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舟山群岛海域沉积物中厌氧氨氧化细菌多样性

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摘要: 本文通过构建 16S rRNA 基因文库和克隆测序研究了舟山海域沉积物中厌氧氨氧化细菌 (Anaerobic ammonium oxidation bacteria, AAOB) 的多样性。从 5 个克隆文库中共获得 297 条 16S rRNA 基因序列, 包含 16 个操作分类单元 (Operational Taxonomic Units, OTUs)。离岸距离较近的 3 个站具有相似的群落结构, 且与离岸较远的 2 个站具有明显差异。系统发育结果显示, *Scalindua* 属是该海域 AAOB 的优势类群, 95.3% 的序列与 *Scalindua* 属 AAOB 具有较近的亲缘关系; 1 条序列与 *Kuenenia* 属具有较近的亲缘关系; 此外还有 15 条序列与已知的 AAOB 相似性较低。相关性分析表明沉积物有机碳含量与多样性指数具有显著正相关, 可能是该海域 AAOB 多样性变化的重要影响因子。

关键词: 厌氧氨氧化细菌; 舟山群岛; 海洋沉积物; 多样性

Diversity of anaerobic ammonium oxidizing bacteria in marine sediments from the Zhoushan Islands

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Abstract: Anaerobic ammonium oxidation (anammox) is an important process regulating the balance of marine nitrogen and ecosystem health, particularly under anoxic conditions. The Zhoushan Islands are located east of the Changjiang river estuary, and collect a high load of anthropogenic nitrogen, which leads to severe eutrophication and seasonal hypoxia. Therefore, bacteria that mediate the anammox process are of major interest in this area. Although the importance of anammox-mediating bacteria is known, few studies on these bacteria have been conducted in the East China Sea. To the best of our knowledge, this study is the first to report the diversity, community composition, and distribution of anammox bacteria in the Zhoushan Islands. Field surveys were conducted in June 2012; triplicate surface sediment samples were collected at each site and stored in sterile plastic bags at -80°C for subsequent DNA extraction and molecular analysis. Total genomic DNA was extracted using the Fast DNA[®] SPIN Kit for soil. Environmental DNA extracted from sediment samples was used as the template for PCR amplification of anammox 16S rRNA genes using primers Amx368f-Amx820r. The purified fragments were cloned and sequenced for phylogenetic and statistical analyses. In total, 297 sequences belonging to 16 operational taxonomic units (OTUs) were obtained from five 16S rRNA gene libraries. The biodiversity of anammox bacteria was examined using rarefaction analysis of the 16S rRNA genes, the Chao1 estimator, and Shannon index calculations. EZ3-1, EZ3-3, and EZ1-5 exhibited higher diversity than EZ1-3 and EZ3-5. A significant positive correlation between Shannon

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index and organic carbon content indicate that sediment organic carbon content plays an important role in modulating anammox bacterial diversity in the Zhoushan Island area. Weighted UniFrac PCoA analysis of the 16S rRNA genes demonstrated spatial heterogeneity in the community composition of anammox bacteria; the anammox bacteria in the study area could be divided into three distinct groups. EZ3-1, EZ3-3, and EZ1-3 exhibited similar community composition, while EZ1-5 and EZ3-5 clustered separately. The composition might be affected by distance from land and water depth. Phylogenetic analysis indicated that anammox bacterial communities were dominated by the genus *Scalindua* (283 of 297 sequences). The *Scalindua* cluster comprised of 245 *Scalindua* sequences with 95.4—98.5% sequence similarity to *Candidatus Scalindua brodae*, and 36 *Scalindua* sequences with 94.5—97.6% sequence identity to the 16S rRNA gene of *Candidatus Scalindua wagneri*. In addition, two *Scalindua* sequences that grouped in the *Scalindua* cluster were distantly related to known *Scalindua* species, indicating that they might represent unidentified species of *Scalindua*. One sequence recovered from the EZ3-5 library was closely related to genus *Kuenenia*, which is traditionally considered an anammox bacterial genus present in freshwater or low-salinity environments. Our results suggest that members of the genus *Kuenenia* may possess the ability to survive in high-salinity marine environments. Additionally, two clusters of unknown sequences (unknown cluster I and II) were not classified under any known anammox bacterial genus, but were most closely related to 16S rRNA gene sequences recovered from arctic sediment and mangrove sediment, respectively.

Key Words: Anaerobic ammonium oxidation bacteria; Zhoushan Islands; Marine sediments, Diversity

微生物的硝化作用能够将环境中的铵盐和亚硝酸盐转化成硝酸盐^[1],因而对保持河口等生境的水质和生态健康具有重要作用。然而,近年来河口生态系统的季节性缺氧现象越发严重,其出现的频率、范围、持续时间和强度都呈现持续上升的趋势^[2]。低氧容易导致好氧的硝化作用中断,使具有生物毒性的亚硝酸盐大量积聚,进而引发一系列生态和环境问题^[3]。

厌氧氨氧化(Anaerobic ammonium oxidation, anammox)是由一类微生物主导,在缺氧条件下以亚硝酸盐为底物进行氨氧化并产生氮气的过程^[4]。当硝化作用受到阻碍时,厌氧氨氧化能够有效地降低亚硝酸盐的浓度,有助于保持水质,维持生态系统的健康。厌氧氨氧化作用最初在废水处理装置中发现^[5],主要受浮霉菌门的一类化能自养细菌调控,这类细菌很难从自然环境中分离纯化^[6],目前已有描述的包括5个属(*Brocadia*, *Kuenenia*, *Scalindua*, *Anammoxoglobus* 和 *Jettenia*)^[7-9]。厌氧氨氧化细菌(AAOB)在自然环境中的分布非常广泛,在淡水沉积物^[10,11]、海水沉积物^[12]和低氧海水^[13]都有发现,厌氧氨氧化作用是水生环境中维持氮平衡的一个重要途径。

舟山群岛位于长江口外海域,受海洋和长江径流的影响,属于咸淡水生态系统的交错群落,具有独特的氮元素生物地球化学循环特征,是厌氧氨氧化作用的重要发生场所。近年来长江口外夏季低氧现象愈加严重,在夏季整个舟山渔场几乎都被低氧区所覆盖^[14,15]。低氧现象严重危害该海域的渔业资源和生态系统健康,破坏该海域的海洋经济^[2]。Dang 等人曾对该海域沉积物中的氨氧化古菌进行过报道^[16],但目前还未有关于舟山群岛海域低氧区 AAOB 的研究。本文采用 16S rRNA 基因文库构建和克隆测序法,研究夏季舟山海域沉积物中的 AAOB 多样性,旨在初步了解其种类组成和分布情况,为揭示海洋沉积环境中 AAOB 对低氧的响应机制提供科学依据。

1 材料和方法

1.1 样品采集

2012年6月在舟山以东海域采集沉积物表层泥样和底层水样,采样站信息见表1。沉积物泥样使用抓斗采泥器采集,取少量沉积物样品装入预灭菌过的微生物采样杯,-20℃冷冻保存,带回实验室分析;底层水样使用 Niskin 采水器采集,水样经 GF/F 滤膜过滤,于-20℃冷冻保存。

1.2 环境参数分析

水样温度和盐度使用 HydroLab 多参数分析仪现场测定,水样营养盐和溶解氧浓度按照《海洋调查规范》^[17]使用分光光度仪测定,沉积物有机碳、总碳和总氮含量参照《海洋监测规范》^[18]使用元素分析仪测定。

表 1 采样站位信息

Table 1 Information of date, locations and water depth of the sampling stations

站号 Station No.	采样日期 Sampling date	经度(°E) Longitude(°E)	纬度(°N) Latitude(°N)	水深/m Depth
ZS1-3	2012.6.25	123	30	49
ZS1-5	2012.6.25	123.5	30	60
ZS3-1	2012.6.24	122.55	30.78	20
ZS3-3	2012.6.24	123	30.67	47
ZS3-5	2012.6.24	123.5	30.67	53

1.3 DNA 的提取和扩增

取少量(1—2 g)沉积物样品,使用 Fast DNA © SPIN Kit for soil(MP,美国)提取沉积物总 DNA。使用 AAOB 的 16S rRNA 基因特异引物^[19]进行 DNA 扩增:Amx368F,5'-TTCGCAATGCCCGAAAGG-3'和 Amx820R,5'-AAAACCCCTCTACTTAGTGCCC-3',50 μl PCR 反应体系包含:5 μl 10×PCR buffer、200nM dNTPs、前后引物各 0.25 μM、1 U Taq 酶和 1 μl DNA 提取物。PCR 反应条件:94 °C 4 min;94 °C 30 s,56 °C 30 s,72 °C 60 s,30 个循环;72 °C 7 min。1%琼脂糖凝胶电泳检测 PCR 扩增结果,并用 QIAquick Gel Extraction Kit(QIAGEN,美国)纯化、回收 DNA 目的扩增产物。

1.4 TA 克隆与测序

PCR 回收产物与 pMD20-T 载体(TaKaRa)连接,转化到感受态细胞 *E. coli* DH5α(TaKaRa),涂布于 LB 平板(含 Amp、IPTG 和 X-Gal),37°C 培养过夜,筛选阳性克隆构建基因克隆文库。将所获的阳性克隆送生工生物工程(上海)测序。

1.5 AAOB 16S rRNA 基因序列分析

测序获得的 16S rRNA 基因序列在 NCBI 数据库中进行 BLAST 比对(<http://www.ncbi.nlm.nih.gov>),下载相似性最高的序列作为参比序列,应用 Clustal X 进行匹配比对。用 DOTUR 软件包将相似性≥97%的序列定义为一个分类单元(OTU)^[20],并构建稀释度曲线。用 MEGA4 软件构建系统发育树^[21]。研究获得序列已提交 GenBank,注册号为 KF029766-KF030062。

1.6 多样性分析

用 DOTUR 软件分析各个克隆文库的生物多样性指标(Shannon 和 Chao 1),根据 OTUs 分析结果计算各个克隆文库的覆盖度,计算公式如下^[22]:

$$\text{Good} = [1 - (n/N)] \times 100$$

式中, n 代表单克隆 OUT 的数量, N 代表文库中克隆总数量。AAOB 群落的生态分布特征使用 Fast UniFrac 进行分析^[23]。

2 结果

2.1 环境特征

研究海域 5 个采样站位根据其环境特征可分为河口和海洋两类(表 2)。EZ3-1 站距离长江口最近,受长江径流影响底层海水温度较高、盐度较低,硝酸盐和硅酸盐浓度是研究站位中最高,沉积物中碳、氮含量也是研究站位中最高。其它 4 个站位环境特征较为均匀,盐度较高、营养盐浓度较低,属典型的海洋环境特征,但 EZ1-3 和 EZ3-3 两个站营养盐浓度和沉积物碳、氮含量略高于 EZ1-5 和 EZ3-5,表明长江径流等对距离较近的

站位仍有一定的影响。研究海域底层水的溶解氧浓度较低,其中 EZ3-3 和 EZ3-5 站位于长江口外低氧区的核心区内^[14],其溶解氧低于其它 3 个站 (<4 mg/L)。

表 2 各采样站位环境参数

Table 2 Physical and chemical parameters of the sampling stations

环境参数 Environmental factors	EZ1-3	EZ1-5	EZ3-1	EZ3-3	EZ3-5
水深 Water depth/m	43	60	16	42	54
温度 ^a Temperature/°C	18.54	18.65	22.86	18.44	19.00
盐度 ^a Salinity	34.36	34.35	31.18	34.34	34.32
溶解氧 ^a Dissolved oxygen/(mg/L)	4.28	4.29	4.16	3.61	3.79
硝酸盐 ^a Nitrate/($\mu\text{mol/L}$)	12.12	11.87	20.04	12.95	11.87
磷酸盐 ^a Phosphate/($\mu\text{mol/L}$)	0.37	0.34	0.60	0.94	0.93
硅酸盐 ^a Silicate/($\mu\text{mol/L}$)	23.21	22.74	24.08	23.33	21.80
有机碳 ^b Organic carbon/%	0.37	0.49	0.52	0.45	0.31
总碳 ^b Total carbon/%	1.29	1.40	1.42	1.48	1.11
总氮 ^b Total Nitrogen/%	0.08	0.07	0.09	0.08	0.05

a: 底层水样; b: 沉积物

2.2 克隆文库与多样性分析

针对 5 个站沉积物样品分别构建克隆文库, 总共获得 297 条 AAOB 16S rRNA 基因序列。根据基因序列相似性 $\geq 97\%$ 归为一个 OTU, 共获得 16 个 OTUs, 5 个文库共有的 OTUs 有 5 个, 文库各自的 OUT 数在 5—8 个之间(表 3)。稀释度曲线显示 5 个基因文库用于测序的克隆数量达到或接近饱和(图 1)。5 个克隆文库的覆盖度较高, 均大于 80%(表 3), 表明本研究所构建的文库基本涵盖研究海域沉积物中的 AAOB 多样性。各个文库的香浓指数和 Chao 1 指数之间有一定差异(表 3), 结果显示 EZ1-5、EZ3-1 和 EZ3-3 文库的多样性较高, EZ1-3 和 EZ3-5 文库的多样性较低, 而 EZ1-3 和 EZ1-5 文库的物种数量较高, EZ3-1、EZ3-3 和 EZ3-5 文库的物种数量较低。

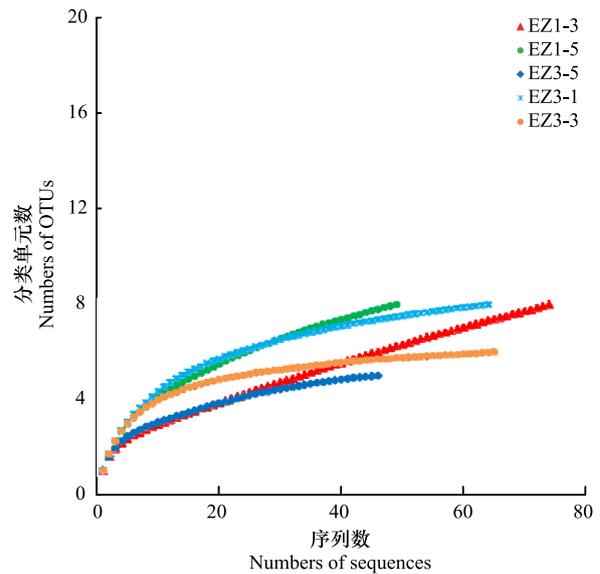


图 1 厌氧氨氧化细菌 16S rRNA 基因稀释度曲线

Fig.1 Rarefaction curves of AAOB 16S rRNA libraries

表 3 厌氧氨氧化细菌基因文库的多样性指数

Table 3 Diversity index of AAOB 16S rRNA libraries

站点 Station No.	分类单元/基因序列 OTUs/Sequences	覆盖度 Coverage	香浓指数 Shannon	Chao1
EZ1-3	8 / 74	89.2%	1.07	13.0
EZ1-5	8 / 49	83.7%	1.48	9.0
EZ3-1	8 / 63	87.3%	1.50	7.0
EZ3-3	6 / 65	90.8%	1.41	6.0
EZ3-5	5 / 46	89.1%	1.05	5.0

使用 FastUniFrac PCoA 分析了研究海域 5 个站位 AAOB 群落组成的空间分布特征。图 2 显示, PCoA 的前两个主坐标轴(PC1 和 PC2)能够解释 5 个采样站位之间 AAOB 群落差异的 70.25%, 根据 AAOB 的群落结

构可以把 5 个站分成 3 类, EZ1-3、EZ3-1 和 EZ3-3 三个站位具有相似的 AAOB 群落结构, EZ1-5 和 EZ3-5 两个站 AAOB 的群落结构与上述三个站具有明显的差别。

2.3 系统发育分析

对从舟山海域沉积物样品中构建的 5 个 AAOB 的 16S rRNA 基因文库进行了系统发育分析, 结果显示, 本研究获得的绝大部分序列 (283 条) 属于 *Scalindua* 属 (图 3), 占总序列数的 95.3%。由图 3 可以看出, 来自 *Scalindua* 属的序列主要聚类于 Brodae 和 Wagneri 2 个分支。Brodae 分支包含 4 个 OUT, 245 条序列, 占 *Scalindua* 属序列数的 86.6%, 与 *Candidatus Scalindua brodae* 的亲缘关系较近。其中, OTU1 包含 47 条序列, 与 *Candidatus S. brodae* 的相似性在 96.0%—97.7% 之间, 主要与来自河口沉积物^[24]、深海热液喷口沉积物^[25] 的 AAOB 非培克隆具有较近的亲缘关系; OTU2 包含 53 条序列, 与 *Candidatus S. brodae* 的相似性在 96.8%—97.9% 之间, 与来自河口沉积物中^[24] 的 AAOB 非培克隆具有较近的亲缘关系; OTU3 包含 26 条序列, 与 *Candidatus S. brodae* (AY254883) 的相似性最高 (>98%), 与来自秘鲁上升流和纳米比亚上升流区的氧最小层 (Oxygen Minimum Zone, OMZ) 中^[26,27] 的 AAOB 非培克隆具有较近的亲缘关系; OTU4 包含 119 条序列, 与 *Candidatus S. brodae* 的相似性在 95.1%—95.6% 之间, 主要与来自长江口、南海和黄河的 AAOB 非培克隆^[28-30] 具有较近的亲缘关系。Wagneri 分支包含 4 个 OTU, 共 36 条序列, 占 *Scalindua* 属序列总数的 12.7%, 与 *Candidatus Scalindua wagneri* 的亲缘关系较近。其中 OTU10 的 10 条序列与 *Candidatus S. wagneri* 的相似性较高 (>97%), 其他 3 个 OTU 的 26 条序列与 *Candidatus S. wagneri* 的相似性在 94.5%—96.6% 之间, 与来自潮间带沉积物、河口沉积物、和近岸低氧沉积物等环境中的 AAOB 非培克隆^[24,31,32] 具有较近的亲缘关系。此外, 2 条来自 EZ3-1 文库的序列 EZ3-1-8 (OTU5) 和 EZ3-1-49 (OTU6) 也聚类于 *Scalindua* 分支内, 但与已知 *Candidatus Scalindua brodae* 和 *Candidatus Scalindua wagneri* 的相似性均较低, 分别为 91% 和 93% 左右, 它们可能代表了该属未知的 AAOB 菌株。

EZ3-5 文库中有一条序列 EZ3-5-46 (OTU11) 聚类于 *Kuenenia* 分支, 与 *Kuenenia* 属的其代表种 *Candidatus Kuenenia stuttgartiensis* 的相似性为 94.6%, 与来自河口、陆源淡水和深海热液喷口的 AAOB^[24,33] 具有较近的亲缘关系。此外, 还有 5 个 OTU 共 15 条序列与已知 AAOB 相似性较低, 这些序列可分为 2 个分支, Unknown I 分支包含 3 个 OTU, 9 条序列, 与来自极地海洋沉积物中的非培克隆^[34] 具有较近的亲缘关系; Unknown II 分支包含 2 个 OUT, 6 条序列, 与来自红树林沉积物中的非培养细菌具有较近的亲缘关系 (图 3)。

3 讨论

厌氧氨氧化细菌 (AAOB) 广泛分布在河流、湖泊、海洋等不同的环境中, 群落组成在不同环境中具有明显的差异性。 *Scalindua* 属是海洋环境中 AAOB 的优势类群^[35], 它们具有较高的盐耐受性, 在多种海洋环境中, 特别是上升流区水柱的 OMZ 和一些次低氧水体中^[35,36] 都有报道。本文研究结果表明舟山群岛海域的 AAOB 是典型的海洋环境群落, *Scalindua* 属是研究海域沉积物中 AAOB 的优势类群, 来自于该属的序列占总序列数的 95.3%。本文研究海域还获得了 1 条与 *Kuenenia* 属 AAOB 亲缘关系较近的 16S rRNA 基因序列。该属主要分布在淡水或陆源生境中^[36,37], 在河口等咸淡水交汇的低盐环境中也有分布^[24,38]。但本文在距离陆地较远的 EZ3-5 站获得了 *Kuenenia* 属序列, 该站位主要受外海水团控制, 盐度较高 (34.32), 营养盐浓度较低, 而在盐

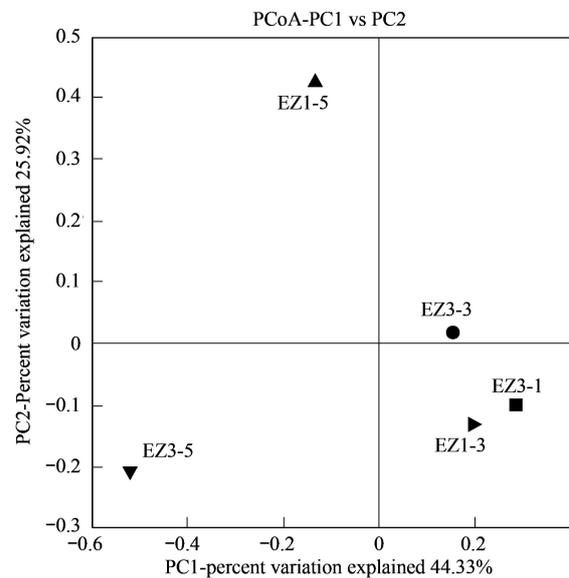


图 2 基于 16S rRNA 基因的厌氧氨氧化细菌群落 PCoA 分析
Fig. 2 The Fast UniFrac weighted PCoA analysis of the anammox communities using 16S rRNA gene sequences

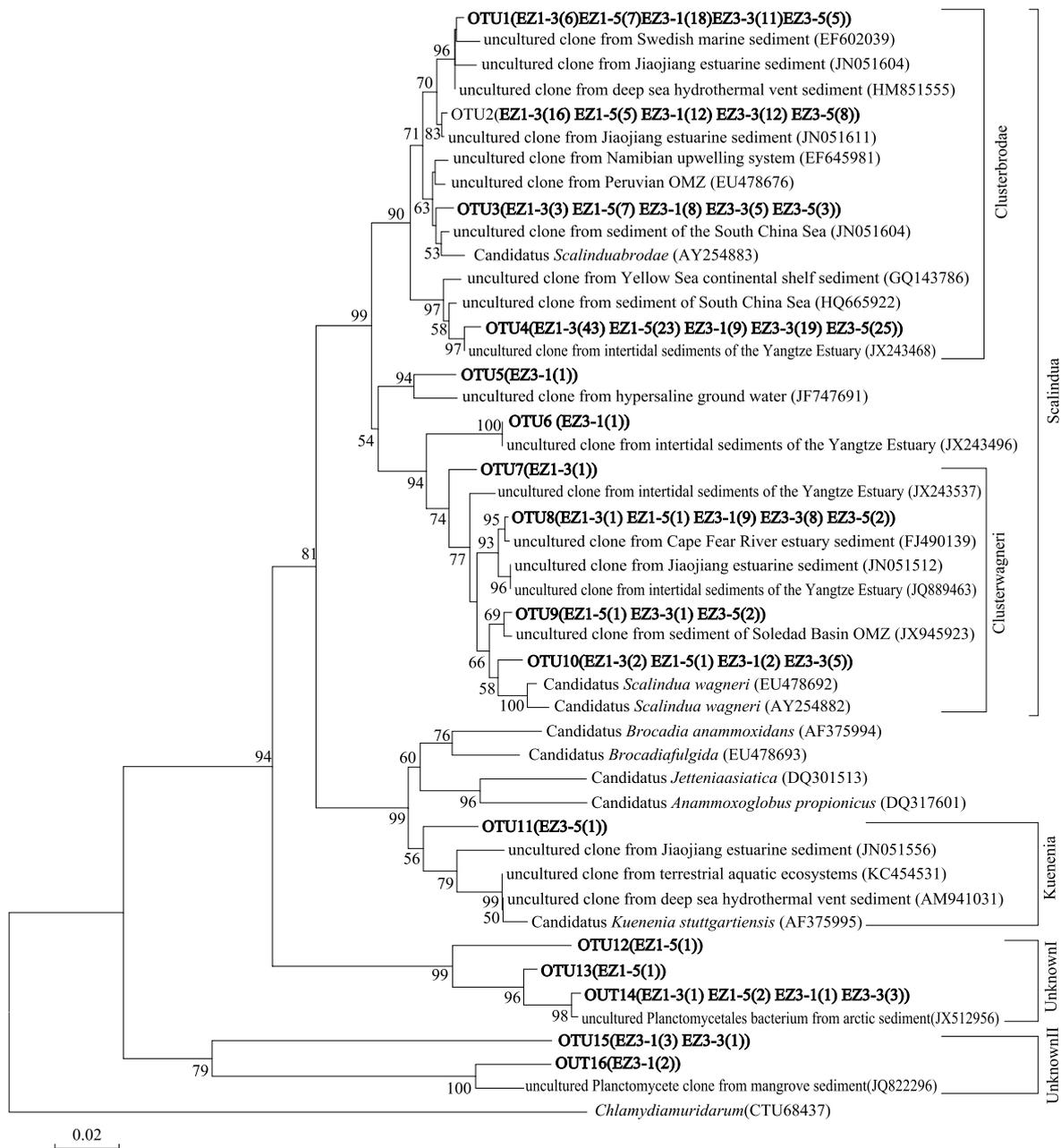


图3 AAOB 16S rRNA 基因系统发育树

Fig.3 Neighbor-joining phylogenetic tree of anammox bacteria-related 16S rRNA gene fragments from the study area

Clon names include the sample name and the number of times a sequence among all of the sequenced clones of samples. Bootstrap values represent 1000 replications and only values above 50% are shown. Branch lengths correspond to sequence differences as indicated by the scale bar. Numbers in parentheses refer to the number of clones were assigned to an OTU

度相对较低的 EZ3-1 站未发现 *Kuenenia* 属序列。近期 Byrne 等在北大西洋中脊的深海热液区的贻贝和烟囱体中也获得了与 *Kuenenia* 属亲缘关系较近的序列^[33], 表明 *Kuenenia* 属的部分 AAOB 对盐度具有较强的耐受能力。本文和 Byrne 等的研究结果表明来自 *Kuenenia* 属 AAOB 的分布范围可能超出我们原来的认识, 一方面这可能受到采样和研究不足的限制, 另一方面需要更深水平的二代测序研究证实。此外, 除了已知的 AAOB 类群, 本研究还发现了一些与来自海洋环境的浮霉菌门非培养克隆相似性较低 (<85%) 的序列 (Unknown I 和 Unknown II)。有研究表明, 海洋环境中可能存在除浮霉菌门以外的其它厌氧氨氧化微生物, 如 β -和 γ -变形菌中的 *Nitrosospira*、*Nitrosomonas* 和 *Nitrosococcus*^[39,40], 甚至在某些海洋环境中存在具有厌氧氨氧化能力的古

菌^[41]。因此,这些序列可能代表了某些未知门类的厌氧氨氧化微生物。

AAOB 多样性和群落结构的分布特征与环境因子有着密不可分的关系。Dale 等认为盐度是影响河口生态系统中 AAOB 群落结构的重要因子^[31],但本文采样站位之间盐度差别不大,除 EZ3-1 略低外,其它站位的盐度十分接近。本文中 EZ3-1、EZ3-3 和 EZ1-3 三个站的群落结构较为接近,而与 EZ1-5 和 EZ3-5 站不同(图 2),表明离岸距离和水深可能是影响表层沉积物 AAOB 群落组成的重要因素^[30]。另一方面,各站位的多样性指数却呈现出不同的分布特征,EZ3-1、EZ3-3 和 EZ1-5 三个站的多样性指数高于 EZ1-3 和 EZ3-5 站(表 2)。相关分析显示沉积物有机碳含量与多样性指数具有显著正相关关系(图 4)。Hou 等研究认为沉积物有机碳含量是河口生态系统中影响 AAOB 多样性的重要环境因子^[29],高有机碳含量能够促进硝酸盐还原作用,进而提高亚硝酸盐浓度,亚硝酸盐是厌氧氨氧化过程中的电子受体,高浓度的亚硝酸盐有利于促进厌氧条件下的氨氧化作用^[42]。Hu 等人在椒江口海域的研究表明 AAOB 多样性指数与沉积物有机碳含量呈正比^[24],Li 等人在南海北部也有类似结果^[30]。本文结果证明在舟山群岛及其临近海域,沉积物有机碳含量可能是影响 AAOB 多样性的重要因子。

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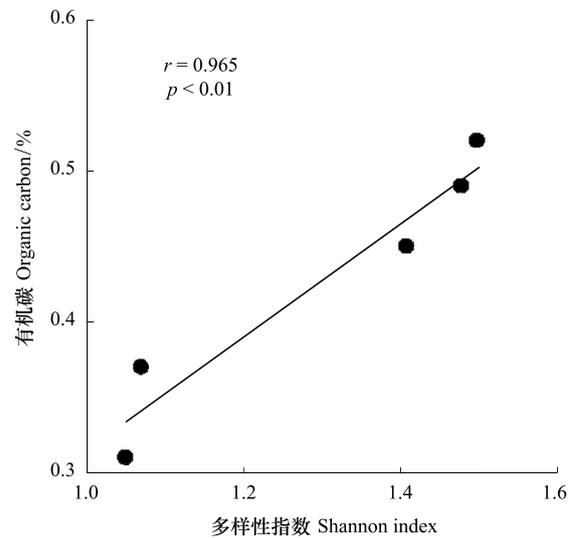


图 4 有机碳与多样性指数相关性

Fig.4 Correlation between organic carbon and Shannon index of the five sampling sites

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