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Denitrification Activity and Relevant Bacteria Revealed by Nitrite Reductase Gene Fragments in Soil of Temperate Mixed Forest

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Denitrification activity and bacterial community constituents were investigated in both well-drained and poorly drained soils of a temperate forest in central Japan by ^{15}N tracer experiments and a cloning–sequencing approach. Denitrification activity was much higher in wet soil than in dry soil, based on $^{15}N^{15}N$ ($^{30}N_2$) and $^{15}N^{15}NO$ ($^{46}N_2O$) production. Labeled nitrate ($^{15}NO_3^-$) was immediately reduced to $^{30}N_2$ in wet soil, whereas it was only reduced to $^{46}N_2O$ in dry soil. Thus, the wet soil at the lower end of the catchment is a functional site for the scavenging for NO_3^- and N_2O. Nitrite reductase gene (*nirK* and *nirS*) fragments from these soils were PCR amplified, cloned, and sequenced. Both *nirK* and *nirS* fragments were detected in the wet soil, whereas only *nirK* fragments were detected in the dry soil. All the *nirK* and *nirS* clones showed less than 90% similarity to known clones. Numerous operational taxonomic units for *nirK* and *nirS* were found in the wet soil. Considerable diversification within the largest clade on the *nirK* phylogenetic tree, which contained no known sequence, was observed in wet soil. Thus, a wet soil environment can provide both the habitat and conditions for the expression of denitrification activity.

Key words: denitrification, forest soil, ^{15}N tracer, *nirK*, *nirS*

The forest ecosystem has been indicated as a potential source of nitrate (NO_3^-) for downstream ecosystems[3]. Increased deposition of nitrogen compounds from the atmosphere may lead to nitrogen saturation in forest ecosystems, causing the leaching of NO_3^- [1]. Denitrification, the respiration process that uses oxidized nitrogen compounds as alternative electron acceptors, is a function that reduces NO_3^- concentrations. Denitrification in forest environments has usually been studied to assess the mass balance of nitrogen; however, our understanding of the bacterial community responsible for denitrification is limited[24–26]. Rich *et al.*[26] carried out a pioneering study of denitrification activity and community composition in the forests of North America, which showed an example of bacterial community constituents influenced by soil environments including a relation between vegetation and denitrification activity based on the *nosZ* gene; however, few studies have investigated both denitrification activity and the relevant bacterial community.

Previous studies of various ecosystems including forest and bacterial strains isolated from different environments[7,42] have indicated that denitrification activity is affected by oxygen partial pressure, which relates to soil water content, and pH, which is constrained by the chemical constituents of the soil. The geographic gradient and variations in the geochemistry of a forest soil may provide differences in environments for denitrification.

The along-slope hydrological dynamics in the catchment of a forest ecosystem produce differences in the water content and chemical constituents of the soil and groundwater, which may affect the constituents of the denitrifying bacterial community and their denitrification activity. The Matsuzawa catchment of the Kiryu Experimental Watershed of Kyoto University, located in a temperate mixed forest of central Japan, was selected for this study because geochemical studies of nitrogen metabolism have been carried out there previously[14,16–18]. Those studies showed that the concentrations of NO_3^- and nitrous oxide (N_2O) differed among positions on the slope. The soil at the lower plot (SG1W) is water-saturated soil, differing in its chemical properties from the surrounding forest soil, whereas the soil in an upper plot (UG31A) is typical water-unsaturated forest soil. Because differences in the water regime are expected to cause differences in the community constituents of denitrifying bacteria and denitrification activities, we tried to elucidate the denitrification in the soils of SG1W and UG31A with the corresponding bacterial communities.

An acetylene inhibition assay has been widely used to study denitrification activity in soil[34,36]. In this assay, denitrification is measured by the production of N_2O, with the inhibition of N_2O reductase to avoid contamination with atmospheric N_2. Thus, the acetylene inhibition assay does not

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allow the measurement of N_2O reductase activity. In contrast, a stable isotope tracer technique using ^{15}N as the substrate allows us to monitor the production of $^{15}N^{15}N$ ($^{30}N_2$) and $^{15}N^{15}NO$ ($^{46}N_2O$) gases simultaneously because the labeled gas produced can be distinguished from the ambient gas. The ^{15}N tracer technique[2] used in this study enabled us to precisely estimate the N_2 and N_2O gases produced via denitrification, clearly distinguishable from these gases produced via nitrification or codenitrification by mass spectrometry.

The reduction of nitrite (NO_2^-) to nitric oxide (NO) is the key step that distinguishes denitrification from nitrate reduction, which does not necessarily ultimately lead to denitrification. NO_2^- is catabolized by two structurally different nitrite reductases: A copper-containing nitrite reductase encoded by *nirK* and a cytochrome cd_1 reductase encoded by *nirS*[22,42]. Bacteria containing either *nirK* or *nirS* are distributed among various phylogenetic groups, predominantly as *Proteobacteria* species. The *nirK* and *nirS* genes have been used to elucidate denitrifier community constituents in various ecosystems, including forest soil[24], marine sediments[5], groundwater[39], and brackish water[30].

In this study, we measured the denitrification activity by a ^{15}N tracer technique[2] employing $^{15}NO_2^-$, as nitrite reductase reduces NO_2^-. However, as a previous study suggested that the abundance of NO_2^- was very low in the area studied, $^{15}NO_3^-$ was employed together with $^{15}NO_2^-$ as an electron acceptor. We also elucidated the community constituents in the soils of SG1W and UG31A by cloning and sequencing *nirK* or *nirS* gene fragments amplified with specific primers[4]. In parallel with those analyses, we measured environmental parameters which may affect denitrification.

Materials and Methods

Study site and sampling

The study site was located in an unchanneled headwater catchment (Matsuzawa catchment, 0.68 ha) in the Kiryu Experimental Watershed (5.99 ha; 34°58'N, 136°00'E) in Shiga prefecture, central Japan. The study site was a mixed forest of Japanese cypress (*Chamaecypais obtuse* Sieb. et Zucc.) and Japanese red pine (*Pinus densiflora* Sieb. et Zucc.). The catchment is underlain by weathered granitic rocks. The soils are predominantly cambisols[17]. Details of the study site have been reported elsewhere[14,16–18]. Soil samples were collected at the SG1W and UG31A plots (Fig. 1) from 16 May 2006 to 17 May 2008, from 0–2 cm below the litter layer. SG1W is located in the saturated zone and UG31A in the unsaturated zone ca. 50 m upslope from SG1W. The soils were sampled in ethanol-rinsed and UV-irradiated bags, brought back to the laboratory on ice within 6 h of sampling, and stored at 4°C. The soils for DNA extraction were immediately frozen at −80°C. Soil moisture, pH, and electrical conductivity (EC) were measured within 8 h of sampling.

Soil samples (10 g) were dried for 18 h at 105°C to determine the initial field soil moisture content of wet soil. Soil pH (H_2O) was measured in soil:MilliQ water slurries (1:2.5 w/w) using a glass electrode (IM-22P, DKK-TOA Corp., Tokyo, Japan). EC was measured in soil:MilliQ water slurries (1:5 w/w) using a glass electrode (CM-14P, DKK-TOA Corp.). Soil subsamples of 10 g dry weight were extracted with 50 mL of 2M KCl, and the ammonium (NH_4^+) concentration was measured by a flow injection analysis system based on the indophenols blue method[15] whereas nitrite (NO_2^-) and nitrate (NO_3^-) were measured by liquid chromatography (Agilent1100, Agilent Technologies, Inc., CA, USA). The total carbon (TC) and total nitrogen (TN) concentrations of the soil samples were measured using the combustion method[6] with an NC analyzer (Sumigraph NC-95A, Sumica Chemical Analysis Service Corp., Tokyo, Japan).

Total direct count (TDC) of Bacteria

Ten grams of fresh soil sampled on 17 May 2008 was fixed with 30 mL of 50% ethanol (Wako Pure Chemical Industries, Ltd., Osaka, Japan) for the enumeration of bacteria. Fixed samples were diluted with MilliQ water to 10 mg dry soil mL^{-1} and stained with SYBR Green I (final concentration, 0.5 μL mL^{-1}; Molecular Probes Inc., Oregon, USA). The bacterial cells were counted by epifluorescence microsopy (BX51, Olympus Corp., Tokyo, Japan).

Soil ergosterol content

The soil ergosterol content was measured as an indicator of fungal biomass[10] by the physical disruption method[9]. In brief, 4 g of moist soil sampled on 17 May 2008 was placed in a 16-mL tube with 4 g of acid-washed glass beads (2 g of 177–250 μm diameter and 2 g of 710–990 μm diameter, AS ONE Corp., Osaka, Japan). After the addition of 6 mL of methanol, the vial was vortexed for 10 s, followed by shaking for 1 h at 320 rpm. The supernatant was retrieved as previously reported[9]. Ergosterol content was determined by HPLC using the same apparatus as a previous report[41].

Tracer experiment to measure denitrification activity

Three grams (dry weight) of fresh soil was added to 67-mL glass vials with Ar-purged sterile water (total sample weight 15 g), and the vials were capped tightly with a butyl rubber stopper and an

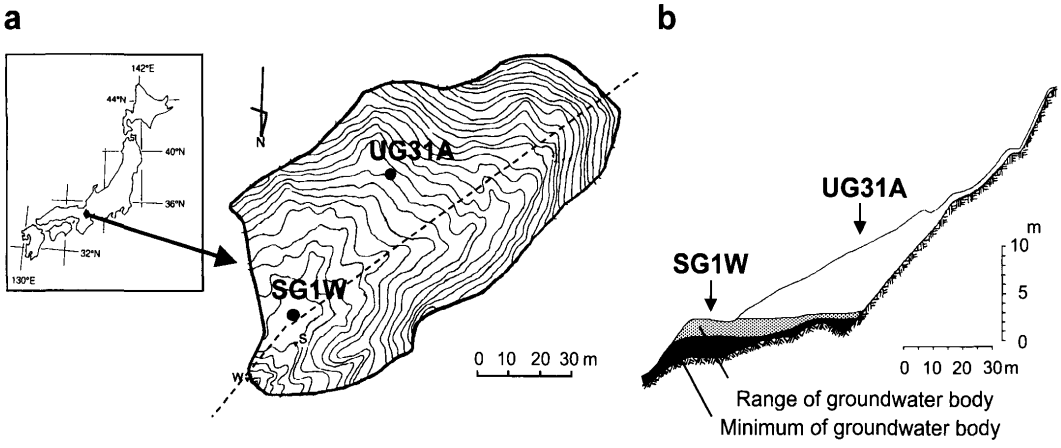

Fig. 1. (a) Locations of the study plots at Matsuzawa catchment. S: spring point. W: gauging weir. (b) the longitudinal section along the dashed line in panel (a). SG1W is located at the lower end of the catchment and UG31A is located in an upper plot of the catchment.

aluminum seal. Anoxic conditions were established as reported[2]. Microoxic conditions were created by adding 1.35 mL of oxygen with a gas-tight syringe to a He atmosphere in the head space of 67-mL vials to which were added the soil slurry, which was equivalent to 0.7 mg O_2 L^{-1} of dissolved oxygen. An oxygen-free stock solution of ^{15}N-labeled $NaNO_3$ (Isotec Inc., OH, USA, ^{15}N atom%: >99% purity) or ^{15}N-labeled $NaNO_2$ (Isotec Inc., ^{15}N atom%: >99% purity) was added with a gas-tight syringe. The soils in the vials were amended with 160 μmol of $^{15}NO_3^-$ or $^{15}NO_2^-$ per vial and incubated at 20°C in the dark. The headspace gas (100 μL) in each vial was collected periodically with a gas-tight syringe under a He stream, and 50 μL of the gas was immediately loaded onto a gas chromatograph-mass spectrometer (GC–MS; Agilent 6890N/5973 GC/MSD system, Agilent Technologies) equipped with a CP-Pora-BOND Q Fused Silica (25 m×0.32 mm) capillary column (Varian, CA, USA) to quantify the N_2 isotopomers ($^{28}N_2$, $^{29}N_2$, $^{30}N_2$), $^{46}N_2O$, O_2, and CO_2. Ultrapure (99.99995%) He gas was used as the carrier gas at a total flow rate of 108 mL min^{-1} with a split ratio of 50. The abundance of each N_2 isotopomer was quantified with standard curves prepared with standard $^{29}N_2$ and $^{30}N_2$ gases.

Potential nitrification

Ten grams (dry weight) of fresh soil sampled on 11 June 2007, 13 July 2007, and 17 May 2008 was placed into glass flasks and amended with either 0 or 20 μg $(NH_4)_2SO_4$-N g^{-1} dry soil. The soils were incubated for 42 days or 44 days at 20°C, with water added periodically to maintain the moisture content at the initial level. The incubated soils were extracted with 50 mL of 2 M KCl at intervals of 2 weeks, and NH_4^+, NO_2^-, and NO_3^- concentrations were measured as mentioned above. Net nitrification was calculated by subtracting the initial NO_3^--N concentration from the final NO_3^--N concentration.

DNA extraction

DNA was extracted from 0.5 g of moist soil from each plot, using an ISOIL for Beads Beating kit (Nippon Gene, Tokyo, Japan), according to the protocol designed for maximum DNA yield.

PCR amplification, cloning, and sequencing of nirK *and* nirS *fragments*

Fragments of the *nirK* and *nirS* genes were amplified using the primer sets nirK1F–nirK5R and nirS1F–nirS6R, respectively[4], with a modified stepdown protocol. The following reaction mixture was used: 5 μL of 10×PCR buffer (Takara Bio, Otsu, Japan), 200 μM of each dNTP (Takara Bio), 1 U of *ExTaq* polymerase (Takara Bio), 20 μg of bovine serum albumin (Sigma, Missouri, USA), and 35 pmol of each primer. The following PCR conditions were used for amplification[24]: A denaturation step at 95°C for 6 min was followed by a touchdown PCR of 10 cycles with denaturation at 95°C for 30 s, primer annealing at 56°C to 51.5°C for 40 s, and extension at 72°C for 1 min, and then by 25 cycles with annealing at 54°C. The amplification was completed with 7 min at 72°C.

The PCR products were cloned into *Escherichia coli* One Shot TOPO10 competent cells with the TOPO TA PCR Cloning Kit (Invitrogen, Carlsbad, CA, USA). To confirm the presence of an insert, plasmid DNA from all clones was amplified with the vector-specific M13F and M13R primers. The sequencing plates were shipped to Takara Bio, where the *nirK* and *nirS* gene fragments were sequenced using the vector-specific T7 primer or T3 primer.

Phylogenetic analysis

The sequences were aligned with the CLUSTAL W package[35]. After alignment, distance matrices were calculated using the Jukes–Cantor algorithm in the program DNADIST from the PHYLIP package[8]. Clones with differences of 5% or less were grouped into one operational taxonomic unit (OTU) using the "furthest neighbor assignment algorithm," implemented in DOTUR[31]. Richness and diversity indices were also calculated using DOTUR, as the non-parametric richness estimator Chao1 and the Shannon–Weiner diversity index, respectively. The Simpson diversity index was calculated using DOTUR, and the reciprocal index was calculated by hand. Sequences homologous to the clones were searched for in the DNA Data Bank of Japan (DDBJ: http://www.ddbj.nig.ac.jp/index-j.html) using BLAST. Phylogenetic trees were constructed using the neighbor-joining algorithm[28] and visualized by the TreeView program[19].

Nucleotide sequence accession numbers

The *nirK* and *nirS* sequences reported in this study have been deposited in DDBJ under accession numbers AB456742 to AB456866 (*nirK*) and AB456867 to AB456954 (*nirS*).

Results

Environmental parameters

The environmental parameters measured for soils from SG1W and UG31A are shown in Table 1. Apparent differ-

Table 1. Environmental parameters of the forest soils sampled from the Matsuzawa catchment at the Kiryu Experimental Watershed, Shiga prefecture, Japan

Plot (slope position)	Soil temp. (°C)	Water content by wet soil (%)	Water content[a] (% MWHC)	pH (H_2O)	EC (mS m^{-1})	NH_4^+-N (mg kg^{-1} dry soil)	NO_2^--N (mg kg^{-1} dry soil)	NO_3^--N (mg kg^{-1} dry soil)	TC (g C kg^{-1} dry soil)	TN (g N kg^{-1} dry soil)	C/N	Date
SG1W (lower)	12.7	61.17	101.7	5.85	1.92	22[b]	b.d.l.[b]	6[b]	130	6.06	21.5	16 May 2006
	—	67.65	129.3	5.89	1.80	112[b]	b.d.l.[b]	b.d.l.[b]	125	5.84	21.4	17 July 2006
	16.2	64.75	113.6	5.41	2.26	8.97	b.d.l.	16.8	148	7.60	19.6	11 June 2007
	21.3	70.31	146.5	6.19	7.79	51.6	b.d.l.	b.d.l.	145	6.86	21.1	13 July 2007
	0.7	69.88	143.5	5.62	5.40	6.55	b.d.l.	b.d.l.	246	10.8	22.8	24 January 2008
	0.0	68.99	137.5	5.87	5.10	5.69	b.d.l.	1.70	165	8.20	20.1	11 February 2008
	—	68.97	137.4	5.35	5.47	5.40	b.d.l.	3.50	184	8.75	21.0	17 May 2008
UG31A (upper)	13.8	46.66	84.25	4.00	2.41	2[b]	b.d.l.[b]	6[b]	137	6.38	21.4	16 May 2006
	—	57.89	132.4	3.78	1.88	—	—	—	132	6.61	20.0	17 July 2006
	18.5	37.38	57.48	4.08	2.15	11.4	b.d.l.	b.d.l.	166	8.46	19.6	11 June 2007
	2.2	40.55	65.69	4.01	5.32	3.30	b.d.l.	b.d.l.	133	6.39	20.8	24 January 2008
	—	23.61	29.76	4.36	5.80	8.58	b.d.l.	b.d.l.	110	4.95	22.3	17 May 2008
p	<0.05	<0.05	<0.1	<0.05	0.291	0.504	—	0.212	0.304	0.346	0.489	

b.d.l., Below detection limit.
—, Not examined.
[a] Maximum water-holding capacity (MWHC) was 1.618 g g^{-1} dry soil for SG1W and 1.038 g g^{-1} dry soil for UG31A (17 May 2008).
[b] Data were obtained by the distillation and titration method. Detection limit was 2 mg kg^{-1} dry soil.

ences between the SG1W and UG31A soils were observed in water content ($p<0.05$) and pH ($p<0.05$), which are likely to influence denitrification activity and denitrifier communities. Maximum water-holding capacity (MWHC) of soils sampled on 17 May 2008 was 1.618 g g^{-1} dry soil (61.80%) for SG1W and 1.038 g g^{-1} dry soil (50.94%) for UG31A. The water content of the SG1W soil ranged from 62.17% to 70.31% (from 101.7% to 146.5% of MWHC), and was higher than that of the UG31A soil, which ranged from 23.61% to 57.89% (from 29.76% to 132.4% of MWHC). The highest water content at both sites was observed at the end of the rainy season (17 July 2006 and 13 July 2007). The soil pH (H_2O) was lower at SG1W than at UG31A. When averaged across sampling dates, the soil pH (mean±SD) was 5.74 ±0.30 at SG1W and 4.05±0.21 at UG31A. The less acidic measurements at SG1W are similar to the values measured in the nearby groundwater (pH 5.60–6.39).

Nitrate and nitrite were little detected in either soil. By contrast, ammonium was accumulated in both soils. High ammonium concentrations were measured at SG1W at the end of the rainy season (17 July 2006 and 13 July 2007), although no significant differences in ammonium concentrations were observed between SGW1 and UG31A ($p=0.504$). No significant differences in total carbon or nitrogen were observed between SG1W and UG31A (Table 1).

TDC in SG1W and UG31A soils (mean±SD) was $4.0\pm0.2\times10^9$ cells g^{-1} dry soil and $3.6\pm0.2\times10^8$ cells g^{-1} dry soil, respectively. Soil ergosterol content, an indicator of fungal biomass, was 0.48 μg g^{-1} dry soil at SG1W and 1.3 μg g^{-1} dry soil at UG31A.

Denitrification activity

Denitrification activities in SG1W and UG31A are shown in Table 2. Very strong denitrification activity was measured in the SG1W soil located at the lower end of the catchment, which is occasionally submerged under water after rain. When the SG1W soil was incubated with $^{15}NO_3^-$ under anoxic conditions, the denitrification activity was two orders of magnitude higher than that observed for UG31A soil of the upper part of the catchment, which was comparatively dry (Table 2).

Fig. 2 shows the denitrification activity measured for SG1W soil sampled on 17 May 2008. The sum of $^{30}N_2$ and $^{46}N_2O$ production was 505 nmol g^{-1} dry soil h^{-1}, comprising $^{30}N_2$ production of 341 nmol g^{-1} dry soil h^{-1} and $^{46}N_2O$ production of 164 nmol g^{-1} dry soil h^{-1}. Therefore, the relative rate of N_2O reduction ($\Delta^{30}N_2/[\Delta^{30}N_2+\Delta^{46}N_2O]$) was 0.68. Denitrification activity was measured five times for the SG1W soils collected from 11 June 2007 to 17 May 2008 and similar denitrification pattern was obtained, which ranged from 254 to 505 nmol ($^{30}N_2+^{46}N_2O$) g^{-1} dry soil h^{-1}. Added $^{15}NO_3^-$ was immediately reduced to $^{30}N_2$ within a few minutes.

When the SG1W soil sampled on 13 July 2007 was incubated with $^{15}NO_3^-$ under anoxic conditions, its denitrification activity was 363 nmol ($^{30}N_2+^{46}N_2O$) g^{-1} dry soil h^{-1}, whereas the $^{30}N_2$ and $^{46}N_2O$ production from $^{15}NO_3^-$ was only 259 nmol ($^{30}N_2+^{46}N_2O$) g^{-1} dry soil h^{-1} under microoxic condi-

Table 2. Denitrification activity measured by ^{15}N tracer experiments

Plot Date	(Oxic condition, amended electron acceptor) Anoxic, $^{15}NO_3^-$				Anoxic, $^{15}NO_2^-$				Microoxic, $^{15}NO_3^-$				Microoxic, $^{15}NO_2^-$			
	$^{30}N_2$ (A)	$^{46}N_2O$ (B)	(A)+(B)	(A)/(A+B) (%)	$^{30}N_2$ (A)	$^{46}N_2O$ (B)	(A)+(B)	(A)/(A+B) (%)	$^{30}N_2$ (A)	$^{46}N_2O$ (B)	(A)+(B)	(A)/(A+B) (%)	$^{30}N_2$ (A)	$^{46}N_2O$ (B)	(A)+(B)	(A)/(A+B) (%)
SG1W																
11 June 2007	*276 (271, 281)	123 (114, 133)	400 (396, 404)	69.1 (67.2, 71.1)	—	—	—	—	—	—	—	—	—	—	—	—
13 July 2007	336 (306–372)	27.3 (25.6–30.0)	363 (336–398)	92.4 (91.1–93.6)	82.5 (58.8–107)	137 (131–140)	219 (197–238)	37.2 (29.8–44.9)	225 (215–234)	33.7 (29.8–38.6)	259 (244–267)	87.0 (85.4–87.8)	—	—	—	—
11 February 2008	184 (181–187)	176 (169–183)	360 (350–366)	51.0 (50.0–51.8)	—	—	—	—	—	—	—	—	—	—	—	—
11 February 2008[a]	141 (124–148)	114 (107–119)	254 (239–269)	55.3 (51.8–58.1)	—	—	—	—	62.8 (60.3–64.3)	92.2 (90.4–94.9)	155 (152–159)	40.5 (39.8–41.3)	—	—	—	—
17 May 2008	341 (305–369)	164 (156–169)	505 (474–525)	67.5 (64.3–70.3)	—	—	—	—	—	—	—	—	—	—	—	—
17 May 2008 (autoclaved)	b.d.l.	b.d.l.	—	—	b.d.l.	2.10 (2.02, 2.17)	2.10 (2.02, 2.17)	0	—	—	—	—	—	—	—	—
UG31A																
11 June 2007	b.d.l.	2.76 (2.55, 2.97)	2.76 (2.55, 2.97)	0	—	—	—	—	—	—	—	—	—	—	—	—
24 January 2008[b]	b.d.l.	3.60 (3.55, 3.65)	3.60 (3.55, 3.65)	0	0.460 (0.226, 0.694)	9.42 (9.16, 9.68)	9.88 (9.85, 9.90)	4.66 (2.28, 7.05)	b.d.l.	2.67 (2.48, 2.87)	2.67 (2.48, 2.87)	0	0.453 (0.291, 0.616)	17.6 (17.4, 17.9)	18.1 (17.7, 18.5)	2.49 (1.64, 3.33)
17 May 2008	b.d.l.	2.73 (2.24–3.03)	2.73 (2.24–3.03)	0	1.87 (1.29–2.20)	22.5 (21.5–23.1)	24.3 (23.7–24.9)	7.69 (5.29–9.29)	b.d.l.	2.22 (2.05–2.49)	2.22 (2.05–2.49)	0	1.33 (0.330–1.87)	37.9 (33.0–42.3)	39.2 (34.8–44.2)	3.42 (0.854–5.16)
17 May 2008 (autoclaved)	b.d.l.	b.d.l.	—	—	b.d.l.	0.819 (0.777, 0.862)	0.819 (0.777, 0.862)	0	—	—	—	—	—	—	—	—

b.d.l., Below detection limit.
—, Not examined.
* Results are expressed as mean values (nmol g^{-1} dry soil h^{-1}), n=3, except for the samples of 11 June 2007, 24 January 2008, and 17 May 2008 (autoclaved), n=2. Individual measurements are shown in parentheses as minimum and maximum values.
[a] Denitrification activity was calculated by N_2 and N_2O production during 5 hours.
[b] Denitrification activity was calculated by N_2 and N_2O production during 48 hours.

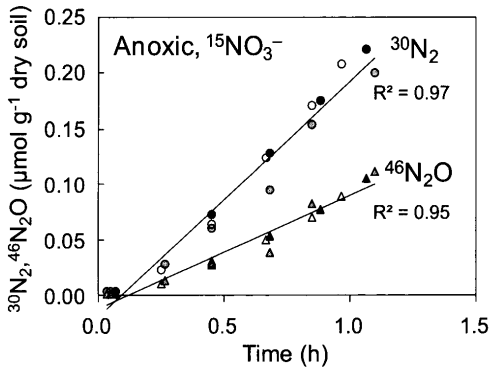

Fig. 2. $^{30}N_2$ (circles) and $^{46}N_2O$ (triangles) production in the SG1W soil sampled on 17 May 2008. The soil was incubated with $^{15}NO_3^-$ under anoxic conditions in triplicate (○, ◍, ●/△, ◬, ▲).

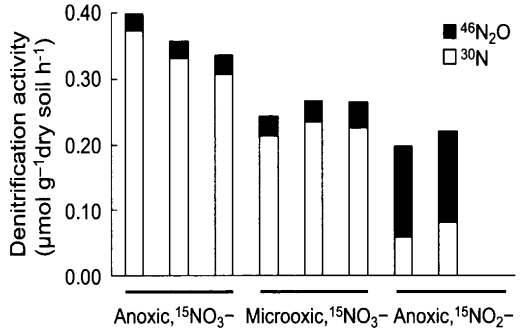

Fig. 3. Denitrification activity comprising the $^{30}N_2$ (open bars) and $^{46}N_2O$ (closed bars) production rates in SG1W soil sampled on 13 July 2007. The soil was incubated with $^{15}NO_3^-$ or $^{15}NO_2^-$ under anoxic or microoxic conditions in triplicate. All measurements were done in 1.3 to 1.6 h.

tions (Fig. 3). A similar pattern was obtained at SG1W on 11 February 2008 (Table 2). When $^{15}NO_2^-$ was added instead of $^{15}NO_3^-$ under anoxic conditions, the denitrification activity decreased from 363 nmol g^{-1} dry soil h^{-1} to 219 nmol ($^{30}N_2$+$^{46}N_2O$) g^{-1} dry soil h^{-1} and N_2O accumulated in the vials ($\Delta^{30}N_2/[\Delta^{30}N_2+\Delta^{46}N_2O]=0.37$).

The effects of oxygen and differences in electron acceptor (nitrate and nitrite) on the denitrification reaction and activity clearly differed between SG1W and UG31A. When the UG31A soil sampled on 17 May 2008 was incubated with $^{15}NO_3^-$ under anoxic conditions, the amount of $^{46}N_2O$ produced was 2.73 nmol g^{-1} dry soil h^{-1}, whereas $^{30}N_2$ did not increase significantly during the incubation period of 7 h (Fig. 4a). To investigate whether the incubation period affected N_2 production, a prolonged experiment was performed for 17 days with a sample collected on 24 January 2008. The initial N_2O production rate did not differ between those two experiments (Table 2). Furthermore, no significant increase in the $^{30}N_2$ concentration was observed in the vials (Table 2), which suggests that N_2O reductase was not active in the UG31A soil. The same phenomenon was observed again on 11 June 2007 (Table 2). In the sample collected on 17 May 2008, the production of $^{46}N_2O$ from $^{15}NO_3^-$ was not affected by the presence of a small amount of oxygen (microoxic conditions) (Fig. 4b). When $^{15}NO_2^-$ was added instead of $^{15}NO_3^-$ under anoxic conditions, the N_2O production rate increased to 22.5 nmol g^{-1} dry soil h^{-1} (Fig. 4c). N_2O production from $^{15}NO_2^-$ increased significantly under microoxic conditions to 37.9 nmol g^{-1} dry soil h^{-1} (Fig. 4d, $p<0.05$). N_2 production from $^{15}NO_2^-$ was just 1.87 nmol g^{-1} dry soil h^{-1} under anoxic conditions and 1.33 nmol g^{-1} dry soil h^{-1} under microoxic conditions. A similar profile of denitrification was found at UG31A on 24 January 2008 (Table 2). When the UG31A soil collected on 17 May 2008 was adjusted to a pH of 7.0 by using 50 mM phosphate buffer (pH 7.0) and 2N NaOH and incubated with $^{15}NO_3^-$ under

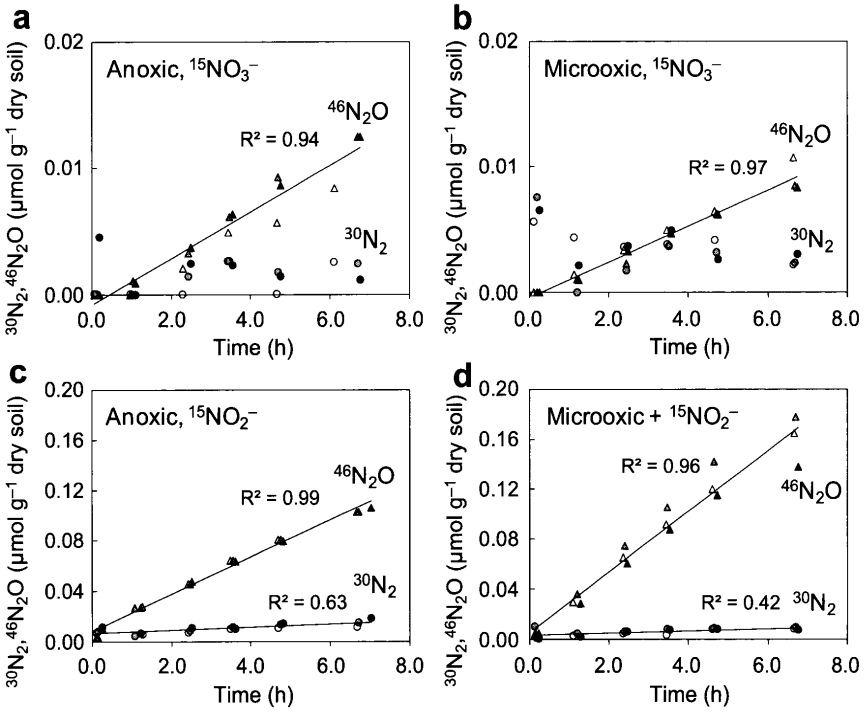

Fig. 4. $^{30}N_2$ (circles) and $^{46}N_2O$ (triangles) production in UG31A soil sampled on 17 May 2008. The soil was incubated with $^{15}NO_3^-$ or $^{15}NO_2^-$ under anoxic or microoxic conditions in triplicate (○, ◍, ●/△, ◬, ▲): (a) with $^{15}NO_3^-$ under anoxic conditions; (b) with $^{15}NO_3^-$ under microoxic conditions; (c) with $^{15}NO_2^-$ under anoxic conditions; (d) with $^{15}NO_2^-$ under microoxic conditions.

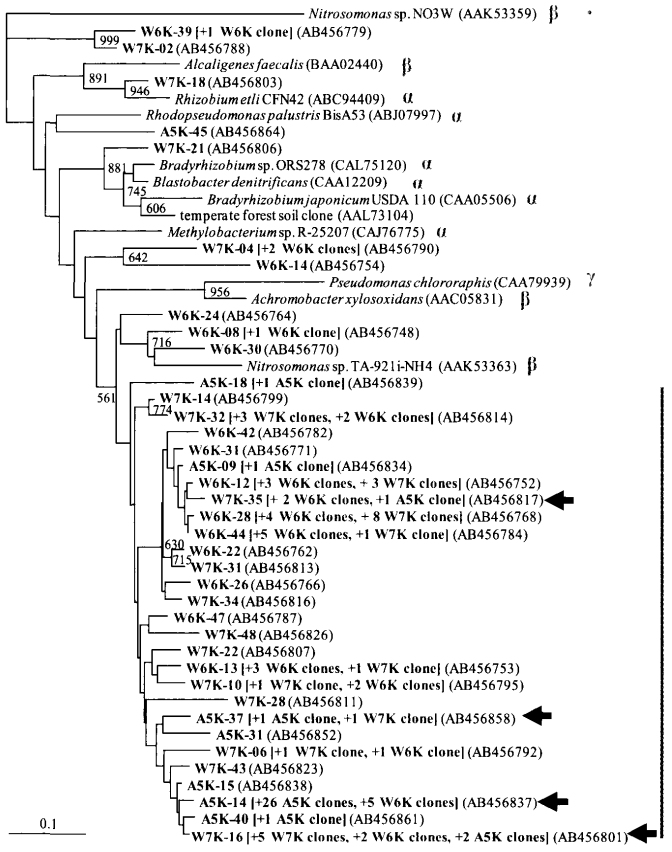

Fig. 5. Phylogenetic tree based on the *nirK* gene products (partial, ~170 amino acids) detected at SG1W on 11 June 2007 (green, W6K), SG1W on 13 July 2007 (blue, W7K), and UG31A on 17 May 2008 (red, A5K). The DDBJ accession numbers of representative clones are shown in parentheses. Bootstrap values above 50% for 1,000 replicates are shown. The sequence of *Nitrosomonas* ap. NO3W NirK was used as the outgroup to root the tree. The largest clade is shown by an orange vertical bar. The OTUs that occurred at both SG1W and UG31A are shown by arrows. Database sequences are shown in black, with DDBJ accession numbers in parentheses. The phylogenetic positions of identified species based on 16S rRNA genes are indicated by α, β, and γ for the subgroups of the *Proteobacteria*.

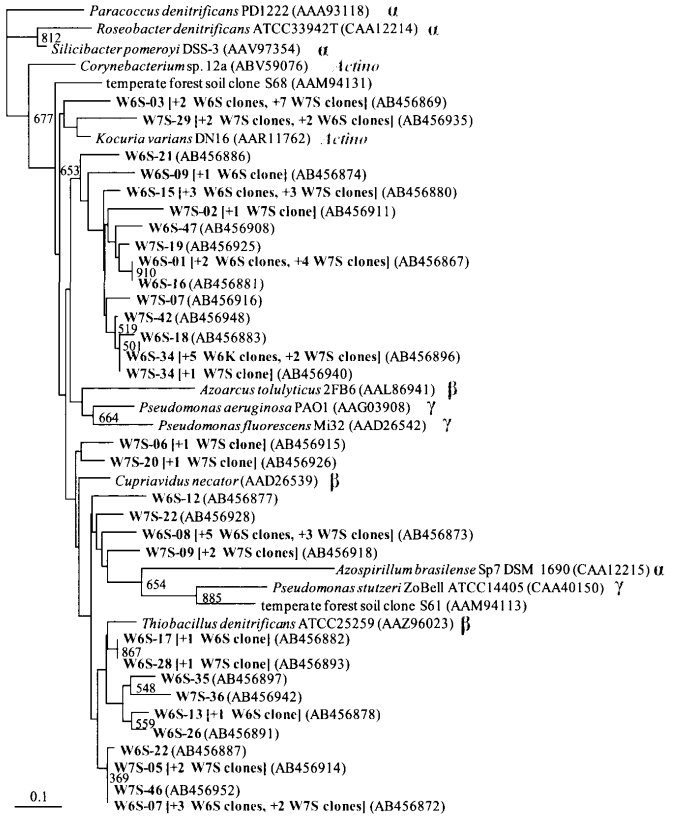

Fig. 6. Phylogenetic tree based on *nirS* gene products (partial, ~110 amino acids) detected at SG1W on 11 June 2007 (green, W6S) and on 13 July 2007 (blue, W7S). The DDBJ accession numbers of representative clones are shown in parentheses. Bootstrap values above 50% for 1,000 replicates are shown. The sequence of *Paracoccus denitrificans* NirS was used as the outgroup to root the tree. Database sequences are shown in black, with DDBJ accession numbers in parentheses. The phylogenetic positions of identified species based on 16S rRNA genes are indicated by α, β, and γ for the subgroups of the *Proteobacteria*, and by *Actino* for the *Actinobacteria*.

anoxic conditions, the N_2O production rate decreased to 1.10 nmol g^{-1} dry soil h^{-1}, and no N_2 was produced, which demonstrated that N_2O reductase activity was not affected by acidic conditions in the experiment shown in Fig. 4a.

When the autoclaved SG1W and UG31A soils collected on 17 May 2008 were incubated with $^{15}NO_2^-$ under anoxic conditions, $^{46}N_2O$ production amounted only to 2.10 nmol g^{-1} dry soil h^{-1} and 0.819 nmol g^{-1} dry soil h^{-1} being 1% and 3% of the measured denitrification, respectively. This indicated that N_2O production from $^{15}NO_2^-$ was mostly carried out through biotic reactions.

Potential nitrification

The incubation experiment carried out with either 0 or 20 μg $(NH_4)_2SO_4$-N g^{-1} dry soil showed an increase in nitrification activity from 17.60 to 220.9 μg NO_3^--N g^{-1} dry soil on incubation from 42 to 44 days, but no NO_3^- production in the UG31A soil.

Phylogenetic analysis of nirK *and* nirS

To analyze the denitrifying bacterial community, gene fragments of the nitrite reductase genes *nirK* and *nirS* were PCR amplified, cloned, and sequenced from the DNA extracted from the UG31A and SG1W soils. A total of 288 clones were sequenced, 144 *nirK* clones and 144 *nirS* clones. Forty eight clones were sequenced for each gene of each soil.

A BLAST search indicated that 125 of the 144 clones

Table 3. Diversity indices and predicted richness of *nirK* and *nirS* gene fragments from SG1W and UG31A, as estimated with the Shannon–Weiner and reciprocal Simpson indices and the Chao1 richness estimator, computed with DOTUR

Gene	Plot	Date	No. of *nir* clones sequenced	No. of OTUs (OTU: 5% cut)	Chao1	Coverage (%)	Shannon-Wiener Index H'	Simpson Index $1/D$
nirK	SG1W	11 June 2007	46	18	22 (19, 38)	82	2.51	9.76
	SG1W	13 July 2007	39	19	65 (31, 190)	29	2.55	11.1
	UG31A	17 May 2008	40	8	9 (8, 16)	89	1.17	2.03
nirS	SG1W	11 June 2007	43	19	26 (21, 51)	73	2.72	17.4
	SG1W	13 July 2007	45	20	23 (21, 36)	87	2.83	20.6

from SG1W and UG31A showed homology to known *nirK* sequences ≥79%. The remaining 19 clones did not match any comparative sequences in the database with sufficient length through the BLAST search. Sequences of non-nitrate reductase genes that showed an E-value of more than 1×10^{-14} in the BLAST analysis were excluded. One hundred twenty-five *nirK* sequences were grouped into 36 OTUs based on the criterion of a ≤5% difference in their nucleic acid sequences. The coverage of the number of OTUs analyzed relative to the expected maximum number of OTUs for *nirK* ranged from 29% to 89% (Table 3).

In the SG1W sample, 85 clones were grouped into 29 OTUs; 18 OTUs were detected from 46 clones collected on 11 June 2007, and 19 OTUs were detected from 39 clones collected on 13 July 2007 (Table 3). Seven of the 29 OTUs contained more than five individual clones, but no OTUs consisting of large numbers of clones were found for *nirK* from SG1W.

The 29 OTUs from SG1W were divided into four distinct clades (Fig. 5) and the largest clade consisted of 72 clones, comprising 85% of the total number of *nirK* clones obtained from SG1W. The clade shown by a vertical bar in Fig. 5 contains these OTUs but includes no identifiable species. In contrast, the clones distributed among the other clades were comprised previously identified species.

No clone showed 90% similarity to a known *nirK* sequence. Although the levels of similarity were not high, the closest known sequences to all the OTUs were those from *Rhodopseudomonas*, *Rhizobium*, *Bradyrhizobium*, and *Nitrosomonas*.

In contrast, only 8 OTUs were detected from 40 *nirK* clones in the UG31A sample collected on 17 May 2008 (shown in red in Fig. 5). Twenty-seven of the 40 clones (68%) were grouped into an OTU, which also contained five clones from SG1W. Almost all the 40 *nirK* clones (except one) obtained from UG31A were classified into the largest clade shown by the vertical bar in Fig. 5. Four OTUs consisting of 50 clones were obtained from both SG1W and UG31A (shown by the arrow in Fig. 5).

In the analysis of the *nirS* gene fragments, the BLAST search indicated that 88 clones from SG1W showed homology to known *nirS* sequences ≥78% (Table 3). No sequences matching the remaining 56 clones were detected with BLAST. All the fragments amplified from UG31A seemed to be false fragments, which did not match with any similar sequence in the database. Sequences of non-nitrate reductase genes that showed an E-value of more than 1×10^{-14} in the BLAST analysis were excluded. The SG1W samples show that 88 *nirS* sequences were grouped into 31 OTUs by differences of ≤5% in their nucleic acid sequences; 19 OTUs were detected from 43 clones collected on 11 June 2007, and 20 OTUs from 45 clones collected on 13 July 2007 (Table 3). The coverage of the number of analyzed OTUs relative to the expected maximum number of OTUs for *nirS* was 73%, and 87% for individual samples.

Clones of the *nirS* gene from SG1W were divided into two large clades, as shown in Fig. 6. No clone had a similarity greater than 90% to a known *nirS* sequence. No dominant group of *nirS* was observed among the 31 distinct OTUs from the SG1W soil.

Both the Shannon–Weiner and reciprocal Simpson indices for *nirK* were higher at SG1W than at UG31A. In a comparison of both gene fragments, the indices did not differ significantly at SG1W between 11 June and 13 July 2007. The diversity indices for *nirS* were greater than those for *nirK* at SG1W.

Discussion

Very high denitrification activity was found in the environment of a mixed forest dominated by Japanese cypress and Japanese red pine. The site of strong activity was at SG1W with wet soil located at the lower end of the catchment. The denitrification activity was 254 to 505 nmol g^{-1} dry soil h^{-1}, which is, to the best of our knowledge, at least three times higher than any previously reported activity measured by the acetylene inhibition assay[11,21,26]. Previous studies in forest soil performed with the acetylene inhibition assay showed denitrification activities ranging from 0.11±0.04 nmol g^{-1} dry soil h^{-1} in a coniferous forest in western North America[26] to 75.7±2.1 nmol g^{-1} soil h^{-1} in a broad-leaved forest in Denmark[21].

In contrast, the denitrification activity measured in the well-drained soil of UG31A was apparently low, ranging from 2.73 to 3.60 nmol N_2O g^{-1} dry soil h^{-1} as an end product, which is in the range of values previously reported for temperate forests[11]. The UG31A environment is strongly influenced by the penetration of air. Therefore, the activity measured under anoxic conditions suggests potential denitrification activity there. In fact, the measured N_2O concentration was low in the unsaturated zone, ranging from 316 to 360 ppbv, whereas the maximum N_2O concentration in the saturated zone amounted to 1,682 ppbv[18].

It has been suggested that denitrification occurs at the lower end of a catchment, which is often submerged due to rainfall or snow melt, creating anoxic conditions. When the submerged conditions at SG1W become anoxic down to about 10 cm of the A horizon, the very high level of denitrification activity discussed above is expected. If strong anoxic conditions do not develop, a reduction in denitrification activity of roughly 30% is expected, as suggested when the denitrification activity was measured under microoxic conditions (Fig. 3). Koba *et al.*[16] also found denitrification at SG1W by using the ^{15}N natural abundance method even though anoxic conditions were not strong. It is suggested that denitrification still occurs at SG1W under wet conditions, even if the site is exposed to oxygen.

We also measured the denitrification activity in the groundwater near SG1W. Groundwater taken from a depth of 250 cm in a well drilled at SG1W showed denitrification activity ranging from 1.4×10^{-4} to 1.7×10^{-3} nmol N_2 mL^{-1} h^{-1} (unpublished data). Thus, the denitrification activity at SG1W was five to seven orders of magnitude higher than that in the groundwater. This suggests that the wet soil at the end of the catchment of a forest is a hot spot for denitrification, with high potential denitrification activity.

We clearly demonstrated that nitrate was immediately reduced to N_2 gas in the soil at SG1W, whereas the addition of $^{15}NO_2^-$ caused an accumulation of N_2O during the experiment, which suggests that nitrite decreased N_2O reductase

activity. However, no nitrite was detected in any soil examined (Table 1). Nitrogen saturation in a forest ecosystem results not only in the supply of nitrate to downstream ecosystems but also the emission of N_2O gas. Wet soil at the lower end of a catchment can reduce the nitrate concentration and scavenge N_2O due to its high potential denitrification activity, which is sustained by the environment and by the constituents of the microbial community. Osaka *et al.*[18] found that the N_2O concentration in groundwater was markedly higher than that in soil gas and was mainly produced in the groundwater itself. The complete denitrification at SG1W revealed in this study supported the finding that the N_2O concentration was low in the soil there. If N_2O is supplied from groundwater to the soil, it is expected to be reduced to N_2.

While N_2 gas was the end product of denitrification at SG1W, the end product at UG31A was mostly N_2O (Fig. 4). It is reported that some bacteria do not bear a N_2O reductase gene in their genome and produce N_2O as an end product (e.g., Sameshima-Saito *et al.*[29]). Thus, it can be expected that those bacteria at UG31A will produce N_2O under the experimental anoxic conditions. N_2O is also produced during fungal denitrification[33], and *Fusarium oxysporum* produced $^{45}N_2O$ when incubated with $^{15}NO_3^-$[32]. However, we detected no $^{45}N_2O$ in this study. Zhaorigetu *et al.*[41] found that N_2O emission was positively correlated with soil ergosterol content of an indicator of fungal biomass. Soil ergosterol content was three times higher at UG31A than SG1W, whereas TDC was one order of magnitude lower at UG31A than SG1W. This suggests that fungal denitrification contributed to N_2O production in the incubated UG31A soil. Although the addition of $^{15}NO_2^-$ enhanced N_2O production at both SG1W and UG31A, total denitrification activity was suppressed at SG1W (Fig. 3). The different response to NO_2^- between SG1W and UG31A suggests the constituents of denitrifying microbes to be different between these two soil environments. Inhibitory effects of NO_2^- are suggested to be different among bacteria and Eukaryote[40]. Although the possible participant microbes are yet to be identified fully, our measurements show the dry and acidic forest soil studied (UG31A) to be a source of N_2O gas. However, our study also indicates that this expected activity is far weaker than the denitrification activity that transforms nitrate and nitrite to N_2 gas, which occurs vigorously in wet soil (SG1W). Whether the entire forest soil system studied is a source or a sink of nitrate and N_2O will only be elucidated by studying the development of habitats appropriate to either N_2O production or N_2 production.

When the soil was incubated with ammonium (0 or 20 μg NH_4-N g^{-1} dry soil) under oxic conditions in a flask that maintained the *in situ* wet conditions, nitrate was detected only in the SG1W soil. This nitrification is a potential source of nitrates for microbes in the surface soil of SG1W. The supply of an electron acceptor through nitrification in the surface environment may support high denitrification activity in the anoxic parts of SG1W. Denitrification is often limited by the availability of electron acceptors in N-limited forest environments[12]. Thus, the absence of nitrification activity observed at UG31A with the same experimental protocol indicated that the supply of electron acceptors is limited.

Whether an environment is anoxic or microoxic and whether the electron acceptor is nitrate or nitrite determines the denitrification reaction and activity of bacterial populations activated by environmental conditions, such as water content, organic compounds, and pH. Differences in the organic compound constituents of an environment are potential sources of the variability in denitrification activity. However, data pertinent to this discussion were not collected in this study.

We found numerous OTUs among the *nirK* and *nirS* gene fragments from SG1W. The Shannon-Weiner and reciprocal Simpson indices for *nirK* were higher at SG1W than at UG31A (Table 3), which suggests that a variety of *nir*-bearing bacteria can express denitrification activity in fluctuating natural environments.

In particular, the finding that *nirK* gene products were distributed among different clades of Fig. 5 suggests a flexible response to changing environments facilitated by the different physiological properties of nitrite reductase.

In contrast, the largest clade in Fig. 5 contained no known *nirK* sequences. The possibility that the 72 clones (23 OTUs) in the largest clade found at SG1W had moved to this site individually or even together in less than 100 years since the formation of this forest ecosystem is not great. These clones might diversify there, though no geographic barrier is apparent, as it is for thermophilic prokaryotes restricted to geothermal environments[20,38]. Diversification within a clade on a phylogenetic tree constructed from *nirK* gene fragments has also been reported for samples from freshwater and brackish environments[30] and marine sediments[5]. However, the *nirK* clones isolated in our study show no strong similarity to those reported sequences. Therefore, the diversification at the clone level observed at SG1W might be unique to this site and might have been created in this area. An active clone will thus be elucidated from this clade.

NirS-bearing bacteria found at SG1W are shown to distribute among different sub-phyla (Fig. 6) based on the finding that the *nirS* gene phylogeny is mostly congruent with the 16S rRNA gene phylogeny at the family or genus level[13,27]. The widely distributed *nirS*-bearing bacteria at SG1W at the sub-phylum level may suggest a flexible response to changing environments.

We did not amplify any *nirS* fragment from the UG31A soil, although we extracted sufficient DNA (20.8 μg g^{-1} of dry soil) to do so. This unsuccessful amplification of *nirS* from UG31A is well consistent with other findings in forested upland soils using the same primers[24]. The low pH in the soil may contribute to the low *nirK* sequence diversity and low *nirS* occurrence, which is true for UG31A.

We have shown that the environment at SG1W provides both the habitat and conditions for the expression of denitrification activity. Clarifying the relationship between denitrification activity and bacterial diversity will extend our understanding of the significance of diversity in ecosystem functioning[23,37]. Therefore, the elucidation of active denitrifiers *in situ* using fluorescent *in situ* hybridization is required for the next step in this direction.

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