1Implications of wetland degradation for the potential denitrifying activity and bacterial populations

2with *nirS* genes as found in a succession in Qinghai-Tibet plateau, China

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19**Abstract:** Alpine wetland in the Zoige Plateau has suffered from serious degradation during the last 20 30 years due to global climate change and anthropogenic impact．Denitrification is a key nitrogen 21 removal process which can be performed by different microorganisms, including bacteria harboring 22 *nirS*-genes. In this study, a degradation succession was used to study the effect on potential 23 denitrification activity (PDA) and on bacterial communities harboring *nirS* genes. Based on the 24 determination of the PDA, the abundance, structural diversity, and phylogenetic identity of the soil 25 bacteria with *nirS* genes were further assessed by qPCR, terminal restriction fragment length 26 polymorphism (T-RFLP), and DNA-sequencing, respectively. The results showed that soil PDA 27 ranged from 8.78 to 52.77 ng N_2O-N g^{-1} dry soil h^{-1}, being lowest in sandy soil and highest in swamp 28 soil. The abundance of *nirS* genes (copies g^{-1} soil) were also the lowest in the sandy soil while highest 29 in the swamp soil. The average Shannon-Wiener diversity index of the *nirS* denitrifying bacterial 30 structural ranged from 2.20 in the meadow soil to 3.07 in the swamp soil. Redundancy analysis (RDA) 31 showed that the *nirS* denitrifying bacterial community correlated with soil water content and available 32 phosphorus, with water content as the major factor in shaping the *nirS* denitrifying bacterial 33 community. The results of this study suggest that the wetland degradation would decrease soil PDA, 34 and abundance and structural diversity of the denitrifying bacteria with *nirS* genes. These findings can 35 contribute to support a theoretical foundation for predicting the potential influences of wetland 36 degradation on soil denitrifying bacteria in alpine wetlands.

37**Keywords**: Wetland degradation; Potential denitrifying activity; *NirS* denitrifying bacterial 38 community; T-RFLP; qPCR

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1. Introduction

Qinghai-Tibet Plateau is a special geographical and ecological area that plays an important role in environmental change. Studying on the dynamics of nitrogen cycle in this area is critical to evaluate the response and feedback of biogeochemistry cycles to global climate change [1]. Zoige wetland on the eastern edge of the Qinghai-Tibetan Plateau is the highest and largest highland peat marsh in China [2]. Most alpine wetlands in the Zoige Plateau have suffered serious degradation, carbon and nitrogen loss and decline of biodiversity due to global climate change [3] and anthropogenic impact [4]. A lowered water table has resulted in loss of humus and peat horizons and transformation of these into meadow soil. Besides, long term overgrazing by cattle in the meadows has decreased vegetation coverage and disturbed their water regulating function. Moreover, draining the mining areas has influenced the water retention ability of peat swamps, and changed the wetland soil moisture conditions [2]. In a typical degradation succession in Zoige wetland, native swamp soil turns first into transition meadow soil and then into degraded sandy soil [5]. Although it is known that wetland degradation affects plant community composition and reduces diversity and above- and below-ground biomass [6], little is known about how the soil microbial community composition and diversity respond to wetland degradation.

Denitrification is a microbiological process which can be mediated by bacteria, archaea or fungi, and more than 50 genera of bacteria have been identified as denitrifying bacteria [7]. It is a key nitrogen removal process in wetlands and frequently limited by available nitrogen rather than carbon [8]. Given that the high phylogenetic diversity among denitrifying bacteria, detection methods based on assessment of genes such as 16S rRNA are not directly useful and therefore requires the use of functional genes which encode for enzymes directly involved in the denitrification process. There are two structurally different but functionally equivalent nitrite reductases: copper (*nirK*) and cytochrome

63cd1-containing (*nirS*) enzymes, which are responsible for conversion of nitrite (NO_2^-) to nitric oxide

64(NO)[9], are frequently-used as the molecular marker for the cultivation independent studies on

65denitrifying bacteria as only denitrifiers possess this enzyme[10]. These functional genes have been

66used as molecular markers for the cultivation independent studies on denitrifying bacterial diversity in

67various environments. Recent studies showed that *nirS* and *nirK*-type denitrifying bacterial

68communities responded differently to environmental parameters such as nitrate and oxygen

69concentrations in rice fields, and probably the *nirK* denitrifying bacteria were active at the beginning

70of anaerobic incubation, while the *nirS* denitrifying bacteria were more active in the fully developed

71anaerobic conditions [11]. Moreover, *nirS* denitrifying bacteria were found to be far more abundant,

72more active, and more diverse than *nirK* denitrifying bacteria [12]. The presence of bacterial

73populations harbouring *nirS* genes may correlate with environmental factors [13]. To date, little is

74known about how the abundance and community composition of the denitrifying bacteria respond to

75Plateau wetland degradation. Due to the expected bigger role of *nirS* denitrifying bacteria and better

76sequence database coverage of *nirS*, studying the response of denitrifying bacterial community, with a

77focus on *nirS* denitrifying bacteria, to wetland degradation in Qinghai-Tibet Plateau is necessary to

78understand how nitrogen cycle responds to the changing climate in this area.

79   To this end, in combination with the measurement of denitrification potential activities, the

80abundance, structural diversity, and phylogenetic identity of the *nirS* denitrifying bacteria in three

81different soils (native swamp, degraded meadow, and sandy soils) were assessed by qPCR, T-RFLP,

82and DNA-sequencing, respectively. The objectives of this study were (1) to investigate the variation in

83*nirS* denitrifying bacterial community in the three different soils associated with wetland degradation

84succession, and (2) to understand the relationships between the *nirS* denitrifying bacterial community

85and soil physicochemical properties. We hypothesized that wetland degradation had a significantly

86 negative effect on soil potential denitrifying activity, and on the abundance, phylogenetic diversity and

87 community composition of the *nirS* denitrifying bacteria.

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89 2. Materials and Method

90 Soil sampling

91   The study site was on the Zoige Plateau, located at the northeastern part of the Qinghai-Tibetan

92 Plateau (101°30′E-103°30′E, 32°20′N -34°00′N) (Table 1). The average altitude at Zoige Plateau is

93 3500 m and the mean annual temperature is 1.1°C, with the lowest monthly temperature of -10.3 °C in

94 January and the highest of 10.9 °C in July. Annual precipitation is 560-860 mm. Swamp soil (SW1,

95 SW2, and SW3) was covered with water all year round, and the vegetation was dominated by

96 hydrophytes and sedge hydro-mesophytes (*Carex muliensis*, *Carex lasiocarpa* and *Carex meyeriana*).

97 Meadow soil (MD1, MD2, and MD3) surface was in a humid state and the vegetation was dominated

98 by mesophytes and hydro-mesophytes (*Kobresia tibetica*). Sandy soil (SD1, SD2, and SD3) surface

99 was continuously dry and had little or only a few *Psammophytes* as vegetation cover [14].

100 Homogeneous silt and clay were the dominant soil parent materials of marsh soils [2]. The soil parent

101 materials of meadow soil were accumulated residues of triassic slate, sandstones and siltstone [15].

102 Sandy soil, the top layer (0-30 cm) of which was sand, was composed of aeolian parent material [16].

103   The soils were sampled using an auger with 20 mm diameter × 100 cm length and only the

104 surface (5-20 cm) soil samples were analyzed in this study. Each of the replicate samples represented a

105 composite sample of 15 subsamples. Surface vegetation and roots were removed before collecting the

106 samples. Samples (approximately 1.5 kg) were divided into two portions: one was air dried before

107 removing roots and stones, and then sieved through a 2-mm mesh screen for physicochemical

108 analyses. The other portion was stored at -80 °C for DNA extraction.

Soil physicochemical parameters and potential denitrifying activity analysis

Soil water content (WC) was determined after oven-drying at 105 °C for 48 h [17]. Soil total nitrogen (TN) and available nitrogen (AN) were determined using the alkaline hydrolysis diffusion method and semi-micro Kjeldahl method, respectively [18]. Soil organic carbon (SOC) and pH were determined by potassium dichromate oxidation-external heating method and potentiometry method, respectively [18]. Total phosphorus (TP) and available phosphorus (AP) were assessed by hydrofluoric acid-perchloric acid digestion-Mo-Sb colorimetry and sodium bicarbonate extraction-Mo-Sb colorimetry methods, respectively [18]. Total potassium (TK) and available potassium (AK) were determined by hydrofluoric acid-perchloric acid digestion-flame photometry and ammonium acetate extraction-flame photometry, respectively [18]. Potential denitrifying activity (PDA) was estimated using the C_2H_2 inhibition method [19].

Soil total DNA extraction

Total DNA was extracted from 0.5 g homogenized soil using a FastDNA SPIN Kit for Soil (MP Biomedicals, Solon, OH, USA). DNA concentration and quality was measured with a Nano-200 spectrophotometer (AoSheng, Hangzhou, China). DNA samples were stored at –20 °C for further analysis. For clone library construction, the template DNA was a mixture of an equal amount of DNA from the three replicates. The triplicate DNA samples were independently used for terminal restriction fragment length polymorphism (T-RFLP) and real-time PCR assays analysis.

Quantitative PCR

The *nirS* gene was quantified by qPCR with three technical replicates per sample. Standard curves to assess *nirS* gene abundance consisted of a 10-fold serial dilution (10^1-10^7) of a plasmid (299 ng μL^{-1}) containing the *nirS* gene fragments from the tested soil as described by Henry et al. [20]. The *nirS* genes were amplified with primer pair nirSCd3aF (5′-AACGYSAAGGARACSGG-3′) and

132nirSR3cd (5′-GASTTCGGRTGSGTCTTSAYGAA-3′) [21]. For qPCR, 12.5 μL ABI Power

133SybrGreen qPCR Master Mix (Applied Biosystems, USA), 0.5 μM of each primer, 10 μM of soil

134DNA and 9.5 μL of sterile water were used to make up a final volume of 25 μL. The reaction was

135carried out on a ABI7500 sequence detection system (Bio-Rad) using the program of 95 °C for 10 min

136followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 1 min and extension at

13772 °C for 30 s. Absolute *nirS* copy number was calculated by interpolating from the standard curve.

138Gene abundances were standardized by the mass of DNA that was extracted per gram of dry soil and

139$\log_{10}$ transformed before analysis.

140**PCR amplification and T-RFLP of the *nirS* gene**

141   The primer pair nirSCd3aF and nirSR3cd [21] was used to amplify the *nirS* gene in a C1000™

142thermal cycler (Bio-Rad, Hercules, CA, USA). Amplification was done in a volume of 50 μL

143containing 10 μM diluted soil DNA, 5 μL of 10× PCR buffer, 200 μM of dNTP mix, 0.5 μM of each

144primer, 18.5 μL of ddH$_2$O and 2.5 U of Taq DNA polymerase (TAKARA, Otsu, Shiga, Japan) with the

145following cycling conditions: 5 min of denaturation at 95 °C, a 21 cycles touchdown program (94 °C

146for 30 s, annealing at 60 °C for 45 s, and 72 °C for 60 s) where the annealing temperature was lowered

1471 °C after every three cycles, 22 cycles with 94°C for 30 s, 53 °C for 45 s, and 72 °C s for 60 s, and a

1487 min extension at 72 °C.The PCR products were analyzed by electrophoresis on 1.0 % agarose gels

149and visualized by UV Transilluminator Model M-26 (UVP, USA) after ethidium bromide staining.

150   For T-RFLP, the forward primers were fluorescently labeled with 6-Fam (6-carboxyfluoresein).

151The *nirS* gene PCR products were digested with *HhaI* endonuclease (NEB, Ipswich, Massachusetts,

152USA) at 37 °C for 6 h, followed by further purifying by Tiangen DNA purification kit (Tiangen,

153China) to clean up the T-RFLP mix. T-RFLP profiles were generated by capillary electrophoresis

154using an ABI Prism 3100 Genetic Analyzer at Sangong Corporation (Shanghai, China). Sizes and

155relative abundances of terminal restriction fragments (T-RFs) were quantified using PeakScan version

1561.0 software (Applied Biosystems, Inc.). Only fragments with a signal greater than 1% of the sum of

157all peak heights were included in the analysis. The peak heights of T-RFs that differed in size by ≤2 bp

158in an individual profile were summed and considered as one fragment. To assign the peaks of samples

159to clone sequences, clones corresponding to different *in silico* T-RFs were also subject to T-RFLP

160analysis with the same procedure mentioned above.

161**Cloning and sequencing**

162   The *nirS* gene PCR products were excised from agarose gels and purified using universal DNA

163Purification Kit (Tiangen Co, Beijing, China) following the manufacturer's instructions. PCR products

164were cloned into pGEM-T vector (Tiangen) and transformed into *Escherichia coli* strain DH-5α,

165followed by blue–white screening. Inserts were amplified with M13F and M13R primers and the size

166of the inserts were estimated by agarose gel electrophoresis. A total of 182 positive clones

167(approximately 60 clones from each site) were randomly selected for restriction screening by digesting

168with *HhaI* endonuclease, and 15 representative clones were sequenced. The *nirS* gene sequences were

169deposited in GenBank under the accession numbers KT783435-KT783449.

170**Statistical analysis**

171   The means and standard deviations of the soil properties and *nirS* gene diversities were calculated

172and tested for normality and homogeneity of variances. Data was analyzed using one-way analysis of

173variance (ANOVA), followed by Tukey's multiple comparison test when the ANOVA results were

174significant. Otherwise, Tamhane's T3 test was adopted using SPSS 21.0 software (IBM SPSS

175Statistics for Windows, Armonk, NY, USA). Two-sided $P< 0.05$ was considered statistically

176significant. To assess the correlation between soil physicochemical properties and *nirS* gene

177abundances, Pearson correlation coefficients were calculated using the Simple Correlation Method in

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178SPSS 21.0. Redundancy analysis (RDA), which was performed using CANOCO version 5, was used

179to assess the relationship between *nirS* denitrifying bacterial community and environmental variables

180[22]. The significance of the RDA results was tested with the Monte Carlo permutation test.

181	The clone sequences were aligned using ClustalX 1.83 [23], and a neighbor-joining tree was

182constructed using MEGA 5.0 [24]. Bootstrap analysis was used to estimate the reliability of the

183phylogenetic reconstruction (1000 replicates). The *nirF* (accession no. D50473) and *nirN* (accession

184no. D84475) gene sequences from *Pseudomonas aeruginosa* were used as out-groups for phylogenetic

185analysis due to that their proteins have recognizable sequence relatedness to *nirS* [25].

186**3. Results**

187**Soil physicochemical properties and potential denitrifying activity**

188	The soil water content (WC) was highest in swamp (79.4%) and lowest in the sandy soil (6.9%)

189(Table 2). Soil pH in meadow was higher than in swamp and sandy soil. Soil organic carbon (SOC)

190content was significantly higher in the swamp soil than in meadow and sandy soils. Soil TN and AN

191ranged from 0.79 to 5.71 g kg^{-1} and 41.22 to 1079.43 mg kg^{-1}, respectively. Swamp soil had the

192highest concentrations of soil total nitrogen (TN) and available nitrogen (AN). The lowest

193concentrations of TN and AN were in the sandy soil. Total phosphorus (TP) and available phosphorus

194(AP) varied from 0.44 to 1.43 g kg^{-1} and 3.45 to 26.01 mg kg^{-1}, respectively, with the highest values

195observed in swamp soil and the lowest in sandy soil. Total potassium (TK) and available potassium

196(AK) ranged from 6.79 to 23.13 g kg^{-1} and 33.42 to 443.45 mg kg^{-1}, respectively. The highest

197concentrations were in meadow soil and the lowest in sandy soil. Soil potential denitrifying activities

198ranged from 8.78 to 52.77 ng N_2O-N g^{-1} dry soil h^{-1}. Activities were highest in swamp soil and lowest

199in sandy soil. The potential denitrifying activity correlated positively with WC, SOC, TN, AN, TP and

200AP, and negatively with TK ($p < 0.01$) (Table 3).

201 *nirS* gene abundance and diversity

202   The soil *nirS* gene copy numbers ranged from 2.77 $\log_{10}$ per gram dry soil in sandy soil to 5.57

203 $\log_{10}$ per gram dry soil in swamp soil (Fig.1), showing that wetland degradation significantly

204 decreased the *nirS* denitrifying bacterial abundance. The *nirS* abundance correlated positively with soil

205 potential denitrifying activity (p<0.01) (Table 3).

206   To assess the diversity of *nirS* carrying microbial populations, the mean values of richness,

207 Shannon-Wiener index (H) and evenness were calculated based on the T-RFLP data (Table 4). The

208 soil *nirS* denitrifying bacterial community ɑ-diversity varied significantly among the swamp soils,

209 meadow soils and sandy soils (*P*<0.05). The average Shannon-Wiener index ranged from 2.20 in the

210 meadow soil to 3.07 in the swamp soil (Table 4).

211 *nirS* denitrifying bacterial community structure

212   The *nirS* T-RFLP profiles from triplicate samples of each soil were similar, indicating that the

213 results were reproducible and representative for the *nirS* denitrifying bacterial communities in the

214 Zoige Plateau wetland soils (Fig.2). Redundancy analysis (RDA) showed that the *nirS* denitrifying

215 bacterial communities in the swamp soils, meadow soils and sandy soils formed separate clusters

216 (Fig.3), indicating that *nirS* denitrifying bacterial communities from these three different soils related

217 to wetland degradation succession were significantly different (P<0.01).

218   The relative abundances of *nirS* gene were also different in the swamp soils, meadow soils and

219 sandy soils (Fig. 2). The relative abundance of the dominant 75-, 80-, and 115 bp T-RFs (>5%) were

220 slightly different among the soils, indicating that the degradation of Zoige wetland had little impact on

221 the core *nirS* denitrifying bacterial community. On the contrary, all the tested three soils had their

222 specific minor *nirS* T-RFs (<5 %). For example, the 50-, 185-, 190- and 230 bp T-RFs were detected

223 only in the swamp soil, the 260- and 280 bp T-RFs only in the meadow soil, and the 20-, 25-, 35- and

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224255 bp T-RFs in the sandy soil only.

Phylogenetic analysis

226 In the phylogenetic analysis, the *nirS* gene sequences were grouped into four clusters based on

227their relationship with *nirS* gene sequences from various habitats and some well characterized

228denitrifying bacteria (Fig. 4). The *nirS* sequences with T-RFs 76, 77, 116, 121, 129, and 227 bp in

229cluster 1 were related to *nirS* gene sequences retrieved from various environments such as estuary

230sediment soil (GI: KM891947) and wetland sediment (GI: KC468857). The sequences in cluster 2

231with T-RFs 60, 113, and 115 bp were also related to the clones retrieved from sludge and sediment,

232and then grouped with the identified *nirS* gene sequence from gammaproteobacterial *Pseudomonas*

233*fluorescens* (GI: AF114792) and *Pseudomonas aeruginosa* (GI: X16452). The sequences in cluster 3

234with T-RFs 260 bp were related to those from betaproteobacterial *Burkholderiaceae*, *Ralstonia* and

235*Thaueraterpenica*. The sequences in cluster 4 with T-RFs 255 and 280 bp were grouped into one clade

236with those from alphaproteobacterial *Paracoccus* sp.

237 The swamp soils, meadow soils and sandy soils harbored different *nirS* denitrifying bacterial

238communities that carried genes related to *nirS* genes retrieved from various habitats. In the swamp

239soil, the most abundant *nirS* genes which accounted for 91.2% of the *nirS* denitrifying bacterial

240communities were related to *nirS* genes retrieved from freshwater sediment habitats. In the meadow

241soil, the *nirS* that accounted for 72% of the total *nirS* detected were related to *nirS* genes isolated from

242wetland and farmland soil. For the sandy soil, the *nirS* genes in cluster 2 were related to *nirS* genes

243from sediment and sludge (Fig. 4).

Relationships between *nirS* denitrifying bacterial community and environmental parameters

245 In the redundancy analysis (RDA), the *nirS* denitrifying bacterial communities from the swamp

246soils, meadow soils and sandy soils were divided into three separate groups (Fig. 3). The RDA results

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247further confirmed that the *nirS* denitrifying bacterial communities in the soils were significantly

248different ($P<0.05$). Among the measured abiotic factors, water content appeared to be the major factor

249in determining the *nirS* denitrifying bacterial community composition ($P<0.05$). Soil *nirS* denitrifying

250bacterial community variance was also significantly linked to available phosphorus ($P<0.05$).

251**4. Discussion**

252**Soil properties in response to wetland degradation**

253   Microbiologically mediated denitrification is the key process of nitrogen removal from wetlands.

254In this study, the physicochemical properties of soils associated with the Zoige wetland degradation

255succession were analyzed. In earlier studies, the hydrological conditions affected the community

256structure, plant productivity, the import of organic matter, and the accumulation of soil organic carbon

257(SOC) and total nitrogen (TN) [26]. Decrease of the soil water content induced by wetland degradation

258increased organic matter decomposition rates and net N mineralization rates [27], leading to the loss of

259soil carbon, nitrogen, and phosphorus [28]. In the process of wetland restoration, concentrations of

260total and available nitrogen and phosphorus significantly decreased with a decrease in water content

261[29]. In our study, water and organic carbon contents and the concentrations of nitrogen and

262phosphorus all decreased with the succession of wetland degradation, indicating that water content is

263the major factor contributing to the decreases in carbon, nitrogen and phosphorus content that

264accompany Zoige wetland degradation. In an anaerobic environment, decomposition of animal and

265plant residues is slow, which possibly explains why SOC was highest in the flooded swamp soil and

266lowest in the sandy soil. An increase in net N mineralization leads to the loss of nitrogen [27, 28], and

267in accordance to that the nitrogen content was highest in the swamp soil. Higher SOC content may

268provide a larger sorption capacity for phosphates [30], plausibly explaining why the phosphorus

269content was higher in the swamp soil than in the meadow and sandy soils.

270 Wetland degradation decreased soil potential denitrifying activity (PDA)

271   High denitrifying capacity depended on the quantity and quality of the organic carbon needed to

272 support bacterial growth [31], and such dependence could be described as a proportional relationship

273 between the denitrification rate and organic carbon supply [32]. Decreased water content led to an

274 increased decomposition rate of soil organic matter, which then decreased the denitrification activity

275 and changed the dominant species of denitrifying bacterial community [33]. In accordance with the

276 previous studies, the Pearson correlation analysis in this study showed that the PDA correlated

277 significantly with SOC. In our study, the decrease in PDA is probably due to the variation in the

278 response of soil parameters to wetland degradation [34]. Key factors that could have varied include the

279 patchiness of particulate organic material in the wetlands [35], vegetation density [36], and the nature

280 and extent of the aerobic/anaerobic interface in the sediments [37].

281 The *nirS* gene abundance decreased in response to wetland degradation

282   The composition of the denitrifying bacterial communities were affected by water regimes and

283 hydrological factors such as water content were dominant in regulating bacterial communities in

284 wetland soils [13]. The effect of water regime on the proportion of denitrification genes in the

285 bacterial community can be explained by the different preferences of the denitrifying bacteria to

286 oxygen conditions in the environment. Most denitrifying bacteria are facultative anaerobes, preferring

287 oxygen as an electron acceptor. If conditions change from aerobic to anaerobic, they begin to use

288 nitrogen oxide as an alternative electron acceptor [38]. Previous studies on different environments

289 have shown that the *nirS* denitrifying bacteria prefer environments with constantly lower oxygen

290 concentrations [25, 38]. Similarly, in the current study, the soil water content appeared to be the most

291 important factor in affecting the *nirS* gene abundance. The swamp soil is generally anaerobic since soil

292 is flooded with water and oxygen availability is restricted, while in the meadow and sandy soils, water

293level fluctuation presumably creates more aerobic conditions.

294The *nirS* denitrifying bacterial community responded to wetland degradation

295   Our main hypothesis that the denitrifying bacterial community structure and diversity would be

296negatively impacted by wetland degradation was not completely supported by the results. The three

297soils with different degradation succession in Zoige wetland hosted distinct denitrifying bacterial

298communities. As judged by the terminal restriction fragments (TRFs), the core of the communities in

299the three tested soils were similar yet more minor TRFs were detected in the swamp and sandy soils

300and each soil had its′ specific TRFs. This suggested that the minor TRFs contributed to the community

301structural differences. Similar result has been observed also in one previous study [39] indicating that

302the core denitrifying bacteria may not be sensitive to wetland degradation whereas some minor

303denitrifying bacteria with low relative abundance are critical when community responses are

304evaluated. Although the diversity index of the denitrifying bacterial communities in the swamp soil

305(native soil) were higher than those in meadow (transitional soil) and sandy (degraded soil), the

306diversity index of the denitrifying bacterial communities in the meadow soil was lower than that in

307sandy soil, indicating a positive effect of sandy soil on denitrifying bacterial diversity. Adsorbed small

308particles on a sand grain may provide binding sites for adsorption of organic matter and microbial cells

309[40], resulting in a larger number of microhabitats available for colonization. This in turn increases the

310potential of multiple species to coexist in close proximity without directly competing for resources and

311potentially increases bacterial diversity [40]. Thus, the increased denitrifying bacterial community

312diversity may be explained by the increased sand content with degradation.

313   In three farmland soils from Finland (62°55′N, 29°30′E), Germany (48°40′N, 11°04′E), and

314Sweden (58°20′N 13°30′E), the majority of the *nirS* gene clones were similar to the *nirS* from

315*Ralstonia* and *Paracoccus* bacteria, both members of the *Proteobacteria* [41]. The *nirS* genes in an

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316agricultural soil from Changsha, China, were also affiliated with *Paracoccus* [42]. In our study, the

317swamp soils, meadow soils and sandy soils in the Zoige Plateau harbored diverse *nirS* species.

318Meadow soil hosted *nirS* denitrifying bacteria closely related to *Thaueraterpenica* and *Paracoccus,*

319and the sandy soil clones were closely related to *nirS* from *Paracoccus* and *Pseudomonas*. The

320majority of swamp soil clones were related to uncultured strains, indicating a dearth of knowledge on

321the denitrifying bacterial community in the Zoige Plateau wetland.

322**Relationship between *nirS* denitrifying bacterial community structure and soil properties**

323   Understanding the environmental variables that affect microbial community structure is a key

324goal in microbial ecology. Soil moisture was found to be an important abiotic factor in affecting the

325*nirS* denitrifying bacterial abundance and community composition [43]. Oxygen was also found to

326have a decisive effect on the structure of denitrifying bacterial communities in the continental margin

327sediments of the oxygen-deficient zone at the Pacific Coast of Mexico [44]. In addition, the relative

328abundances of *nirS* terminal restriction fragments responded variously to the O_2, NO_3^-, NO_2^- and depth

329gradients in the water column of the oxygen minimum zone in the Eastern South Pacific [45]. In our

330study, soil physicochemical parameters were important in shaping *nirS* denitrifying bacterial

331communities, and soil water content and available phosphorus were the most important factors in

332shaping *nirS* denitrifying bacterial communities in Plateau wetland.

333   Phosphorus was one of the soil parameters which correlated positively with the *nirS* gene

334abundances in this study. It was reported that the availability of phosphorus greatly affected soil

335microbial community [46]. A similar relationship between the abundance of *nirK* denitrifying bacteria

336and available phosphorus has been also observed in a spruce forest soil, where the available

337phosphorus content was closely connected with the nutrient availability [47]. In contrast, a negative

338relationship has been observed for the abundance of *nir* denitrifying bacteria at scales compatible with

339land management [46]. Given that phosphorus is a vital constituent component for microorganisms

340[46], it is not surprising to find a positive correlation across this and other studies [47, 48]. There may

341be a complex relationship between nitrogen-cycling and phosphorus-cycling microorganisms in

342wetland soil. It should be noted that the T-RFLP in this study could only reveal the most dominant

343*nirS* denitrifying bacterial community. To fully reveal the variation of the denitrifying bacterial

344community in the soils associated with Zoige wetland degradation, approaches based on high

345throughput targeted amplicon sequencing should be used.

346   In summary, the soil properties, potential denitrifying activity, and *nirS* denitrifying bacterial

347abundance, structural diversity and phylogenetic identity were different in the three tested soils

348associated with the Zoige wetland degradation succession. Although wetland degradation decreased

349the *nirS* gene phylogenetic diversity, the degraded soil (sandy) harbored relative higher *nirS* diversity

350index than the transition soil (meadow). Among the analyzed soil properties, soil water content was

351the most important factor in shaping *nirS* denitrifying bacterial community composition. The results of

352this study can contribute to support a theoretical foundation for predicting the potential influences of

353wetland degradation on soil denitrifying bacteria in alpine wetlands.

Acknowledgments

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358 References

359 [1] Q.L. Xiong, K.W. Pan, L. Zhang, Y.J. Wang, W. Li, X.J. He, H.Y. Luo, Warming and nitrogen

360 deposition are interactive in shaping surface soil microbial communities near the alpine timberline

361 zone on the eastern Qinghai–Tibet Plateau, Southwestern China, Appl. Soil. Ecol. 101 (2016) 72-83.

362 [2] L.L. Huo, Z.K. Chen, Y.C. Zou, X.G. Lu, J.W. Guo, X.G. Tang, Effect of Zoige alpine wetland

363 degradation on the density and fractions of soil organic carbon, Ecol. Eng. 51 (2013) 287-295.

364 [3] J.H. Bai, Q.Q. Lu, J.J. Wang, Q.Q. Zhao, O.Y. Hua, W. Deng, A. Li, Landscape pattern evolution

365 processes of alpine wetlands and their driving factors in the Zoige Plateau of China, J. Mt. Sci. 10

366 (2013) 54-67.

367 [4] X.J. Guo, W. Du, X. Wang, Z.F. Yang, Degradation and structure change of humic acids

368 corresponding to water decline in Zoige peatland, Qinghai-Tibet Plateau, Sci. Total. Environ. 445-

369 446 (2013) 231-236.

370 [5] J. Tang, X. Ding, L.M. Wang, Effects of wetland degradation on bacterial community in the Zoige

371 Wetland of Qinghai-Tibetan Plateau (China), World. J. Microbiol. Biotechnol. 28 (2012) 649-657.

372 [6] S.B. Wu, P. Kuschk, H. Brix, J. Vymazal, R.J. Dong, Development of constructed wetlands in

373 performance intensifications for wastewater treatment: A nitrogen and organic matter targeted

374 review, Water. Res. 57 (2014) 40-55.

375 [7] W. Zumft, The denitrifying prokaryotes, Prokaryotes, 1 (1992) 554-582.

376 [8] P.G. Hunt, T.A. Matheny, K.C. Stone, Denitrification in a coastal plain riparian zone contiguous to

377 a heavily loaded swine wastewater spray field, J. Environ. Qual. 33 (2004) 2367-2374.

378 [9] S. Ullah, T.R. Moore, Biogeochemical controls on methane, nitrous oxide, and carbon dioxide

379 fluxes from deciduous forest soils in Eastern Canada, J. Geophys. Res. G: Biogeosci 116 (2011)

380 2156-2202.

381 [10]G. Braker, J.Z. Zhou, L.Y. Wu, A.H. Devol, J.M. Tiedje, Nitrite reductase genes (*nirK* and *nirS*) as

382 functional markers to investigate diversity of denitrifying bacteria in Pacific northwest marine

383 sediment communities, Appl. Environ. Microbiol. 66 (2000) 2096-2104.

384 [11]Q. Yuan, P.F. Liu, Y.H Lu, Differential responses of *nirK*- and *nirS*-carrying bacteria to

385 denitrifying conditions in the anoxic rice field soil, Environ.Microbiol. Rep. 4 (2012)113-122.

386 [12] A.C. Mosier, and C. A. Francis, Denitrifier abundance and activity across the San Francisco Bay

387 estuary. Environ.Microbiol. Rep. 2 (2010) 667-676.

388 [13]T. Ligi, M. Truu, J. Truu, H. Nõlvak, A. Kaasik, W.J. Mitsch, Ü. Mander, Effects of soil chemical

389 characteristics and water regime on denitrification genes (*nirS*, *nirK*, and *nosZ*) abundances in a

390 created riverine wetland complex, Ecol. Eng. 72 (2014) 47-55.

391 [14] G.S. Zhang, J.Q. Tian, N. Jiang, X.P. Guo, Y.F. Wang, X.Z. Dong, Methanogen community in

392 Zoige wetland of Tibetan plateau and phenotypic characterization of a dominant uncultured

393 methanogen cluster ZC-I, Environ.Microbiol, 10(2008) 1850-1860.

394 [15]X.Y. Zhang, X.G. Lv, S.P. Shen, Dynamic changes of Ruoergai Plateau wetland ecosystem service

395 value, Chin. J. Appl. Ecol. 20 (2009) 1147-1152. (In Chinese)

396 [16]Y.X. Liang, G.M. Yi, K.Y. Chu, X.J. Xia, C.P. Chang, J.X. Liu, M.Y. Yang, H. Yu, B. Fu, M.H.

397 Chen, The research of the relationship between shrink age of Zoige's wetlands, deterioration and

398 desertification of Zoige's grasslands and north sandy arid region, Chin. J. Nat. 29 (2007) 233-238.

399 (In Chinese)

400 [17]C.C. Shi, J. Tu, Remote sensing monitory study on land desertification in Ruoergai Plateau of

401 Sichuan Province during 40 years, South. West. Chin. J. Agric. Sci. 22 (2009) 1662-1664. (In

402 Chinese)

403 [18]R.K. Lu, The methods of agriculture chemical analysis. China Agricultural Sicentech Press.

404   Beijing. 1999 pp, 146-165. (In Chinese)

405 [19]C. Dambreville, S. Hallet, C. Nguyen, T. Morvan, J.C. Germon, L. Philippot, Structure and

406   activity of the denitrifying community in a maize-cropped field fertilized with composted pig

407   manure or ammonium nitrate, FEMS. Microbiol. Ecol. 56 (2006) 119-131.

408 [20]S. Henry, E. Baudoin, J.C. Lopez-Gutierrez, F. Martin-Laurent, A. Baumann, L. Philippot,

409   Quantification of denitrifying bacteria in soils by *nirK* gene targeted real-time PCR, J. Microbiol.

410   Meth. 59 (2004) 327–335

411 [21]I.N. Throback, K. Enwall, A. Jarvis, S. Hallin, Reassessing PCR primers targeting *nirS*, *nirK* and

412   *nosZ* genes for community surveys of denitrifying bacteria with DGGE, FEMS. Microbiol.Eco. 49

413   (2004) 401-417.

414 [22]C.J.F. tterBraak, P. Smilauer, Canoco 5, Windows release.www. canoco5. com. 2012.

415 [23]J.D. Thompson, T.J. Gibson, F. Plewniak, F. Jeanmougin, D.G. Higgins, The CLUSTAL_X

416   windows interface: flexible strategies for multiple sequence alignment aided by quality analysis

417   tools, Nucleic. Acids. Res. 25 (1997) 4876-4882.

418 [24]K. Tamura, D. Peterson, N. Peterson, G. Stecher, M. Nei, S. Kumar, MEGA5: molecular

419   evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum

420   parsimony methods, Mol. Biol. Evol.28 (2011) 2731-2739.

421 [25]W.G. Zumft, Cell biology and molecular basis of denitrification. Microbiol. Mol. Biol. Rev. 61

422   (1997) 533-616.

423 [26]J.M. O'Brien, W.K. Dodds, Saturation of nitrate uptake in prairie streams as a function of acute

424   and chronic nitrogen exposure, J. North. Am. Benthol. Soc. 29 (2010) 627-635.

425 [27]M.R. Shaw, J. Harte, Response of nitrogen cycling to simulated climate change: differential

426   responses along a subalpine ecotone, Global. Change. Biol. 7 (2001) 193-210.

427 [28] S. Fontaine, G. Bardoux, L. Abbadie, A. Mariotti, Carbon input to soil may decrease soil carbon

428   content. Ecol. Lett. 7 (2004) 314-320.

429 [29] J.R. Tian, P.J. Zhou, Phosphorus fractions of floodplain sediments and phosphorus exchange on

430   the sediment–water interface in the lower reaches of the Han River in China, Ecol. Eng., 30 (2007)

431   264-270.

432 [30] Y.Q. Wang, X.C. Zhang, C.Q. Huang, Spatial variability of soil total nitrogen and soil total

433   phosphorus under different land uses in a small watershed on the Loess Plateau, China. Geoderma,

434   150 (2009) 141-149.

435 [31] M.M.R. Jahangir, M.L. Khalil, P. Johnston, L.M. Cardenas, D.J. Hatch, M. Butler, M. Barrett, V.

436   O'flaherty, K.G. Richards, Denitrification potential in subsoils: A mechanism to reduce nitrate

437   leaching to groundwater, Agr. Ecosyst. Environ. 147 (2012) 13-23.

438 [32] T.L. Ingersoll, L.A. Baker, Nitrate removal in wetland microcosms, Water. Res. 32 (1998) 677-

439   684.

440 [33] Z. Chen, X. Luo, R. Hu, M.N. Wu, J.S. Wu, W.X. Wei, Impact of long-term fertilization on the

441   composition of denitrifier communities based on nitrite reductase analyses in a paddy soil, Microb.

442   Ecol. 60 (2010) 850-861.

443 [34] S.S. Kaushal, P.M. Groffman, P.M. Mayer, E. Striz, A.J. Gold, Effects of stream restoration on

444   denitrification in an urbanizing watershed, Ecol. Appl. 18 (2008) 789-804.

445 [35] T.B. Parkin, Soil microsites as a source of denitrification variability, Soil. Sci. Soc. Am. J. 51

446   (1987) 1194-1199.

447 [36] P.A.M. Bachand, A.J. Horne, Denitrification in constructed free-water surface wetlands. Part II:

448   effects of vegetation and temperature, Ecol. Eng. 14 (2000) 17-32.

449 [37] K.R. Reddy, W.H. Patrick, F.E. Broadbent, Nitrogen transformations and loss in flooded soils and

450   sediments, Crit. Rev. Environ. Control. 13 (1984) 273-309.

451 [38] D.W. Graham, C. Trippett, W.K. Dodds, J.M. O'Brien, E.B.K. Banner, I.M. Head, M.S. Smith,

452   R.K. Yang, C.W. Knapp, Correlations between in situ denitrification activity and *nir*-gene

453   abundances in pristine and impacted prairie streams, Environ. Pollut. 158 (2010) 3225-3229.

454 [39] B. Petr, Š. Jaroslav, M. Věra, D. Petra, C. Tomáš, V. Vendula, Responses of the extracellular

455   enzyme activities in hardwood forest to soil temperature and seasonality and the potential effects of

456   climate change, Soil. Biol. Biochem. 56 (2013) 60-68.

457 [40] J.F. Chau, A.C. Bagtzoglou, M.R. Willig, The effect of soil texture on richness and diversity of

458   bacterial communities, Environ.Forensics. 12 (2011) 333-341.

459 [41] G. Braker, P. Dörsch, L.R. Bakken, Genetic characterization of denitrifier communities with

460   contrasting intrinsic functional traits, FEMS. Microbiol. Ecol. 79 (2012) 542-554.

461 [42] Y.N. Chen, W. Zhou, Y.P. Li, J.C. Zhang, G.M. Zeng, A.Z. Huang, J.X. Huang, Nitrite reductase

462   genes as functional markers to investigate diversity of denitrifying bacteria during agricultural waste

463   composting, Appl. Microbiol. Biotechnol. 98 (2014) 4233-4243.

464 [43] A.L. Peralta, J.W. Matthews, A.D. Kent, Microbial community structure and denitrification in a

465   wetland mitigation bank, Appl. Environ. Microbiol. 76 (2010) 4207-4215.

466 [44] X.D. Liu, S.M. Tiquia, G. Holguin, L.Y. Wu, S.C. Nold, A.H. Devol, K. Luo, A.V. Palumbo, J.M.

467   Tiedje, J.Z. Zhou, Molecular diversity of denitrifying genes in continental margin sediments within

468   the oxygen-deficient zone off the Pacific coast of Mexico,Appl. Environ. Microbiol. 69 (2003)

469   3549-3560.

470 [45] M. Castro-González, G. Braker, L. Farías, O. Ulloa, Communities of *nirS*-type denitrifiers in the

471   water column of the oxygen minimum zone in the eastern South Pacific, Environ. Microbiol. 7

472   (2005) 1298-1306.

473 [46] K. Enwall, I.N. Throbäck, M. Stenberg, M. Söderström, S. Hallin, Soil resources influence spatial

474 patterns of denitrifying communities at scales compatible with land management. Appl. Environ.

475 Microbiol. 76 (2010) 2243-2250.

476 [47] E. Báttard, T. Rechová, D. Vaněk, T. Picek, H. Šantrůčková, Effect of pH and dissolved organic

477 matter on the abundance of *nirK* and *nirS* denitrifiers in spruce forest soil, Biogeochemistry. 101

478 (2010) 123-132.

479 [48] C. Yin, F.L. Fan, A.L. Song, Denitrification potential under different fertilization regimes is

480 closely coupled with changes in the denitrifying community in a black soil, Appl. Microbiol.

481 Biotechnol. 99 (2015) 5719-5729.

Figure legends

Fig. 1. Abundances of *nirS* genes in swamp soil (SW), meadow soil (MD) and sandy soil (SD). Different letters above the bars indicate significant differences based on protected LSD test ($P<0.05$).

Fig. 2. Average relative abundances of *nirS* terminal restriction fragments (T-RFs) in swamp soil (SW), meadow soil (MD) and sandy soil (SD). The relative abundance of T-RFs is given as a percentage of the total peak height. Fragment sizes (bp) of the experimental T-RFs are indicated on the right.

Fig. 3. Ordination diagram from redundancy analysis (RDA) on the basis of relative abundance of *nirS* T-RFs in swamp soil (SW), meadow soil (MD) and sandy soil (SD). AP, available phosphorus; WC, water content; TN, total nitrogen.

Fig. 4. Neighbor-joining phylogenetic tree of *nirS* gene sequences derived from swamp soil (SW), meadow soil (MD) and sandy soil (SD) in the Zoige Plateau. *nirN* gene (D84475) and *nirF* gene (D50473) from *Pseudomonas aeruginosa* were used as outgroups. Bootstrap values greater than 50% of 1000 resamplings are shown. The scale indicates the number of nucleotide substitutions per site. Numbers before the sample code indicate the sequenced *nirS* gene clones. Numbers in parentheses indicate the Genbank accession numbers.
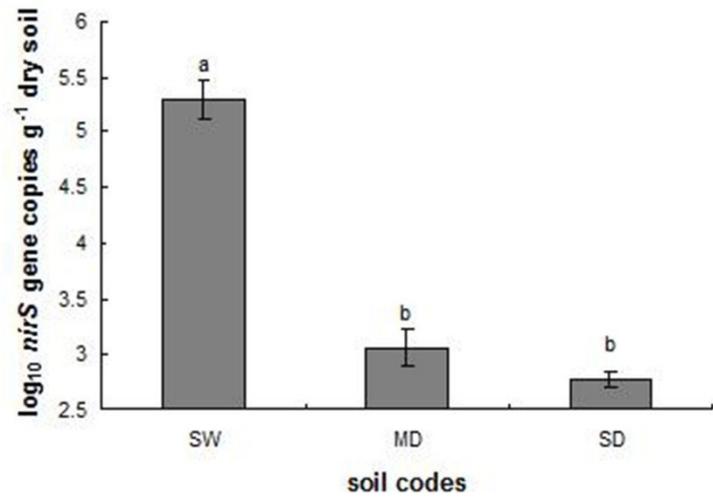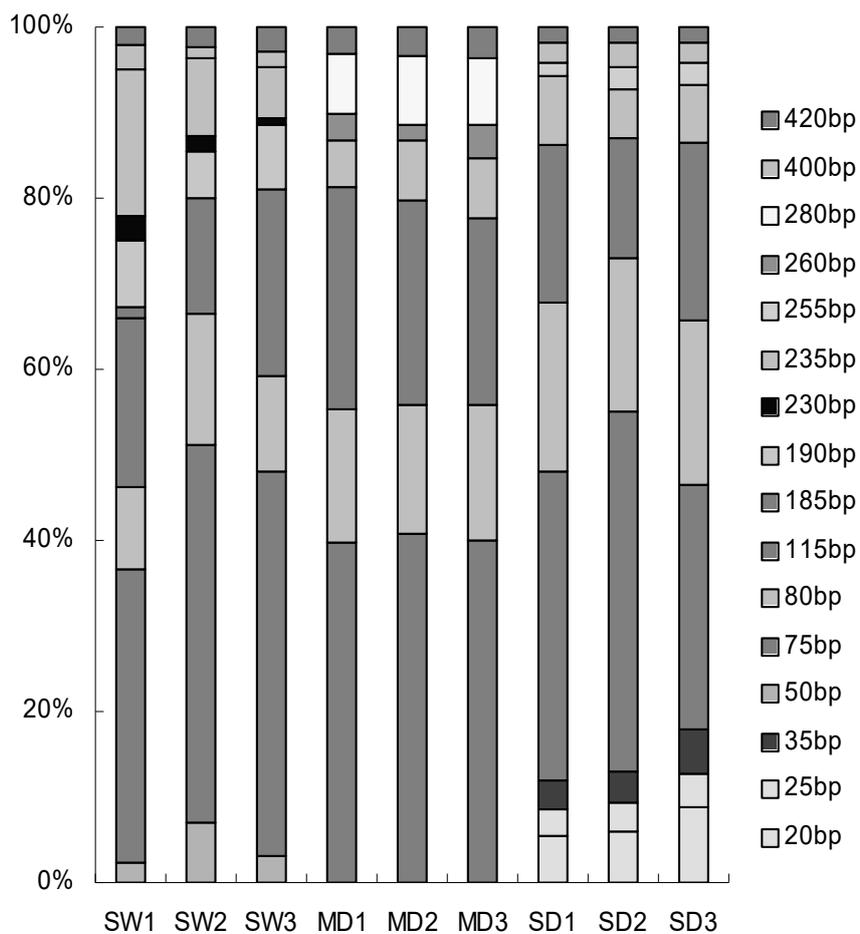
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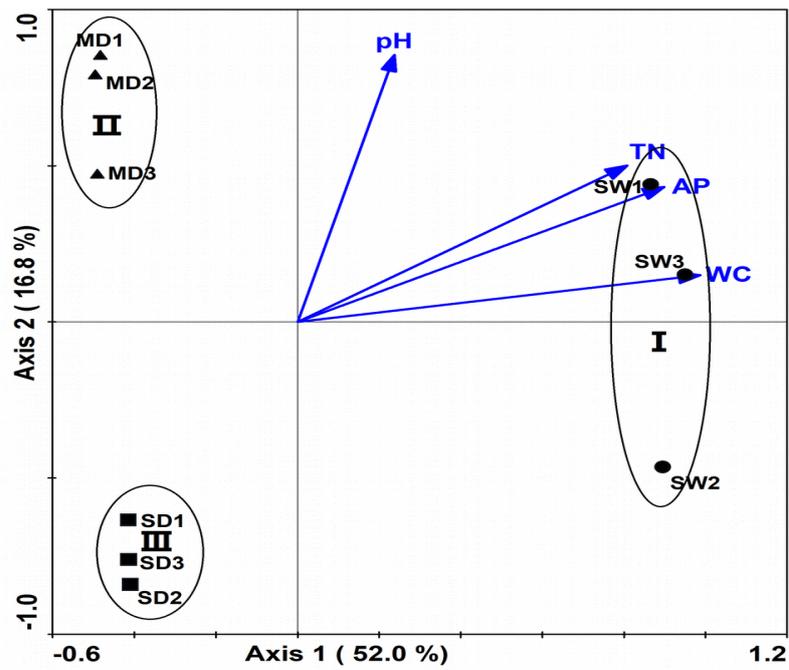
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●Swamp soil
▲Meadow soil
■Sandy soil
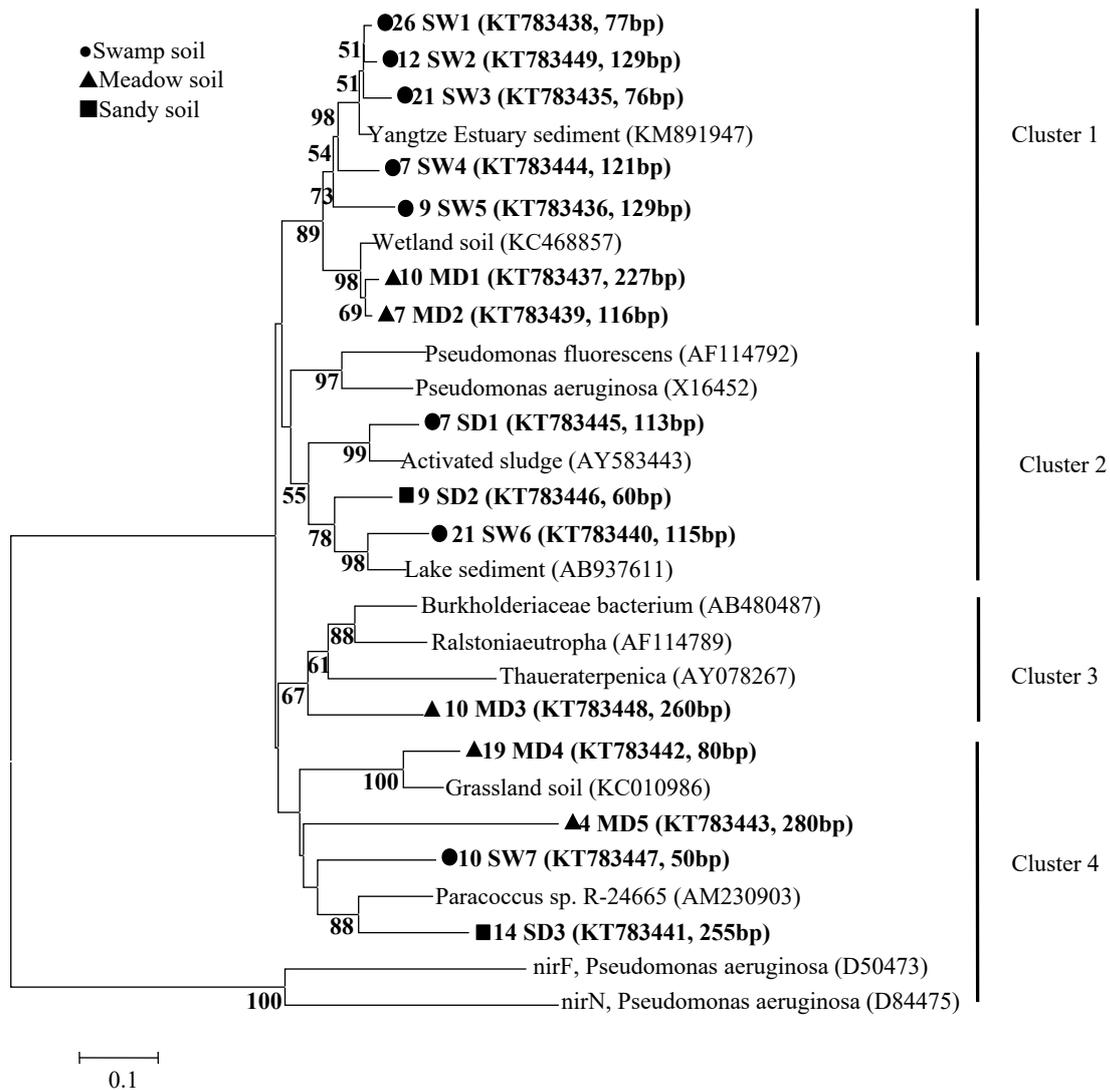
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518 before the sample code indicate the sequenced *nirS* gene clones. Numbers in parentheses indicate the

519 Genbank accession numbers.

Table 1. Sampling sites in Zoige Plateau wetland

Sample code*	Sampling site	Longitude	Latitude	Altitude (m)	Soil temperature (°C)
SW1	Axi	102°49′ 02.8″N	33°54′57.0″E	3427	5
SW2	Heihe	102°48′ 48.2″N	33°54′58.2″E	3432	6
SW3	Fenqu	102°48′ 59.4″N	33°55′21.2″E	3432	5
MD1	Axi	102°52′ 08.5″N	33°47′37.4″E	3465	4
MD2	Huahu	102°56′ 94.9″N	33°38′99.6″E	3481	4
MD3	Ruoergai	103°13′ 81.7″N	33°11′99.8″E	3585	4
SD1	Tangke	102°27′ 07.6″N	33°17′55.4″E	3445	5
SD2	Tangke	102°43′40.5″N	33°31′32.8″E	3522	5
SD3	Tangke	102°40′35.5″N	33°25′16.9″E	3528	4

528* SW: swamp soil; MD: meadow soil; SD: sandy soil.

529Table 2. Physicochemical parameters of soil samples from Zoige Plateau wetland

WC (%)	pH	SOC (g·kg⁻¹)	TN (g·kg⁻¹)	TP (g·kg⁻¹)	TK (g·kg⁻¹)	AN (mg·kg⁻¹)	AP (mg·kg⁻¹)	AK (mg·kg⁻¹)	PDA N g⁻¹
74.96± 0.33 a	7.53± 0.02a	279.14± 2.77a	5.15± 0.44a	1.43± 0.13a	7.44± 0.45a	993.00± 14.65a	26.01± 1.36a	99.83± 0.79a	33.5 2.14
76.53± 2.34a	7.66± 0.01a	283.09± 4.30 a	5.71± 0.03a	1.41± 0.00a	6.79± 0.10a	1079.43± 4.04a	22.63± 0.16a	96.67± 0.83a	52.7 3.53
79.40± 1.36a	7.56± 0.01a	281.25± 5.67a	5.29± 0.15a	1.32± 0.01a	7.30± 0.09a	1025.88± 7.00a	24.72± 1.40 a	98.33± 2.83a	48.6 2.37
16.12± 0.82c	7.84± 0.01b	66.34± 1.88 c	3.22± 0.36c	0.98± 0.11c	21.34± 1.57c	246.33± 6.11c	12.40± 0.14c	365.42± 4.37c	17.2 4.14
14.59± 0.12 c	7.75± 0.01c	55.69± 1.54b	2.99± 0.34b	0.44± 0.05c	23.13± 0.44c	195.32± 5.05c	11.87± 0.29c	103.14± 4.25c	19.3 2.42
16.56± 0.71c	7.98± 0.01c	72.83± 1.47b	3.67± 0.41c	0.95± 0.33c	22.25± 0.23c	277.35± 7.65c	11.17± 0.23b	443.45± 5.28c	20.1 1.17
7.23± 0.51d	6.74± 0.02c	8.55± 0.24d	0.86± 0.15d	0.84± 0.01d	17.73± 0.44d	65.44± 3.03d	4.52± 0.76d	51.24± 0.44d	14.5 0.24
7.01± 0.31d	6.71± 0.01d	8.24± 0.17c	0.81± 0.01c	0.76± 0.11d	16.66± 0.19d	61.23± 2.14d	3.45± 0.12d	33.42± 1.13d	12.1 0.78
6.89± 0.51d	6.71± 0.01d	8.02± 0.06c	0.79± 0.03d	0.72± 0.03d	16.89± 0.14d	41.22± 1.12d	3.78± 0.15c	41.27± 2.55d	8.78 1.03

530Mean ± standard error (n=3). Values within the same column followed by the same letter do not differ

531at *P* < 0.05. WC: water content; OC: organic carbon; TN: total nitrogen; TP: total phosphorus; TK:

532total potassium; AN: available nitrogen; AP: available phosphorus; AK: available potassium. PDA:

533Potential denitrifying activity. For sample codes, see Table1.

534Table 3. Pearson correlation between soil physicochemical properties, potential denitrifying activity

535(PDA) and *nirS* gene abundance

	WC	pH	SOC	TN	TP	TK	AN	AP	AK	PDA
PDA	0.946**	0.466	0.945**	0.897**	0.811**	-0.804**	0.956**	0.895**	-0.086	1
Abundance	0.943**	0.292	0.931**	0.816**	0.841**	-0.897**	0.946**	0.842**	-0.238	0.963**

536WC: water content; SOC: soil organic carbon; TN: total nitrogen; TP: total phosphorus; TK: total

537potassium; AN: available nitrogen; AP: available phosphorus; AK: available potassium. **: $P < 0.01$.

538

539Table 4. *nirS* gene diversity indices of soil samples from Zoige Plateau wetland

Soil codes	Shannon-Wiener index (H)	Richness	Evenness
SW1	2.86±0.06b	3.30±0.10c	0.58±0.02b
SW2	2.74±0.04bc	4.25±0.07a	0.37±0.15c
SW3	3.07±0.03a	3.89±0.10b	0.59±0.12b
MD1	2.22±0.04e	2.17±0.03f	0.72±0.01ab
MD2	2.31±0.02e	1.80±0.02g	0.81±0.00a
MD3	2.20±0.06e	1.97±0.03g	0.76±0.14ab
SD1	2.67±0.02cd	2.83±0.03c	0.69±0.00ab
SD2	2.57±0.02d	2.77±0.05cd	0.68±0.01ab
SD3	2.59±0.05cd	2.61±0.07e	0.74±0.009ab

540Mean ± standard error (n=3). Values within the same column followed by the same letter do not differ

541at *P*< 0.05. For sample codes, see Table1.