cover the whole tree and it can be concluded that soil harbours substantial *nirS* diversity. Earlier conclusions that *nirS* denitrifiers are preferentially found in marine ecosystems and that *nirK* denitrifiers are predominant in soil [25,28] should be revised. By using the new primer set in future studies, new insights on community composition of the undiscovered *nirS* diversity in soil will be gained.

Environmental clones as well as pure cultures were compared to the *nirK* sequences from the DGGE bands (Fig. 5). Five putative clusters were recognised, but only two of them were supported by the bootstrap analysis. The tree was similar to that reported by Priemé et al. [28], who also used *aniA* as outgroup. The distribution of environmental *nirK* clones from this study over the whole tree supports the appropriate choice of primers. Also, the *nirK* clones showed low identity to sequences from cultured denitrifying bacteria. Looking at all the environmental clones, there appears to be habitat specificity to some extent. Various soil clones are found in the central part of the tree, a sub-cluster in the upper part is dominated by *nirK* amplicons from activated sludge and several marine clones are located at the bottom.

The phylogram in Fig. 6 of *nosZ* genes from different environmental clones as well as pure cultures was an alignment of only 190 bp to allow a comparison with the *nosZ* sequences published by Scala and Kerkhof [20], Stres et al. [26] and the unpublished *nosZ* sequences from coastal seawater (Accession Nos. AB089825, AB089829 and AB089832). The bootstrap analysis supported four clusters and the tree resembled those reported by others [26,29]. The sequences derived in this study divided into the four major clusters as well as in almost all of the subclusters within the largest cluster, showing that the nosZ-F:nosZ1622R primer combination acts as broad range primers for *nosZ*. Most of our sequences were related to other environmental clones, although UZ4 showed 93% identity to *Ralstonia solanacearum* GMI1000. Environmental clones dominated the upper cluster and within this cluster soil clones were located at the top and marine clones were found in the middle.

4. Conclusions

Primer design is the most critical aspect of microbial environmental diversity surveys and this study clearly demonstrated the need to re-evaluate primers for PCR amplification of denitrifying bacteria. This was especially true for primers targeting *nirS*, With the new primer set, we demonstrated that *nirS* denitrifiers were common in soil and this has not been shown before. DGGE of *nirK* and *nosZ* genes proved to be a good tool for screening and comparing denitrifying communities in different types of environmental samples, but the resolution was insufficient to resolve *nirS* gene fragments.

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