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Anaerobic Ammonium Oxidation in Acidic Red Soils

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Anaerobic ammonium oxidation (anammox) has been proven to be an important nitrogen removal process in terrestrial ecosystems, particularly paddy soils. However, the contribution of anammox in acidic red soils to nitrogen loss has not been well-documented to date. Here, we investigated the activity, abundance, and distribution of anammox bacteria in red soils collected from nine provinces of Southern China. High-throughput sequencing analysis showed that *Candidatus* Brocadia dominates the anammox bacterial community (93.03% of sequence reads). Quantification of the hydrazine synthase gene (*hzsB*) and anammox 16S rRNA gene indicated that the abundance of anammox bacteria ranged from 6.20×10^6 to 1.81×10^9 and 4.81×10^6 to 4.54×10^8 copies per gram of dry weight, respectively. Contributions to nitrogen removal by anammox were measured by a ^{15}N isotope-pairing assay. Anammox rates in red soil ranged from 0.01 to 0.59 nmol N g^{-1} h^{-1}, contributing 16.67–53.27% to N_2 production in the studied area, and the total amount of removed nitrogen by anammox was estimated at 2.33 Tg N per year in the natural red soils of southern China. Pearson correlation analyses revealed that the distribution of anammox bacteria significantly correlated with the concentration of nitrate and pH, whereas the abundance and activity of anammox bacteria were significantly influenced by the nitrate and total nitrogen concentrations. Our findings demonstrate that *Candidatus* Brocadia dominates anammox bacterial communities in acidic red soils and plays an important role in nitrogen loss of the red soil in Southern China.

Keywords: red soils, anaerobic ammonium oxidation, high-throughput sequencing, *Candidatus* Brocadia, nitrogen removal

INTRODUCTION

Anaerobic ammonium oxidation (anammox) is a principal microbe-driven process that removes excess N from an ecosystem and oxidizes NH_4^+ with NO_2^- or NO_3^- to form N_2 gas under anaerobic autotrophic condition (Mulder et al., 1995). The discovery of anammox challenged the established concept attributing atmospheric dinitrogen gas (N_2) from fixed nitrogen in the environment to heterotrophic denitrification. The anammox process is mediated by bacteria belonging to the monophyletic order of *Candidate* Brocadiales, of the phylum *Planctomycetes* (Jetten et al., 2010). Presently, there are five different genera of anammox bacteria that have been described, including *Candidatus* Brocadia (Strous et al., 1999; Kartal et al., 2008), *Candidatus* Jettenia (Quan et al., 2008),

Candidatus Kuenenia (Schmid et al., 2000), *Candidatus* Anammoxoglobus (Kartal et al., 2007), and *Candidatus* Scalindua (Schmid et al., 2003; Kuypers et al., 2005).

Anammox bacteria are broadly distributed in many kinds of natural environments, including aquatic and terrestrial ecosystems. *Candidatus* Scalindua was almost the only genus found in marine sediments, such as South China Sea (Hong et al., 2011a), Bohai Sea (Dang et al., 2013), and Arabian Sea (Woebken et al., 2008). In contrast to marine environments, all of these genera of anammox bacteria have been detected in soil ecosystems (Wang et al., 2015; Yang et al., 2017). Zhu et al. (2011) found that the 16S rRNA gene sequence of fertilized paddy soil were related to four different genera of anammox bacteria. Anammox bacteria related to *Candidatus* Brocadia, Kuenenia, and two novel unidentified clusters were found to dominate in the 12 typical paddy soils collected in southern China (Yang et al., 2015). Shen et al. (2017) detected three genera of anammox bacteria by Illumina-based 16S rRNA gene sequencing in a vegetable field, including *Candidatus* Kuenenia, Brocadia, and Jettenia. Nonetheless, another study showed that anammox bacteria in rice paddy soils were consisted of mainly *Candidatus* Scalindua (Wang and Gu, 2013), which was regarded as the dominant genus in marine environments. In addition, anammox have been reported to be an important nitrogen removal pathway in soil ecosystems, which accounts for 0.4–37% of the total N_2 production in soil ecosystems (Zhu et al., 2011; Yang et al., 2015; Shan et al., 2016, 2018; Xi et al., 2016). Yang et al. (2015) suggested that ∼10% of applied N-based fertilizers was lost via the anammox process. Until now, however, there has been limited evidence for the existence and role of anammox bacteria in acidic and natural red soils.

Red soil is widely distributed and covers an area of nearly 2.04×10^6 km² in southern China, accounting for 6.5% of the total farmland area, and is one of China's most important agricultural soils (Xu et al., 2003). On the other hand, red soils are acidic (pH: 4.2–5.9), nutrient deficient (total N ranges from 1.4 to 2.0 g kg⁻¹, Total P: 0.6–0.9 g kg⁻¹), poor in organic matter (ranging from 10 to 100 g kg⁻¹) and have low water-holding and supplying capacity (Wilson et al., 2004). Fe (total iron: 11.7–148.0 g kg⁻¹) and Al oxides are often the dominant clay minerals in red soils. Increasing evidence shows that soil characteristics (e.g., soil moisture, pH, and nutrient conditions) may significantly affect the activity and community of soil ammonia oxidation bacteria and archaea (Nicol et al., 2008; Erguder et al., 2009; Gleeson et al., 2010; Gubryrangin et al., 2011; Liu et al., 2017). We hypothesized that the activity, abundance, and diversity of anammox bacteria in red soil would be lower than in other natural habitats. Besides, the anammox population in red soil is expected to be related to a limited number of genera that can be adapted to acidic and nutrient-deficient conditions.

In this study, 10 representative red soil samples from nine provinces of southern China were collected to (i) analyze the diversity and structure of anammox bacteria by Illumina-based 16S rRNA gene sequencing; (ii) determine the abundance of anammox bacteria by quantitative PCR (qPCR) analysis of the hydrazine synthase gene (*hzsB*) and 16S rRNA

gene; (iii) evaluate the potential rates and contribution of anammox and denitrification to N_2 production using the ¹⁵N-labeling approach; (iv) analyze the relations between the physicochemical characteristics of red soil and activity, abundance, and community structure of anammox bacteria. This study provides a new understanding of the community composition and N loss contribution of anammox bacteria in non-fertilized acidic red soils.

MATERIALS AND METHODS

Samples Collection of Red Soil
A total of 10 red soil samples from nine provinces were collected from the natural fields in southern China (**Figure 1** and **Table 1**) during July 2017. These fields were not affected by a substantial amount of nitrogen fertilizer. Red soil samples (depth: 0–20 cm, **Supplementary Figure 1**) of four replicates (ca. 500 g each subsample) were collected from 10 natural red soils. Soil samples were immediately placed in sterile plastic bags, sealed, and transported to the laboratory on ice. The collected samples were subsequently divided into three parts. The first part was incubated to determine anammox and denitrification activities immediately, the second part was stored at 4°C for subsequent analysis of physicochemical properties, and the third part was stored at −80°C for DNA extraction and molecular analyses.

Analyses of Red Soil Physicochemical Properties
pH of red soil was measured at a soil/Milli-Q water ratio of 1:2.5 with a pH analyzer (Mettler Toledo S220, Switzerland). Dissolved inorganic nitrogen (NH_4^+, NO_2^-, and NO_3^-) was extracted from the soil using 2 mol/L KCl at a liquid to solid ratio of 100. The extraction process was as follows. Freeze-dried and sieved 100 mg of a sediment sample was placed in a polyethylene centrifuge tube, and a 2 mol/L KCl solution was added to it. It was agitated on a rotary shaker for 8 h. After that, the sediment was centrifuged for 10 min at 3500 rpm, and the supernatant was used to measure the dissolved inorganic nitrogen concentration. Concentrations of NH_4^+, NO_2^-, and NO_3^- in the supernatants were determined by spectrometric detection methods described by Wu et al. (2016) and Guan et al. (2017). Total nitrogen (TN), organic carbon (%), and the C:N ratio in each sample were analyzed on an elemental analyzer (IsoPrime 100, Elemental, Germany) after leaching with 0.1 M HCl to remove sedimentary carbonate.

Measurement of Potential Anammox and Denitrification Rates
The potential rates of anammox and denitrification in the red soils were measured as described by Risgaard-Petersen et al. (2003) and Hou et al. (2012) with a slight modification, and their relative contributions to N_2 production were calculated based on these rates. Briefly, 30 g of field moist soils was transferred to a 250 mL glass bottle with helium-purged water
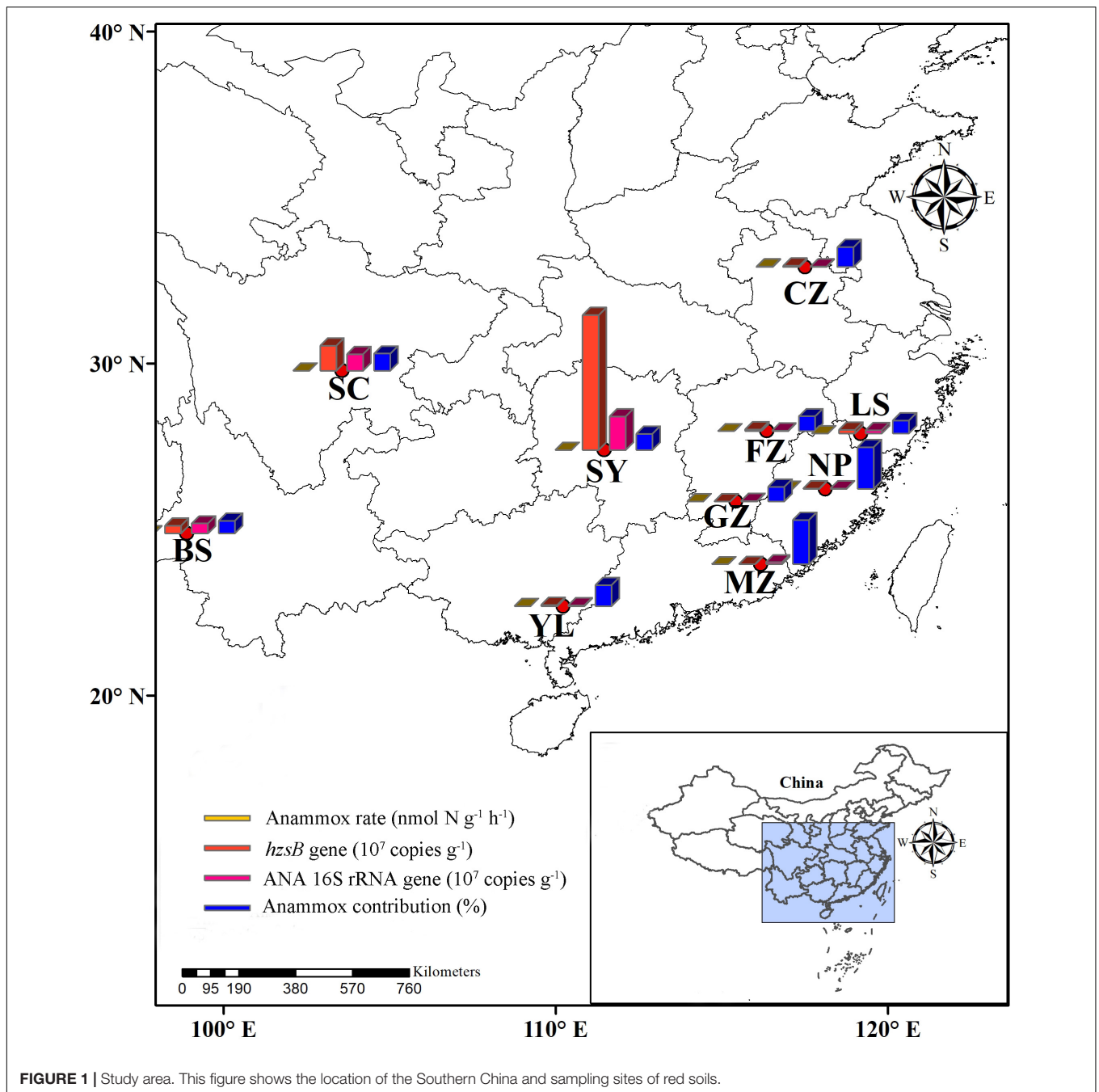
FIGURE 1 | Study area. This figure shows the location of the Southern China and sampling sites of red soils.

at a volume ratio of 1:5. The mixture was purged with helium for 30 min. The resulting soil slurries were transferred into gas-tight 12.5 mL helium-flushed glass vials (Labco Exetainters, United Kingdom) under helium. Next, these slurries were incubated for approximately 24 h to remove residual NO_x^- and dissolved oxygen at *in situ* sampling temperature. The ^{15}N atom% (F_n, represents the fraction of ^{15}N in total NO_3^-) was calculated by taking into account ^{15}N atom% of stock solutions and any residual ambient $^{14}NO_3^-$ as determined by difference, ranging from 0.80 to 0.98. After pre-incubation, these vials with slurries were divided into three groups, which

were spiked through the septum with helium-purged stock solutions of (1) $^{15}NH_4^+$ (^{15}N at 99.6%), (2) $^{15}NH_4^+ + ^{14}NO_3^-$, and (3) $^{15}NO_3^-$ (^{15}N at 99%). The final concentration of ^{15}N in each vial was ~100 μM. Samples for dissolved gas analysis were preserved with 200 μL of a 50% $ZnCl_2$ solution and analyzed within 8 h. Membrane inlet mass spectrometry (MIMS, Hiden) was employed to measure the concentrations of $^{29}N_2$ and $^{30}N_2$ produced during the incubation period. Finally, the developed methods were used to calculate the rates of both anammox and denitrification and their potential contribution to N_2 production.

TABLE 1 | Characteristics of the collected red soils in Southern China.

Province	City	Samples	Position	pH	NH_4^+ (mg kg^{-1})	NO_3^- (mg kg^{-1})	NO_2^- (mg kg^{-1})	TN (mg kg^{-1})	C (%)	C:N
Yunnan	Baoshan	BS	98°52′ E, 24°53′ N	6.02	69.12	49.17	4.59	570	0.433	7.60
Anhui	Chuzhou	CZ	117°30′ E, 32°54′ N	5.78	29.72	300.61	4.59	120	0.064	5.33
Jiangxi	Fuzhou	FZ	116°20′ E, 27°57′ N	5.18	45.11	14.27	4.77	180	0.116	6.44
Jiangxi	Ganzhou	GZ	115°25′ E, 25°50′ N	4.40	41.23	14.27	3.69	200	0.201	10.05
Zhejiang	Lishui	LS	119°10′ E, 27°53′ N	4.55	42.26	63.56	3.33	130	0.178	13.69
Guangdong	Meizhou	MZ	116°10′ E, 23°57′ N	4.82	39.79	5.49	5.40	70	0.062	8.86
Fujian	Nanping	NP	118°45′ E, 27°32 ′N	5.08	38.80	28.55	2.25	120	0.115	9.58
Sichuan	Leshan	SC	103°32′ E, 29°47′ N	5.06	27.25	3.29	2.25	70	0.056	8.00
Hunan	Shaoyang	SY	111°27′ E, 27°23′ N	4.65	45.18	25.50	5.22	570	0.366	6.42
Guangxi	Yulin	YL	110°12′ E, 22°41′ N	4.56	65.25	10.37	2.70	170	0.238	14.00

DNA Extraction and PCR Amplification

Approximately 0.3 g of red soil was used for genomic DNA extraction using the PowerSoil DNA Isolation Kit (Mobio, United States). DNA quality and quantity were checked on a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, United States). Anammox bacteria were targeted by the barcoding primers A438f and A684r. The forward primer of A438f was attached to a unique 8 bp barcode sequence. More details of the primers and PCR amplification program are given in **Supplementary Table 1**. The amplified products were verified by electrophoresis in a 1.0% agarose gel and then purified using a MiniBEST agarose gel DNA extraction kit (TaKaRa, Beijing, China) before high-throughput sequencing.

High-Throughput Sequencing and Analysis

Data analysis of high-throughput raw sequences was conducted in the Mothur software v.1.35.1 following the standard protocol[1] (Schloss et al., 2009). The obtained reads were processed by removing tags and primers before the sequence read numbers per sample were grouped together. The quality-trimmed sequences were aligned to the newly developed anammox 16S rRNA gene databases. The chimeric sequences were identified and removed by the Chimera-uchime. High-quality anammox bacterial sequences were used to generate a distance matrix and cluster with the average neighbor algorithm. Representative sequences for each operational taxonomic unit (OTU) as defined by 97% sequence identity were obtained for further diversity analyses (Zhao et al., 2013). Phylogenetic analysis of the representative anammox bacterial 16S rRNA gene sequences from each OTU was conducted with the MEGA 7.0 software. A heat map was constructed based on the abundance of top 30 OTUs. Principal coordinates analysis (PCoA) was conducted by Normalized weighted Unifrac (Lozupone et al., 2006). Canonical correspondence analysis (CCA) was performed using the CANOCO 5.0 software. The plots in this study were created in SigmaPlot (version 12.5).

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[1]http://www.mothur.org

qPCR Analysis of *hzsB* and 16S rRNA Genes of Anammox Bacteria

The abundance of the *hzsB* gene and 16S rRNA gene of anammox bacteria was determined in triplicate on a Bio-Rad iQ5 thermal cycler (Bio-Rad Laboratories) with primer sets HSBeta396F-HSBeta742R and A438f-A684r, respectively. More details of qPCR assays are given in **Supplementary Table 1**. Plasmids carrying the targeted gene fragments were extracted from *Escherichia coli* DH5α hosts using a Plasmid Mini Preparation Kit (TaKaRa, Beijing, China). Standard curves were built using 10-fold serial dilution of the plasmid with target anammox bacterial genes: *hzsB* and 16S rRNA gene. Specificity of the amplified products was checked by examination of a single melting peak and the presence of a unique band of the expected size in a 2% agarose gel stained with ethidium bromide. The results with efficiency and correlation coefficient above 90% and 0.97 were employed in this study.

Statistical Analysis

The relations among the activity, abundance of anammox bacteria, and different environmental factors were examined by Pearson correlation analyses using Statistical Analysis System (SAS 9.4).

Nucleotide Sequence Accession Numbers

The raw Illumina reads of 16S rRNA gene sequences of anammox bacteria were deposited in the NCBI short-read archive under the Accession No. SRP140525.

RESULTS

Physicochemical Properties of Red Soils

The concentrations of NH_4^+, NO_3^-, NO_2^-, TN, and C (%) and the ratio C:N of red soils from nine provinces of Southern China are listed in **Table 1**. The pH values of all collected red soil samples were relatively low, ranging from 4.40 to 6.02. High concentrations of NH_4^+ were characteristic of all red soils, ranging from 27.25 to 69.12 mg kg^{-1}. NO_3^- concentrations (ranging from 3.29 to 300.61 mg kg^{-1}) were lower than those of NH_4^+

but peaked in sample CZ (300.61 mg kg^{-1}) in Anhui Province. The concentrations of NO_2^- were relatively low and ranged from 2.25 to 5.40 mg kg^{-1}. The soil TN and C% contents were lower than those in other agricultural soils, which varied from 70 to 570 mg kg^{-1} and 0.056 to 0.433%, respectively. Such differences may be the result of less application of fertilization in natural red soils. Most of the samples had a low C:N ratio (ranging from 5.33 to 10.05). Nonetheless, samples LS and YL showed relatively higher C:N ratios (13.69 and 14.00). The physicochemical properties of red soils in this study were in the same range as other reported values for red soils (Wilson et al., 2004).

## The Potential Rates of Anammox and Contribution to N$_2$ Production

The potential anammox and denitrification rates were determined in red soils based on the incubation of $^{15}NO_3^-$. The potential anammox rates ranged from 0.01 ± 0.00 to 0.59 ± 0.07 nmol N g^{-1} dry red soil h^{-1}, while denitrification rates ranged from 0.01 ± 0.01 to 1.63 ± 0.19 nmol N g^{-1} dry red soil h^{-1} (**Figure 2** and **Supplementary Table 2**). Denitrification rates were higher than anammox rates, suggesting that denitrification dominated the total nitrogen loss in red soils. The highest anammox and denitrification rates were both observed in CZ in Hunan province. The red soil collected in SC, YL, MZ, and GZ showed relatively lower potential anammox rates. The relative contribution of anammox to N$_2$ production varied from 16.67 to 53.27%.

Abundance of Anammox Bacteria

The *hzsB* gene and anammox 16S rRNA gene were used to estimate the abundance of anammox bacteria in red soils by the qPCR method. The abundance of anammox bacteria ranged
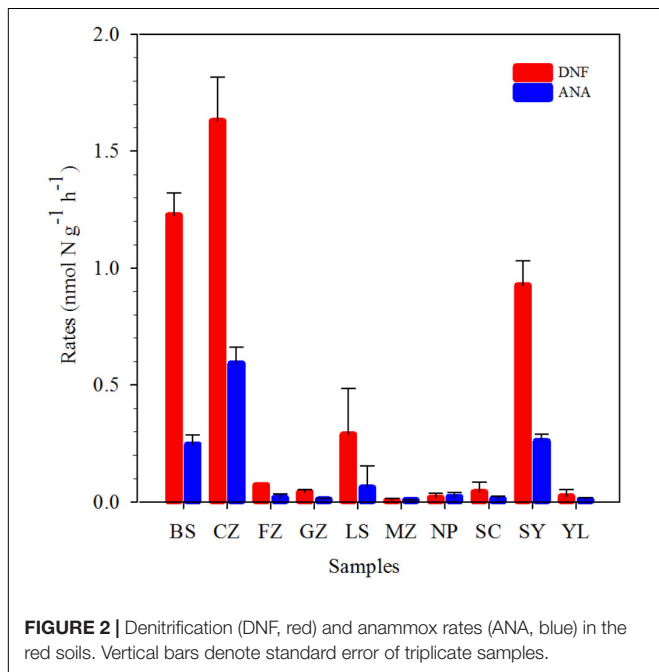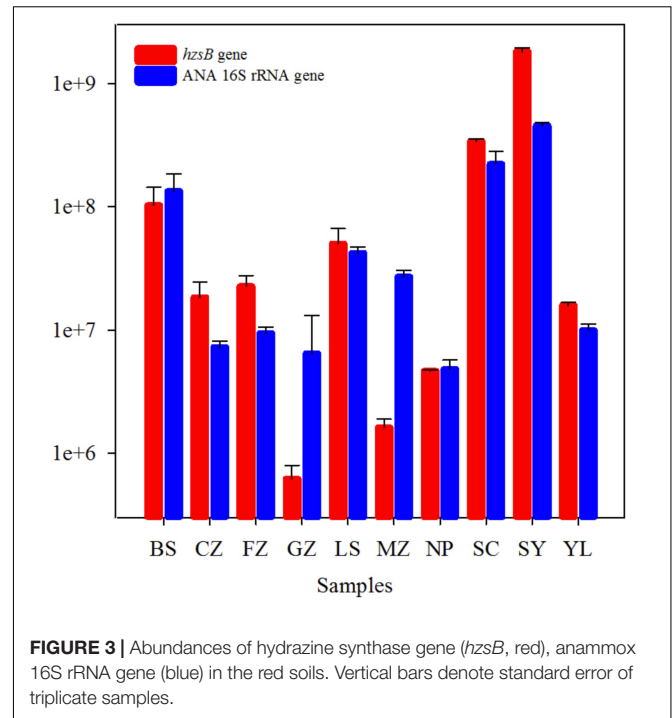
FIGURE 3 | Abundances of hydrazine synthase gene (*hzsB*, red), anammox 16S rRNA gene (blue) in the red soils. Vertical bars denote standard error of triplicate samples.

from 6.20 × 10^5 to 1.81 × 10^9 (*hzsB* gene) and 4.81 × 10^6 to 4.54 × 10^8 (anammox 16S rRNA gene) copies per gram of dry weight (**Figure 3**). The highest abundance of anammox bacteria was observed in sample CZ, while other samples had values between 10^5 and 10^8 copies/g. *hzsB* was slightly more abundant than the 16S rRNA gene of anammox bacteria (the hzsB/anammox 16S rRNA gene ratio was 2.18) in red soils. The presence of anammox bacteria was ascertained in the current study by the positive correlation between *hzsB* and ANA 16S rRNA gene ($r = 0.818$, $P < 0.001$, $n = 10$).

Community Composition and Phylogenetic Analysis of Anammox Bacteria

A total of 0.14 million of anammox raw sequences (15000 sequence reads per sample except NP, which contained only 7222 sequences reads) were subjected to denoising and trimming of sequences. After quality controls, ~12000 reads per sample was filtered as high-quality reads for further analysis (**Table 2**).

Based on the 0.03 distance level, 138 OTUs (77 ± 7 OTUs per sample, $n = 10$) of the anammox 16S RNA gene were obtained (**Table 2**). The highest number of OTUs was seen in sample GZ (84 OTUs). The Chao1 and ACE richness estimators of anammox bacteria ranged from 89.169 to 107.521 and 107.027 to 164.916, respectively. Higher richness of anammox bacteria was found in sample BS. The Shannon index showed that the highest diversity of anammox bacteria was detected in sample NP (0.624), whereas sample MZ (0.263) had the lowest diversity among red soils. The evenness of anammox bacteria was low in red soil and ranged from 0.060 to 0.150. Good's coverage values varied from 0.995 to 0.996, suggesting that the libraries were adequately

FIGURE 2 | Denitrification (DNF, red) and anammox rates (ANA, blue) in the red soils. Vertical bars denote standard error of triplicate samples.

TABLE 2 | Estimates of sedimentary 16S rRNA gene sequence richness and diversity in different red soils.

Samples	High-quality reads	OTUs[a]	Chao1[a]	ACE[a]	Shannon[a]	Evenness[b]	Coverage
BS	12450	74	89.169	107.027	0.334	0.077	0.996
CZ	11823	77	89.366	130.129	0.542	0.125	0.995
FZ	12425	78	93.469	135.885	0.444	0.102	0.995
GZ	12280	84	105.999	164.916	0.530	0.120	0.995
LS	12501	82	96.499	140.765	0.365	0.083	0.995
MZ	12461	80	95.796	138.615	0.263	0.060	0.995
NP	5824	64	97.833	132.182	0.624	0.150	0.995
SC	12500	81	99.562	148.988	0.320	0.073	0.995
SY	12349	75	91.721	126.715	0.361	0.084	0.996
YL	12404	82	107.521	137.841	0.493	0.112	0.995

[a]Richness and diversity were determined based on 0.03 distance.
[b]Evenness was calculated by dividing Shannon index by Ln (OTUs).

large. Rarefaction curves (**Supplementary Figure 2**) and a rank-abundance curve (**Supplementary Figure 3**) for the anammox bacterial 16S rRNA gene at 97% similarity showed that the high-throughput sequencing could supply enough bioinformation to investigate the community composition and diversity of anammox bacteria in the current study.
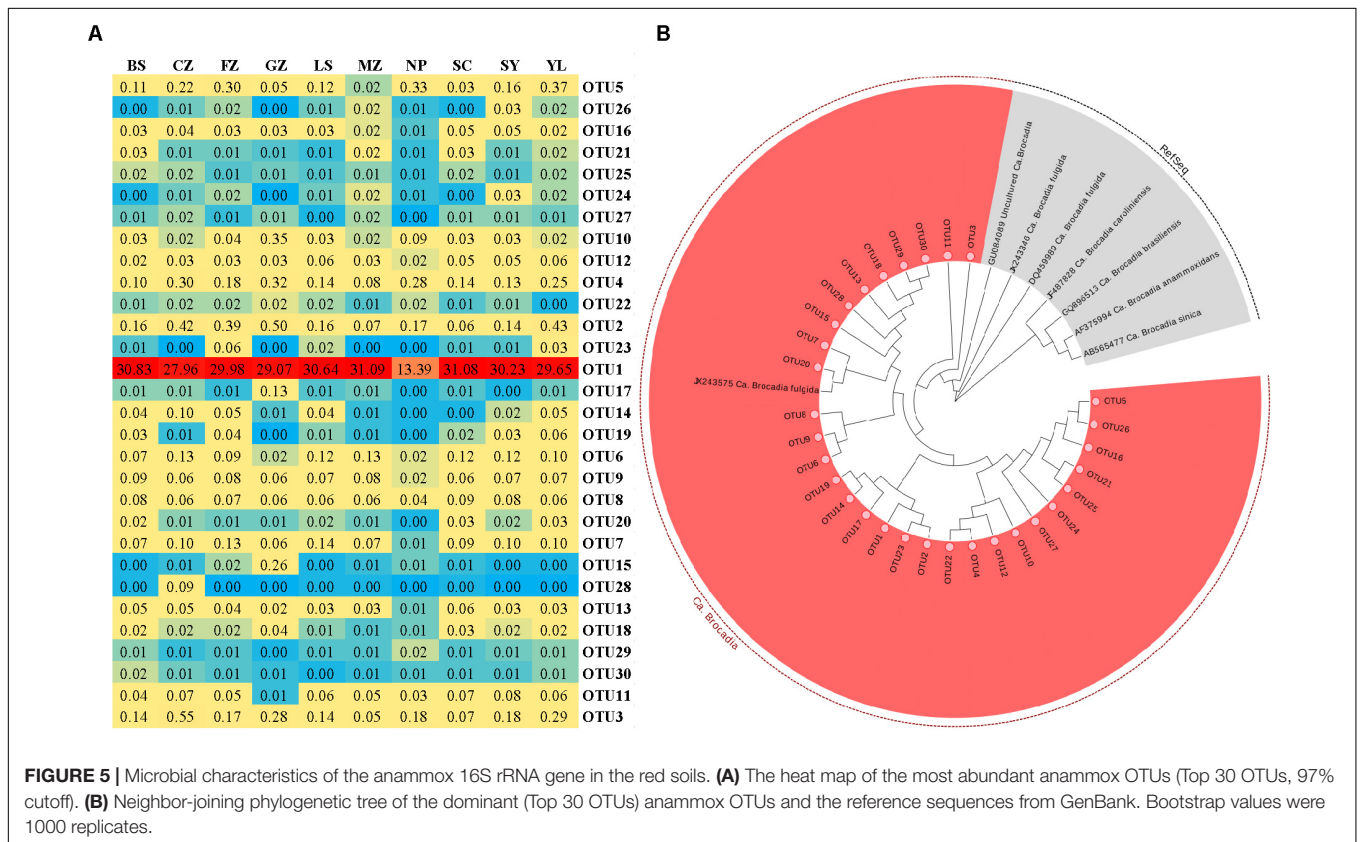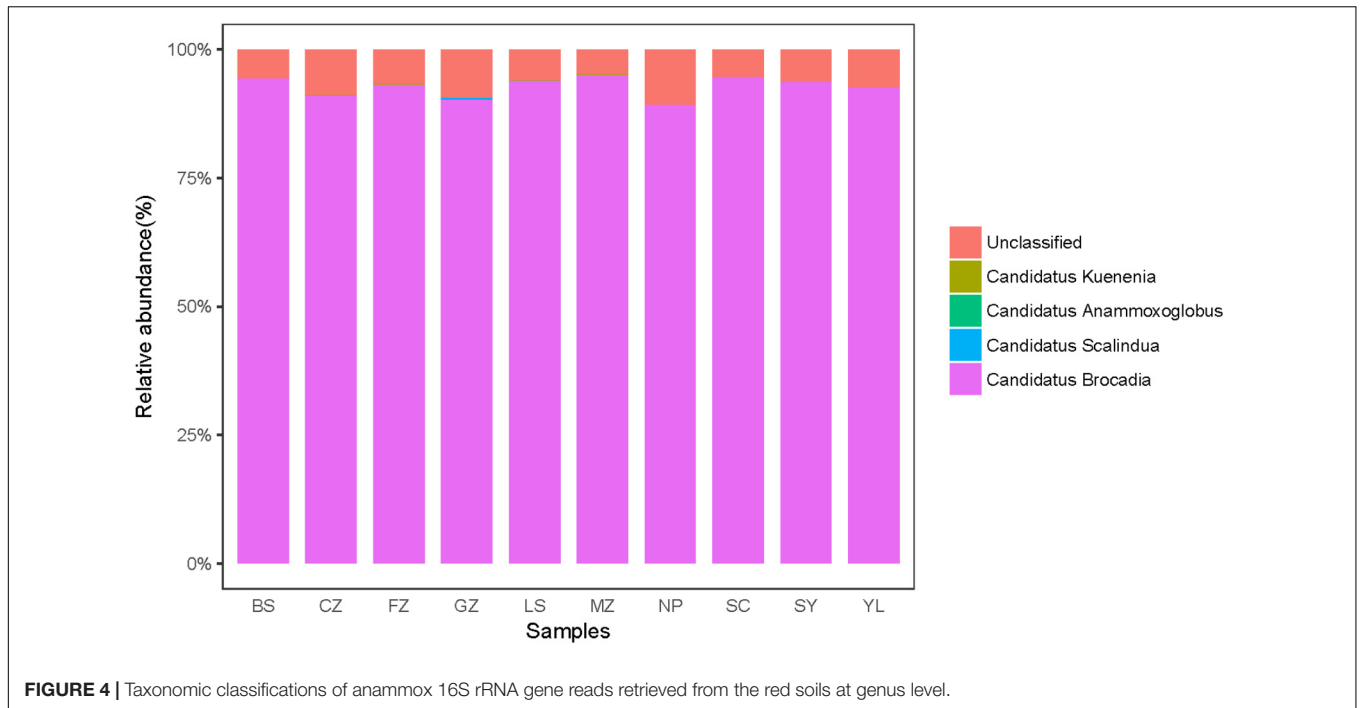
The community structure of anammox bacteria in red soil at the genus level is depicted in **Figure 4**. Gene sequences of anammox bacteria mainly belonged to *Candidatus* Brocadia (93.03%), *Candidatus* Scalindua (0.09%), *Candidatus* Anammoxoglobus (0.01%), *Candidatus* Kuenenia (0.04%) and unclassified (6.83%). *Candidatus* Brocadia (93.03%) was the dominant genus in acidic and natural red soils, whereas *Candidatus* Scalindua was more abundant in sample GZ. A heatmap (**Figure 5A**) and phylogenetic tree (**Figure 5B** and **Supplementary Figure 4**) of dominant anammox OTUs (top 30 OTUs, 99% sequences were obtained) indicated that the top 30 OTUs were affiliated with only one cluster, which was closely related to *Candidatus* Brocadia fulgida. OTU 1 was the most abundant sequence in all the samples and had average relative abundance of 27.39.

Correlation Analysis of Anammox Potential Rates, Gene Abundances, and Red Soil Properties

Pearson correlation analysis was used to illustrate the correlations among anammox potential rates, gene abundances, and red soil properties (**Figure 6** and **Supplementary Table 3**). The results indicated that anammox rates positively correlated with NO_3^- (coefficient = 0.889, $P < 0.001$). *hzsB* abundances positively correlated with TN (coefficient = 0.637, $P < 0.05$) and anammox 16S rRNA abundances (coefficient = 0.942, $P < 0.001$). Anammox 16S rRNA abundance also positively correlated with TN (coefficient = 0.652, $P < 0.05$). In addition, C% positively correlated with NH_4^+ (coefficient = 0.779, $P < 0.001$) and TN (coefficient = 0.993, $P < 0.001$) in red soils. As revealed by CCA, pH and NO_3^- were found to be the main factors affecting the distribution of anammox bacteria in the red soils (**Supplementary Figure 5**).
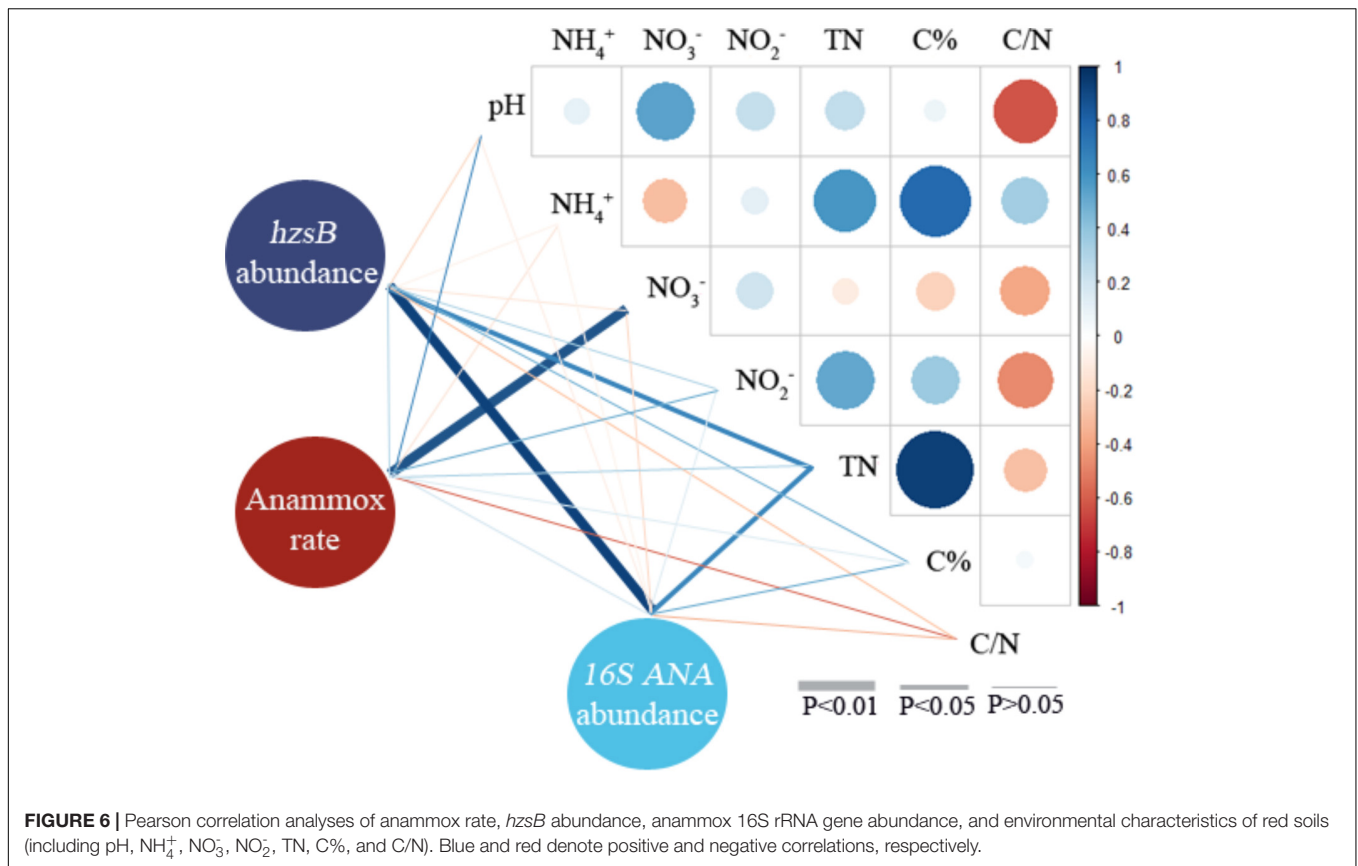
DISCUSSION

Recently, increasing evidences showed that anammox bacteria play a significant role in nitrogen loss in agricultural soils, particularly paddy soils (Zhu et al., 2011; Wang and Gu, 2013; Wang et al., 2014; Nie et al., 2015; Yang et al., 2015). In the present study, we present the evidence of anammox process for the first time in 10 different acidic red soils of Southern China by combining the ^{15}N-labeling approach, qPCR analysis, and high-throughput sequencing. The potential anammox rates, ranging from 0.01 ± 0.00 to 0.59 ± 0.07 nmol N g^{-1} dry red soil h^{-1} (**Figure 2** and **Supplementary Table 2**), were examined in all the selected red soils, revealing the same range as that found in agricultural soils (Long et al., 2013; Nie et al., 2015) and forest soils (Xi et al., 2016), but lower than other values reported for fertilized paddy soils and agricultural fields (Zhu et al., 2011; Yang et al., 2015; Shen et al., 2016, 2017). Red soils in China were found to be characterized by low pH, TN, and organic carbon, in contrast to other fertilized agricultural soils (**Table 1**). The physicochemical properties of red soils were found to be the most important factors influencing the potential anammox and denitrification rates, especially soil organic carbon and TN. Soil organic carbon can enhance microbial activities and improve physical and chemical conditions of the soil (Pulleman et al., 2000; Zhang et al., 2009). These results indicated that the characteristics of red soil may influence the activity of anammox bacteria. Other studies also showed that flooding and fertilization conditions made the paddy soils a suitable habitat for anammox bacteria (Shen et al., 2017). Zhu et al. (2011) found many microniches were existed in a paddy soil; thus, it could support the various ecophysiologies of the different anammox genera and facilitate the anammox activity. In addition, it should be noticed that slurry incubations may underestimate the *in situ* anammox activity due to the following reasons. Firstly, the incubation conditions at higher moisture may affect the microbial community, which results in underestimation of the denitrification and anammox rate. Finally, the incubation time of 24 h may not be enough to eliminate the initial $^{14}NO_3^-$ in sample CZ (Fn: 0.80) and LS (Fn: 0.87), which may underestimate the potential rates. Different potential rates of

FIGURE 4 | Taxonomic classifications of anammox 16S rRNA gene reads retrieved from the red soils at genus level.

FIGURE 5 | Microbial characteristics of the anammox 16S rRNA gene in the red soils. **(A)** The heat map of the most abundant anammox OTUs (Top 30 OTUs, 97% cutoff). **(B)** Neighbor-joining phylogenetic tree of the dominant (Top 30 OTUs) anammox OTUs and the reference sequences from GenBank. Bootstrap values were 1000 replicates.

anammox were observed in 10 samples from nine provinces of Southern China (**Figure 2**). Cultivation history, land use patterns, and a cropping system are the important factors influencing

the physicochemical properties of red soil in different provinces (Shen et al., 2013). Anammox contributed 16.67 to 53.27% to red soil N_2 production (**Figure 2** and **Supplementary Table 2**),

FIGURE 6 | Pearson correlation analyses of anammox rate, *hzsB* abundance, anammox 16S rRNA gene abundance, and environmental characteristics of red soils (including pH, NH_4^+, NO_3^-, NO_2^-, TN, C%, and C/N). Blue and red denote positive and negative correlations, respectively.

suggesting that anammox should be recognized as a significant N sink in red soil. On the base of the soil density, the average potential anammox rate, and red soil area in southern China, N loss attributed to anammox was estimated to reach~ 2.33 Tg N per year. This result was comparable to previously reported values (2.50 Tg N per year) in paddy soil in Southern China (Yang et al., 2015), demonstrating the importance of anammox in red soil ecosystems. Pearson correlation analyses showed that the potential anammox rates positively correlated with NO_3^- (coefficient = 0.889, *P* < 0.001) (**Figure 6** and **Supplementary Table 3**). Previous study on freshwater sediments also suggested that NO_3^- is a factor controlling anammox rates (Yoshinaga et al., 2011). Anammox bacteria are favored in environments where NO_3^- is available (Trimmer et al., 2003; Engström et al., 2005; Yang et al., 2015).

The abundance of anammox bacteria ranged from 6.20×10^5 to 1.81×10^9 *hzsB* copies/g and 4.81×10^6 to 4.54×10^8 (anammox bacterial 16S rRNA gene), which fall within the ranges reported in Pearl River Estuary ($1.4-20 \times 10^8$ *hzsB* copies/g; Wang et al., 2012), Yangtze Estuary ($3.67-822 \times 10^5$ 16S rRNA copies/g; Zheng et al., 2016), and paddy soils ($0.7-1.4 \times 10^7$ *hzs* copies/g; Nie et al., 2015). Anammox bacterial abundances in red soils were confirmed by means of the anammox *hzsB* gene and 16S rRNA gene (**Figure 3**), and abundances of these two target genes correlated (*r* = 0.818, *P* < 0.001, *n* = 10). In this study, TN concentration was identified as an important factor influencing the anammox bacterial abundance in selected red

soils (**Figure 6** and **Supplementary Table 3**). It can be speculated that higher TN concentration may provide an environment favorable for the distribution and growth of anammox bacteria (Shen et al., 2016). In line with other studies (Metz et al., 2003; Etchebehere and Tiedje, 2005; Yang et al., 2017), a positive correlation between anammox activity and abundance (*hzsB* or 16S rRNA gene) was not observed. These results indicated that anammox rates are limited not by the presence of the population capable of anammox but by the availability of NO_3^- or NH_4^+ as substrate. If anammox bacterial abundance correlated with their activities, it suggested that anammox bacterial abundance has a potential to predict anammox activity (Dale et al., 2009). Concentrations of NO_3^- and/or NH_4^+ have been reported to significantly affect the anammox rate (Shen et al., 2013). Lisa et al. (2015) found that physiological nature of an organism and the environmental conditions of the system are the two important factors influencing whether the genetic potential may or may not predict the potential for that process. Furthermore, the presence of anammox bacteria detected by gene amplification is not an indication of their level of activity as would be indicated by active community (cDNA) qualification.

Some studies have reported that *Candidatus* Scalindua is the exclusive anammox genus in marine ecosystems (Schmid et al., 2007; Hong et al., 2011a,b). In this study, *Candidatus* Brocadia dominated the anammox community in acidic red soils. In total, 93.03% sequence reads of anammox bacteria were related to *Candidatus* Brocadia (**Figure 4**) and the dominant

OTUs (TOP 30) were all affiliated with *Candidatus* Brocadia fulgida (**Figure 5**). *Candidatus* Brocadia was reported to be the most common anammox genus in terrestrial ecosystems, including paddy soils (50%, Yang et al., 2015) and Chinese agricultural soils (75%, Shen et al., 2013). Some studies showed that *Candidatus* Brocadia possesses diverse metabolic pathways (Gori et al., 2011) and better adaptability to terrestrial soils than do other anammox genera (Shen et al., 2013). Kartal et al. (2008) found that *Candidatus* Brocadia fulgida can use short-chain organic acids as alternative electron donors to reduce NO_2^- to N_2. *Candidatus* Brocadia belongs to the r-strategists, making it suitable for the higher substrate concentration, and it will outcompete other anammox genera (Strous, 1999; Gao et al., 2018). CCA suggests that pH and NO_3^- were the main factors affecting the distribution of anammox bacteria in the red soils (**Supplementary Figure 5**). According to the composition of anammox bacteria communities of different samples (**Table 2** and **Figure 5A**), higher Shannon (0.542) and evenness indexes (0.125) were detected in sample CZ with higher pH (5.78) and NO_3^- values (300.61 mg kg^{-1}). This situation may lead to the higher diversity of anammox bacteria observed in samples with higher pH and NO_3^- content. Here, the presence and activity of anammox bacteria were detected successfully in acidic red soils (pH ranged from 4.40 to 6.02). The reported optimal pH range for *Candidatus* Brocadia fulgida is 7.2 to 8.3 (Oshiki et al., 2016). Our study extended the pH range in which anammox bacteria can survive and their activity can be detected. A special membrane probably present in the cell of anammox bacteria, so they can survive in acidic environments. Yang et al. (2015) also found that pH significantly correlates with the anammox bacterial composition in paddy soils. Additionally, NO_3^- content had a significant contribution to anammox bacterial community structure, which may be attributed to an increased supply of NO_2^- via reduction of NO_3^-. Similar results have also been observed in the sediments of the Dongjiang River (Sun et al., 2013) and Mai Po Nature Reserve (Li et al., 2011).

Thus, both the presence and activity of anammox bacteria were for the first time shown in 10 different acidic red soils from nine provinces in Southern China. These data expand the knowledge of the distribution and N loss contribution of anammox bacteria in acidic red soils. Our high-throughput sequencing analysis indicates that *Candidatus* Brocadia dominates the anammox bacterial community. Anammox contributed 16.67 to 53.27% to red soil N_2 production, which was the vital N sink (2.33 Tg N per year) in red soils of Southern China. These results demonstrate the lower diversity but higher contribution of anammox bacteria to the removal of fixed N from acidic red soils.

AUTHOR CONTRIBUTIONS

JW, YH, XH, LJ, and XW performed the research. JW, YH, SC, GC, YL, TH, YHH, and XL analyzed the data. JW and YH wrote the paper. All co-authors substantially contributed to commenting and revising it and read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

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