八门湾红树林土壤放线菌多样性及抗病原菌活性分析*

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摘要 红树林土壤中蕴藏着丰富的放线菌资源, 是新菌种的重要来源。为获得更多新的放线菌资源以用于开发新的生物活性化合物, 了解放线菌的多样性是非常重要和必要的。研究采用扩增子高通量测序和传统培养法对八门湾红树林土壤放线菌进行了非培养和培养水平多样性研究, 同时对其抗病原菌活性进行了分析。对于非培养水平多样性研究, 与细菌通用引物相比, 利用放线菌相关引物可以提高放线菌丰度的检测水平, 其百分比含量提高 2.47 倍; 可以检测到放线菌门更多的目、科和属; 对于在目水平上的放线菌类群组成来说, Acidimicrobiales、Corynebacteriales、Gaiellales、Kineosporiales、Solirubrobacterales 是优势类群, 但是在用两对不同引物得到的结果中, 其百分含量差异较大。放线菌相关引物更适合环境样品中放线菌多样性的分析。对于培养水平多样性研究, 分离到 256 株放线菌, 属于 7 个目、9 个科、14 个属, Shannon-Wiener 多样性指数为 1.32, *Streptomyces* (42.58%)和 *Micromonospora* (42.19%)是优势属; 与模式菌株的相似性小于 98.5%的有 13 株, 其中菌株 HA161004 和 HA161010 与 *Amycolatopsis thermoflava* N1165^T 相似性分别为 95.56%和 96.80%, 是潜在新种。病原微生物拮抗活性测定表明, 来自 9 个属的 92 株代表性菌株具有抗病原菌活性, 其中对 *Colletotrichum gloeosporioides*、*Fusarium oxysporum*、*Escherichia coli*、*Staphylococcus aureus* 和 *Vibrio neocaledonicus* 有拮抗活性的菌株数量分别为 71、25、6、9 和 19 株。研究为选择更合适的引物以便更客观地反映环境中放线菌多样性提供了数据支持, 同时也为后续红树林放线菌资源收集、活性评价和开发利用提供菌种保障。

关键词 放线菌; 多样性; 抗菌活性; 红树林

中图分类号 R915 doi: 10.11693/hyhz20210800176

红树林生态系统是指位于热带和亚热带海岸潮间带, 包括种类丰富的动物群落、红树木本植物群落、微生物群落的复杂而独特的生态系统。红树林生态系统覆盖了世界上60%~75%的海岸线, 是生产力水平最高的四个海洋生态系统之一(Alongi, 2008)。红树林作为重要的近岸湿地生态系统, 具有防风消浪、促淤护岸、调节大气、净化海水和美化景观等作用, 对海洋环境保护和生态平衡有重要的作用(Alongi, 2008)。红树林土壤具有高湿度、高盐度、强还原性、强酸性、营养成分丰富等特点, 因此微生物群落具有极高的多样性和独特性(Alongi, 1996)。

放线菌广泛分布于陆地(Hayakawa *et al*, 2000; Xu *et al*, 2014)和海洋生境(Demain *et al*, 2009; 洪葵, 2013)。由于红树林生态环境的独特性, 被认为是分离放线菌新种资源的理想生境(Ser *et al*, 2016b; Huang *et al*, 2018; 候师师等, 2020; Asha *et al*, 2021)。已有研究表明红树林土壤中存在丰富多样的放线菌资源(洪葵, 2013; Claverías *et al*, 2015), 部分菌株已被鉴定为

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收稿日期: 2021-08-04, 收修改稿日期: 2021-09-12

新种(Demain *et al*, 2009; Suthindhiran *et al*, 2010; Arumugam *et al*, 2017; Huang *et al*, 2018)。放线菌因其具有产生多种生物活性次级代谢产物的能力而备受关注(Demain *et al*, 2009), 具有多种抗菌、抗氧化、抗癌、神经保护和酶抑制剂等功能(Amrita *et al*, 2012; Ser *et al*, 2016b; Arumugam *et al*, 2017), 在医药、工业、农业、生态等领域有广泛应用(Xu *et al*, 2014)。然而, 近年来从陆地来源的放线菌中发现活性化合物效率低(Ser *et al*, 2016b), 因此, 从海洋生境中寻找新的放线菌资源及其天然产物越来越受到关注, 其中红树林已成为寻找新的放线菌资源及其天然产物的热点生境。

为了挖掘出更多新的放线菌菌株以满足活性物质开发, 首先需要了解它们的多样性。以往的研究主要集中于传统培养方法对红树林放线菌多样性的研究(Mevs *et al*, 2000; Janssen *et al*, 2002; Page *et al*, 2004; Arumugam *et al*, 2017; Law *et al*, 2017; Huang *et al*, 2018)。本研究将利用两种不同引物(放线菌相关引物和细菌通用引物)对八门湾红树林土壤放线菌在非培养水平多样性进行对比分析; 利用传统培养法对红树林土壤放线菌在可培养水平多样性进行分析, 同时分析放线菌的抗病原菌活性, 为后续从红树林生境中挖掘新的放线菌资源提供前期数据支持, 也为放线菌的开发利用提供菌种资源。

1　材料与方法

1.1　样品采集

样品采集地点位于海南省文昌市八门湾红树林湿地, 以*Bruguiera sexangular*和*Xylocarpus mekongensis*为优势树种的红树林区, 设立3个采样点, 于2011年12月采集5~30 cm深的黑褐色泥质土壤样品, 在每个采样点采集3份重复土壤样品(每份约500 g土壤), 重复样品采集点呈正三角形分布（两点间隔5 m）, 然后将三个重复样品混匀, 将所有样品混合均匀, 置于冰盒带回实验室, 用于后续实验。

1.2　DNA 提取和高通量测序

土壤总 DNA 的提取按照试剂盒 FastPrep® SPIN Kit for Soil (MP Biomedicals, 美国)步骤进行。利用两对引物对 16S rDNA 进行扩增, 引物分别是: 放线菌相关引物(可提高环境样品中放线菌的检出率)ACT235F (CGCGGCCTATCAGCTTGTTG) 和 ACT878R (CCGTACTCCCCAGGCGGGG), 细菌通用引物 341F (5′-CCTACGGGAGGCAGCAG-3′) 和 1073R (5′-ACGAGCTGACGACARCCATG-3′) (Farris *et al*, 2007)。PCR 反应体系为(20 μL 混合体系): 4 μL 5× FastPfu Buffer, 2 μL 2.5 mmol/L dNTPs, 0.8 μL 引物 1 (5 μmol/L), 0.8 μL 引物 2 (5 μmol/L), 0.4 μL FastPfu Polymerase, 10 ng 模板 DNA。PCR 反应程序: 95 °C 10 min; 95 °C 30 s, 55 °C 30 s, 72 °C 30 s; 72 °C 10 min (Liu *et al*, 2017)。利用 AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, 美国)胶回收试剂盒对 PCR 产物纯化, 利用 QuantiFluor™ -ST (Promega, 美国)进行定量, 然后送至上海美吉医药科技有限公司进行高通量测序(Roche Genome Sequencer GS FLX+ Titanium platform)。对获得的原始序列进行拼接、过滤除杂和去除嵌合体等质量控制后得到最终分析的有效序列, 通过 UPARSE (version 7.1 http:// drive5.com/uparse/)对有效序列以 97%的一致性进行聚类分析, 获得 OTU (操作分类单元, operational taxonomic unit)(Yan *et al*, 2006; Dias *et al*, 2011)。将 OTU 序列与 SILVA 数据库进行比对确定 OTU 的系统发育信息(Yan *et al*, 2006; Dias *et al*, 2011; Mendes *et al*, 2012), 使用 QIIME(版本 1.17)处理序列(Caporaso *et al*, 2010), 进行多样性和群落结构分析。将从高通量测序数据中选取放线菌门序列做进一步分析。高通量测序序列在 NCBI 数据库的序列接收号为 SRX506963 和 SRX3938853。

1.3　放线菌的分离和 16S rDNA 序列分析

土壤在室温下自然风干, 然后研磨成粉末。沉淀物粉末采用以下两种方法处理: 一种方法是将 5 g 沉淀物粉末悬浮在 10 mL 林格氏液中, 并将水加热至 55 °C; 另一种方法是将 5 g 沉淀物粉末在 120 °C 下加热 1 h, 然后悬浮在 10 mL 林格氏液中。将沉淀物悬浮液在 28 °C 摇床(180 r/min 转速)摇动 30 min。利用稀释涂布平板法, 将稀释(10^{-2} 到 10^{-4})的土壤悬浮液涂布在琼脂分离培养基上分离放线菌菌株。分离培养基有: 高氏合成一号培养基(20 g 可溶性淀粉, 1 g KNO_3, 0.5 g K_2HPO_4, 0.5 g $MgSO_4·7H_2O$, 0.1 g $FeSO_4·7H_2O$, 15 g 琼脂, 1 L 海水, pH 7.2); 腐殖酸维生素培养基(1.0 g 腐殖酸, 0.5 g Na_2HPO_4, 1.7 g KCL, 21 g $CaCL_2$, 0.5 g $MgSO_4·7H_2O$, 0.01 g $FeSO_4·H_2O$, 复合维生素, H_2O 1 L, 20.0 g 琼脂, pH 7.2; 复合维生素: 0.5 mg 肌醇, 0.5 mg 泛酸, 0.5 g 氨基苯酸, 0.25 mg 生物素, 0.5 mg VB1, 0.5 mg VB2, 0.5 mg VB3, 0.5 mg VB6); 土壤提取物培养基(50%土壤提取物, 2%琼脂, 1 L 海水, pH 6.8)。在分离培养基中, 添加细菌与真菌

抑制剂: 50 μg/mL 重铬酸钾、100 μg/mL 制霉菌素、20 μg/mL 萘啶酮酸。将平板置于 28 °C 恒温培养箱中倒置培养 2~4 周, 挑取符合条件的单菌落划线于 ISP2 培养基上直至菌株纯化。纯化菌株的 16S rDNA 序列使用细菌通用引物 PCR 扩增, 引物为 27f (5′-AGAGTTTGAT CMTGCCTCAG-3′) 和 1492r (5′-TACGGYTACCTTG TTACGACTT-3′), 16S rDNA 序列提交至 EzBioCloud (http://www.ezbiocloud.net/) 进行系统发育相似性比对。多样性分析采用 Shannon-Wiener 多样性指数:

$$H' = \sum_{i=1}^{n} P_i \times \ln P_i$$

其中 $P_i = N_i/N$ 即第 i 种占总个体数 N 的比例。

1.4 抗病原菌活性测定

为了分析抗菌活性, 菌株在 ISP2 培养基中 28 °C 摇床(200 r/min)培养, 7~14 d 后, 收集发酵液并离心, 取上清液通过 0.45 μm 和 0.2 μm 硝酸纤维素过滤器过滤, 然后使用纸片扩散法测定抗菌活性(Kamei *et al*, 2003)。试验中使用的致病微生物菌株为 *Colletotrichum gloeosporioides*、*Escherichia coli*、*Fusarium oxysporum*、*Staphylococcus aureus* 和 *Vibrio neocaledonicus*。

2 结果与讨论

2.1 在非培养水平上放线菌多样性分析

利用放线菌相关引物进行扩增子高通量测序, 经过质控检测后, 共获得44 003条有效序列用来进一步分析, 测序深度指数 Good's coverage 为 0.986 6~0.989 6, 表明测序量足够大, 可以反映样品中绝大部分的微生物物种信息(附表1)。对于细菌群落多样性, 共检测到33个门, 前10个门分别是: Actinobacteria (41.56%)、Proteobacteria (29.48%)、Gemmatimonadetes (6.99%)、Chloroflexi (5.71%)、Acidobacteria (4.23%)、Nitrospirae (1.48%)、Bacteroidetes (1.31%)、Chlorobi (1.17%)、Cyanobacteria (1.10%)和Firmicutes (0.97%) (图1)。对于放线菌多样性, 共有18297条序列属于Actinobacteria门, 共包括15个目, 分别是: Acidimicrobiales (30.10%)、Corynebacteriales (17.25%)、Gaiellales (9.90%)、Kineosporiales (9.82%)、Solirubrobacterales (6.70%)、Frankiales (5.57%)、Micrococcales (5.11%)、Micromonosporales (4.60%)、Propionibacteriales (3.12%)、Streptomycetales (1.26%)、Pseudonocardiales (0.99%)、PeM15 (0.35%)、Streptosporangiales (0.27%)、Euzebyales (0.11%)、Coriobacteriales (0.05%)和未分类放线菌(4.80%) (图2); 29个科(不包括未培养和未分类的科), 32个属, 其中百分比大于1%的属包括 *Mycobacterium* (16.2%)、*Acidothermus* (4.9%)、*Ilumatobacter* (2.3%)、*Gaiella* (1.5%)、*Quadrisphaera* (1.7%)、*Microbacterium* (1.7%)、*Intrasporangium* (1.3%)、*Luedemannella* (1.5%)、*Nocardioides* (1.4%)和*Streptomyces* (1.3%) (附表2)。

利用细菌通用引物进行扩增子高通量测序, 经过质控检测, 共有24 821条有效序列用来进一步分析, 测序深度指数Good's coverage为0.979 4~0.989 0, 表明测序量足够大, 可以反映样品中绝大部分的微生物物种信息(附表1)。对于细菌群落多样性, 共检测到30个门, 其中前10个门分别是: Proteobacteria (49.40%)、Actinobacteria (16.79%)、Chloroflexi (9.46%)、Acidobacteria (7.20%)、Gemmatimonadetes (2.74%)、Nitrospirae (2.24%)、Bacteroidetes (2.06%)、Cyanobacteria (1.90%)、Chlorobi (1.89%)、Firmicutes (1.71%)(Fig. 1)。对于放线菌多样性, 共有4140条序列属于Actinobacteria门, 共包括13个目, 分别是: Gaiellales (28.03%)、Acidimicrobiales (27.57%)、Solirubrobacterales (22.82%)、Kineosporiales (7.10%)、Micrococcales (2.73%)、Frankiales (2.51%)、Propionibacteriales (1.11%)、Corynebacteriales (0.94%)、Micromonosporales (0.94%)、Streptomycetales (0.51%)、PeM15 (0.27%)、Coriobacteriales (0.22%)、Euzebyales (0.17%)、Streptosporangiales (0.10%)、未分类放线菌(4.97%) (图2); 23个科(不包括未培养和未分类的科)(附表2); 17个属, 其中百分比大于1%的属包括 *Gaiella* (6.43%)、*Kineosporia* (3.31%)、*Acidothermus* (2.10%)和*Ilumatobacter* (1.62%) (附表2)。

通过对放线菌相关引物和细菌通用引物获得的数据对比分析可知: (1) 与细菌通用引物相比, 利用放线菌相关引物可以检测到更多的目、科和属; (2) 对于在门水平上的细菌群落组成来说, Actinobacteria、Proteobacteria、Gemmatimonadetes、Chloroflexi、Acidobacteria都是优势类群, 但是其百分含量差异较大; 利用放线菌相关引物可以提高Actinobacteria丰度的检测水平, 其百分比含量提高了2.47倍; Gemmatimonadetes的百分比含量提高2.55倍; 此外, 对于minor-groups(所占百分比小于1%的类群)中

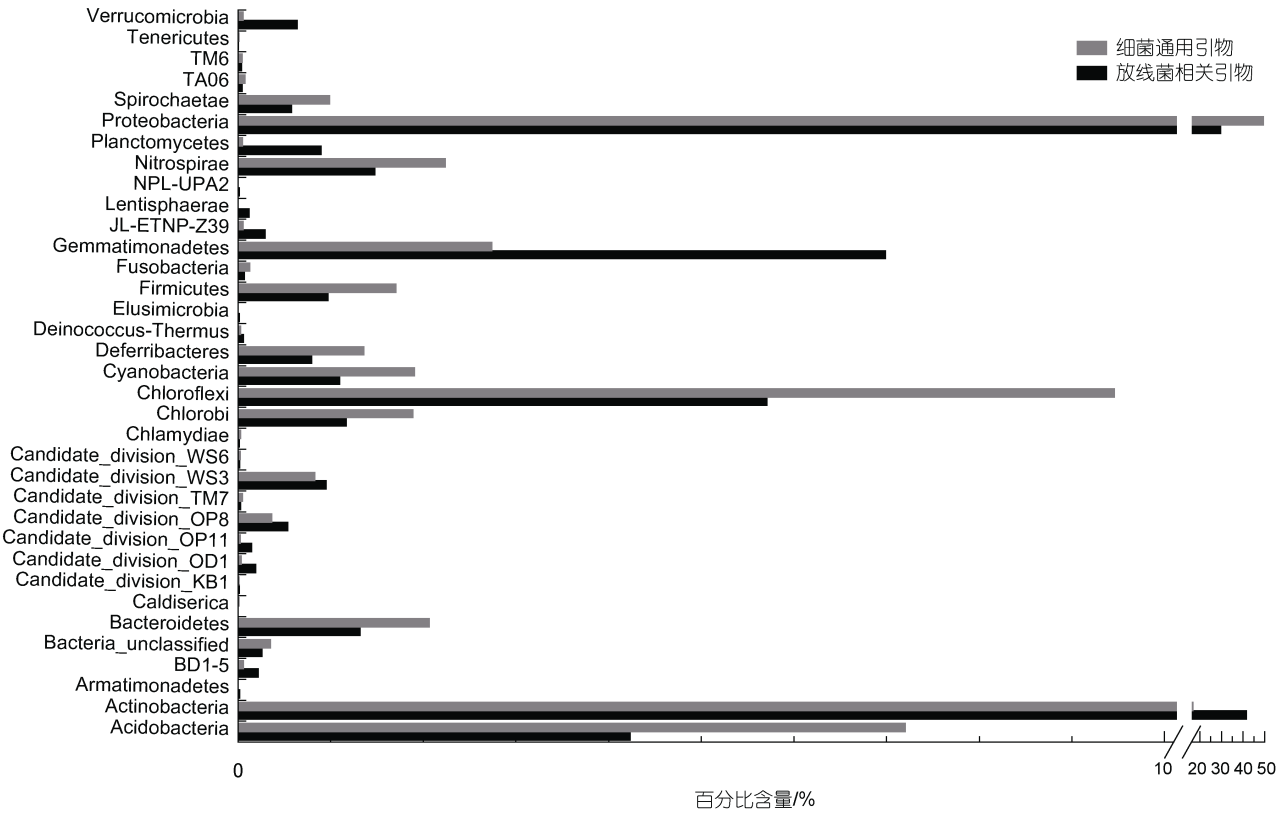

图 1　利用放线菌相关引物和细菌通用引物对八门湾红树林土壤细菌多样性的分析(门水平)

Fig.1　Diversity of bacteria in the phylum level from soil of Bamenwan mangrove by pyrosequencing methods with Actinobacteria-specific primer and the universal bacterial primer

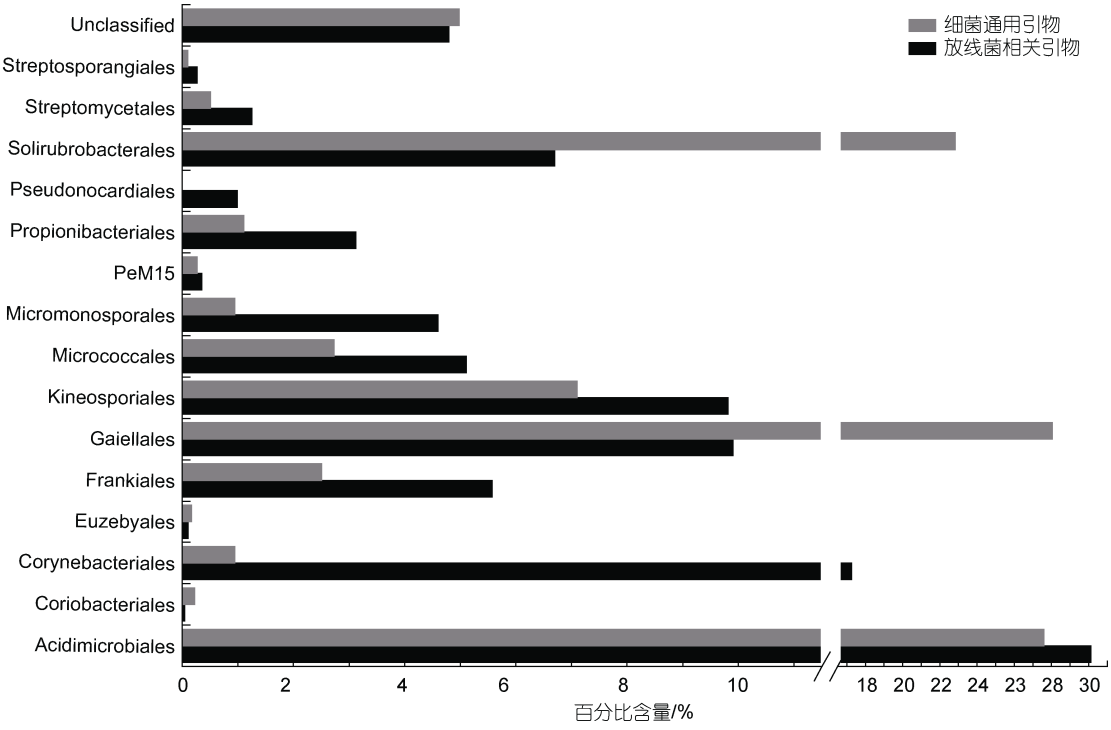

图 2　利用放线菌相关引物和细菌通用引物对八门湾红树林土壤放线菌多样性的分析(目水平)

Fig.2　Diversity of Actinobacteria in the order level from soil of Bamenwan mangrove by pyrosequencing with Actinobacteria-specific primer and the universal bacterial primer

Planctomycetes和Verrucomicrobia检出丰度也分别提高18和11倍; 其中三个minor-groups (Lentisphaerae、Elusimicrobia和Armatimonadetes)只在利用放线菌相关引物进行测序时检测到(图1); (3) 对于在目水平上的放线菌类群组成来说, 5个目(Acidimicrobiales、Corynebacteriales 、 Gaiellales 、 Kineosporiales 、Solirubrobacterales)是丰度相对较高的类群, 在两对不同引物得到的结果中, 其丰度差异较大; 利用放线菌相关引物检出的Corynebacteriales的百分含量提高了 18.32 倍 , Micromonosporales 提 高 了 4.89 倍 , Propionibacteriales、Streptosporangiales、Streptomycetales和Frankiales分别提高了2.81、2.77、2.48和2.22倍。相比之下, 利用细菌通用引物检出Solirubrobacterales和Gaiellales的百分比更高, 分别提高3.4和2.83倍(图2)。

通过以上比较分析表明, 利用放线菌相关引物可以提高放线菌的百分比含量, 更多目、科和属被检测到, 这与前人的研究一致(Mcveigh *et al*, 1996; Heuer *et al*, 1997; Lüdemann *et al*, 2000; Stach *et al*, 2003; Schäfer *et al*, 2010)。以前的研究报道, 某些微生物类群, 特别是具有高G+C含量的类群(如放线菌)在16S rRNA基因库中被低估(Hill *et al*, 2006)。细菌16S rDNA通用引物不能扩增环境样品中所有放线菌的该基因片段, 而专门设计的放线菌相关引物可大大提高环境样品中放线菌16S rDNA的扩增概率(Mcveigh *et al*, 1996; Heuer *et al*, 1997; Lüdemann *et al*, 2000; Stach *et al*, 2003)。Stach等(2003)报道, 利用放线菌相关引物扩增环境样品时, 约75% PCR产物序列属于放线菌门, 本研究中为41.56%, 这说明放线菌相关引物更适合于环境样品中放线菌多样性的分析。另外, 在非可培养水平上, 利用放线菌相关引物专门针对红树林放线菌多样性的研究未见报道, 目前文献中主要利用细菌通用引物研究红树林细菌多样性, 其中放线菌(Actinobacteria)所占比例较低, 所占百分比在以下研究地点分别为: 香港Mai Po Ramsar湿地为2.1% (Jiang *et al*, 2013)和3.5% (Wang *et al*, 2012), 巴西São Paulo State、Rio de Janeiro state Ilha Grande和Bahia state Porto Seguro红树林湿地为5.4%~12.2% (Andreote *et al*, 2012)、8.4%和7.5% (Thompson *et al*, 2013), 马来西亚Rantau Abang红树林湿地为4.55% (Chan *et al*, 2015)、深圳湾红树林湿地2.2% (Zhang *et al*, 2015), 本研究中利用细菌通用引物检测到的放线菌百分比是16.79%, 而用放线菌相关引物检测到的百分比为41.56%, 可见放线菌相关引物可提高自然环境中放线菌类群的检测水平。此外, 对于Corynebacteriales类群, 放线菌相关引物检出的百分率(17.25%)高于细菌通用引物(0.94%), 这可能是细菌通用引物对该类群也存在一定的低估所致。

本研究中利用放线菌相关引物和细菌通用引物检测微生物多样性时, 4个目(Acidimicrobiales、Gaiellales、Kineosporiales和Solirubrobacterales)和3个属(*Gaiella*、*Acidothermus*和*Ilumatobacter*)相对丰度都较高。Acidothermaceae科中的*Acidothermus*属中只有一个种*Acidothermus cellulolyticus*, 代表菌株具有嗜热、嗜酸性和分解纤维能力(Mohagheghi *et al*, 1986), 而红树林土壤呈酸性, 且含有大量来自植物落叶的纤维素和木质素, 这说明这类细菌对环境的适应性, 及其可能参与了红树林有机碳的代谢。*Mycobacterium* 是利用放线菌相关引物检测时发现的丰度最高的属, 这类微生物广泛存在于酸性环境中, 与红树林土壤酸性环境相适应; 该属物种最主要特征是细胞壁比其他细菌厚, 疏水、蜡质且富含霉菌酸(Faller *et al*, 2004), 细胞壁由疏水霉酸酯层和由阿拉伯半乳聚糖连接在一起的肽聚糖层组成, 对植物的耐寒性有重要贡献, 细胞壁这些组成成分的生物合成途径也成为结核病新药的潜在靶点, 可见, 红树林放线菌是蕴藏着丰富的可用于海洋药物开发的放线菌资源。

2.2 在培养水平上放线菌多样性

通过传统培养法, 本研究共分离得到放线菌256株, 其中通过水浴55 °C加热土壤样品分离到207株, 120 °C加热1 h土壤样品分离到49株。利用不同培养基分离到的菌株数量也不同, 利用高氏合成一号培养基分离得到的菌株数量最多(共105株), 其次是利用腐殖酸维生素培养基分离到80株, 利用土壤提取物培养基分离到71株。将纯化菌株与数据库中的模式菌株做16S rDNA相似性分析, 相似性100%的有58株细菌, 相似性99.0%~99.9%有155株, 相似性98.5%~98.9%有25株, 相似性98.0%~98.5%有11株, 相似性低于98%的有2株, 分别是菌株HA161004和HA161010, 这两株菌与*Amycolatopsis thermoflava* N1165^{T}相似性为95.56%和96.80% (表1), 表明是潜在新种(Tindall *et al*, 2010)。在分离放线菌时, 对于相似性低于98.5%的13株潜在新种放细菌, 从不同样品处理方法来看, 其中4株是通过将土壤样品水浴55 °C加热分离到, 9株是通过将土壤样品120 °C加热1 h分离到; 从不同培养基来看, 其中6株利用腐殖酸维生素培养基分离到, 7株利用土壤提取物培养基分离到, 由此可知, 为获得

表 1　八门湾红树林土壤可培养放线菌的 16S rDNA 序列分析及拮抗病原菌活性分析

Tab.1　16S rDNA sequence analysis and antimicrobial activity of representative actinobacterial strains isolated from Bamenwan mangrove soil

菌株名称	序列接收号	最相似模式菌及相似性	抗病原菌活性	菌株名称	序列接收号	最相似模式菌及相似性	抗病原菌活性
HA13696	MF573412	*Actinomadura formosensis* JCM 7474(T) 98.83%	C	HA13690	KJ467040	*Micromonospora narathiwatensis* BTG4-1(T) 99.25%	C
HA161003	MF573492	*Allostreptomyces psammosilenae* YIM DR4008(T) 98.75%	—	HA161155	MF573431	*Micromonospora sediminicola* SH2-13(T) 99.78%	C/V
HA161006	MF573495	*Amycolatopsis dongchuanensis* YIM 75904(T) 99.87%	C	HA13626	MF573358	*Micromonospora siamensis* TT2-4(T) 98.88%	C/V
HA161004	MF573493	*Amycolatopsis thermoflava* N1165(T) 95.56%	—	HA13677	MF573397	*Micromonospora siamensis* TT2-4(T) 99.03%	—
HA161010	MF573499	*Amycolatopsis thermoflava* N1165(T) 96.80%	—	HA13691	MF573408	*Micromonospora siamensis* TT2-4(T) 99.63%	—
HA161018	MF573504	*Amycolatopsis thermoflava* N1165(T) 99.43%	—	HA13597	MF573336	*Micromonospora chalcea* DSM 43026(T) 99.86%	V
HA161043	MF573511	*Amycolatopsis tucumanensis* ABO(T)99.87%	C	HA13609	MF573345	*Micromonospora chalcea* DSM 43026(T) 99.47%	V
HA13601	MF573339	*Blastococcus jejuensis* KST3-10(T) 98.87%	F	HA13604	MF573341	*Micromonospora tulbaghiae* TVU1(T) 100.00%	C
HA13583	MF573329	*Jishengella endophytica* 202201(T) 99.74%	—	HA13628	MF573360	*Micromonospora tulbaghiae* TVU1(T) 99.26%	C
HA13611	MF573346	*Jishengella endophytica* 202201(T) 99.78%	—	HA13645	KJ467029	*Micromonospora tulbaghiae* TVU1(T) 99.55%	C
HA13651	KJ467031	*Jishengella endophytica* 202201(T) 99.16%	—	HA161200	MF573441	*Micromonospora tulbaghiae* DSM 45142(T) 99.27%	—
HA161222	MF573445	*Krasilnikoviella muralis* T6220-5-2b(T) 99.72%	C	HA13701	MF573417	*Micromonospora maritima* D10-9-5(T) 99.78%	C
HA13687	MF573405	*Microbispora bryophytorum* NEAU-TX2-2(T) 99.55%	V	HA13695	MF573411	*Micromonospora* gifhornensis DSM 44337(T) 99.60%	C
HA13712	MF573425	*Microbispora rosea* subsp. *Rosea* ATCC 12950(T) 99.25%	C	HA13698	MF573414	*Micromonospora* gifhornensis DSM 44337(T) 99.78%	C
HA13587	MF573330	*Micromonospora aurantiaca* ATCC 27029(T) 100.00%	—	HA13615	KJ467025	*Micromonospora* sediminis MS426(T) 99.40%	—
HA13600	MF573338	*Micromonospora aurantiaca* ATCC 27029(T) 99.92%	—	HA161257	MF573473	*Nocardia grenadensis* NBRC 108939(T) 100.00%	C/F
HA13638	MF573368	*Micromonospora aurantiaca* ATCC 27029(T) 99.85%	—	HA13589	MF573332	*Nonomuraea ferruginea* IFO 14094(T) 99.17%	—
HA13655	KJ467032	*Micromonospora aurantinigra* TT1-11(T) 100.00%	C/F	HA13710	MF573424	*Nonomuraea ferruginea* IFO 14094(T) 99.44%	—
HA13664	MF573388	*Micromonospora carbonacea* DSM 43815(T) 99.63%	C/F	HA13713	MF573426	*Nonomuraea ferruginea* IFO 14094(T) 99.26%	—
HA13702	KJ467042	*Micromonospora carbonacea* DSM 43815(T) 98.65%	F	HA13684	MF573403	*Nonomuraea maritima* FXJ7 203(T) 99.63%	—
HA13675	MF573395	*Micromonospora carbonacea* DSM 43815(T) 99.13%	C/F	HA13665	MF573389	*Nonomuraea turkmeniaca* IFO 13155(T) 99.18%	—
HA161188	MF573437	*Micromonospora chaiyaphumensis* DSM 45246(T) 99.22%	—	HA161260	MF573476	*Rhodococcus rhodochrous* NBRC 16069(T) 98.07%	C
HA13612	KJ467024	*Micromonospora chalcea* DSM 43026(T) 99.32%	C	HA161015	MF573502	*Streptomyces albogriseolus* NRRL B-1305(T) 99.86%	C
HA13614	MF573348	*Micromonospora chalcea* DSM 43026(T) 99.47%	C/V	HA161132	MF573544	*Streptomyces albogriseolus* NRRL B-1305(T) 99.87%	C
HA13616	MF573349	*Micromonospora chalcea* DSM 43026(T) 99.78%	C/F	HA161236	MF573455	*Streptomyces albogriseolus* NRRL B-1305(T) 100.00%	C
HA13620	MF573352	*Micromonospora chalcea* DSM 43026(T) 99.85%	C/F/V	HA161081	MF573520	*Streptomyces antibioticus* NBRC 12838(T) 100.00%	F
HA13640	MF573369	*Micromonospora chalcea* DSM 43026(T) 99.77%	C/F	HA161167	MF573551	*Streptomyces atrovirens* NRRL B-16357(T) 99.04%	F
HA13667	MF573390	*Micromonospora chalcea* DSM 43026(T) 99.85%	C	HA161229	MF573451	*Streptomyces badius* NRRL B-2567(T) 100.00%	V
HA13679	MF573399	*Micromonospora chalcea* DSM 43026(T) 99.55%	C/F	HA13685	MF573404	*Streptomyces cellostaticus* NBRC 12849(T) 99.12%	C
HA161291	MF573489	*Micromonospora chalcea* DSM 43026(T) 99.61%	C/E	HA161231	MF573453	*Streptomyces chumphonensis* K1-2(T) 99.87%	M/V
HA13650	KJ467030	*Micromonospora chalyaphumensis* MC5-1(T) 99.33%	—	HA161264	MF573477	*Streptomyces chumphonensis* K1-2(T) 99.74%	M/V
HA13606	MF573342	*Micromonospora chokoriensis* 2-19/6(T) 99.70%	C	HA161239	MF573458	*Streptomyces coelicoflavus* NBRC 15399(T) 100.00%	C/M

续表

菌株名称	序列接收号	最相似模式菌及相似性	抗病原菌活性	菌株名称	序列接收号	最相似模式菌及相似性	抗病原菌活性
HA13632	MF573364	*Micromonospora chokoriensis* 2-19/6(T) 98.88%	V	HA161048	MF573514	*Streptomyces coeruleorubidus* ISP 5145(T) 98.55%	C
HA13658	MF573382	*Micromonospora chokoriensis* 2-19/6(T) 99.48%	C	HA13683	MF573402	*Streptomyces diastaticus* subsp.*ardesiacus* NRRL B-1773(T) 99.63%	C
HA13681	KJ467038	*Micromonospora chokoriensis* 2-19/6(T) 98.81%	V	HA161096	MF573532	*Streptomyces griseoflavus* LMG 19344(T) 99.23%	—
HA13613	MF573347	*Micromonospora coxensis* 2-30-B/28(T) 99.55%	C/E	HA161116	MF573428	*Streptomyces griseoflavus* LMG 19344(T) 99.38%	—
HA13693	KJ467041	*Micromonospora coxensis* 2-30-B/28(T) 99.48%	C/E	HA161125	MF573542	*Streptomyces griseoincarnatus* LMG 19316(T) 100.00%	F
HA13635	MF573366	*Micromonospora eburnea* LK2-10(T) 99.33%	C/F	HA161256	MF573472	*Streptomyces hiroshimensis* NBRC 3839(T) 98.58%	F
HA13642	MF573371	*Micromonospora eburnea* LK2-10(T) 98.94%	C	HA161045	MF573513	*Streptomyces matensis* NBRC 12889(T) 98.31%	V
HA13656	MF573381	*Micromonospora eburnea* LK2-10(T) 99.02%	C/F	HA161164	MF573435	*Streptomyces panacagriGsoil* 519(T) 98.12%	M
HA13670	KJ467036	*Micromonospora eburnea* LK2-10(T) 98.72%	C	HA161021	MF573506	*Streptomyces paucisporeus* CGMCC 4.2025(T) 98.70%	F
HA13708	MF573423	*Micromonospora eburnea* LK2-10(T) 98.88%	C	HA13688	MF573406	*Streptomyces psammoticus* NBRC 13971(T) 98.90%	C/F
HA13618	MF573351	*Micromonospora echinospora* ATCC 15837(T) 99.63%	C	HA161080	MF573519	*Streptomyces puniceus* NRRL ISP-5058(T) 99.35%	C/F
HA13666	KJ467034	*Micromonospora echinospora* ATCC 15837(T) 99.09%	V	HA161220	MF573443	*Streptomyces qinglanensis* 172205(T) 99.88%	C
HA13672	MF573392	*Micromonospora echinospora* ATCC 15837(T) 99.46%	C/V	HA161221	MF573444	*Streptomyces qinglanensis* 172205(T) 100.00%	C
HA13686	KJ467039	*Micromonospora echinospora* ATCC 15837(T) 98.80%	C	HA161270	MF573559	*Streptomyces qinglanensis* 172205(T) 99.87%	C
HA161162	MF573433	*Micromonospora echinospora* ATCC 15837(T) 99.55%	V	HA161119	MF573539	*Streptomyces rameus* LMG 20326(T) 100.00%	—
HA161191	MF573439	*Micromonospora echinospora* ATCC 15837(T) 99.57%	E/C	HA161138	MF573550	*Streptomyces rubiginosohelvolus* NBRC 12912(T) 99.76%	—
HA13637	KJ467027	*Micromonospora halophytica* DSM 43026(T) 98.71%	F	HA161225	MF573447	*Streptomyces sanyensis* 219820(T) 99.87%	C/F/M
HA13588	MF573331	*Micromonospora humi* P0402(T) 99.09%	—	HA161227	MF573449	*Streptomyces sanyensis* 219820(T) 100.00%	C/F
HA13592	MF573334	*Micromonospora humi* P0402(T) 99.40%	—	HA161166	MF573436	*Streptomyces spinoverrucosus* NBRC 14228(T) 98.46%	C
HA13668	MF573391	*Micromonospora humi* P0402(T) 99.04%	—	HA161133	MF573545	*Streptomyces spongiicola* HNM0071(T) 99.85%	—
HA13624	MF573356	*Micromonospora krabiensis* MA-2(T) 99.25%	C	HA161240	MF573459	*Streptomyces sundarbansensis* MS1/7(T) 99.73%	C/M
HA13648	MF573376	*Micromonospora krabiensis* MA-2(T) 100.00%	C	HA161094	MF573530	*Streptomyces sundarbansensis* MS1/7(T) 99.61%	C/M
HA13659	MF573383	*Micromonospora krabiensis* MA-2(T) 99.28%	C	HA161249	MF573467	*Streptomyces thermocarboxydus* DSM 44293(T) 99.87%	C
HA161199	MF573440	*Micromonospora krabiensis* MA-2(T) 99.24%	C	HA161001	MF573491	*Streptomyces thermoviolaceus* subsp. *Thermoviolaceus* DSM 40443(T) 98.40%	M/V
HA13594	MF573335	*Micromonospora marina* JSM1-1(T) 99.55%	C	HA161279	MF573480	*Streptomyces wuyuanensis* CGMCC 4.7042(T) 99.87%	C/F/M
HA13619	KJ467026	*Micromonospora marina* JSM1-1(T) 99.40%	C/V	HA161019	MF573505	*Streptomyces yanglinensis* 1307(T) 98.56%	C/F
HA13625	MF573357	*Micromonospora marina* JSM1-1(T) 99.63%	C/V	HA161029	MF573509	*Streptomyces yanglinensis* 1307(T) 98.70%	C/F
HA13631	MF573363	*Micromonospora matsumotoense* IMSNU 22003(T) 99.51%	C	HA13602	MF573340	*Streptosporangium amethystogenes* subsp.*amethystogenes* DSM 43179(T) 99.25%	—
HA13639	KJ467028	*Micromonospora matsumotoense* IMSNU 22003(T) 98.54%	C/E	HA161205	MF573442	*Tsukamurella sinensis* HKU51(T) 99.76%	—
HA13643	MF573372	*Micromonospora matsumotoense* IMSNU 22003(T) 98.62%	C/E				

注: C: *Colletotrichum gloeosporioides*; E: *Escherichia coli*; F: *Fusarium oxysporum*; M: *Staphylococcus aureus*; V: *Vibrio neocaledonicus*; —: 无拮抗活性

更多的新种资源, 需要尝试不同的样品处理方法和多种培养基。

国内外对于红树林土壤放线菌资源的分离收集已有大量研究(Usha *et al*, 2010; Rosmine *et al*, 2016; Ser *et al*, 2016a, 2016b; Ariffin *et al*, 2017; Arumugam *et al*, 2017; Law *et al*, 2017; Huang *et al*, 2018; Asha *et al*, 2021), 从红树林中分离鉴定的放线菌共有8亚目11科24属(Xu *et al*, 2014)。本研究分离得到的放线菌属于7个目, Micromonosporales (46.48%)、Streptomycetales (42.92%)、Actinomycetales (4.69%)、Streptosporangiales (3.13%)、Corynebacteriales (1.95%)、Frankiales (0.39%)和Micrococcales (0.39%); 9个科; 14个属, *Actinomadura*、*Allostreptomyces*、*Amycolatopsis*、*Blastococcus*、*Micromonospora*、*Jishengella*、*Krasilnikoviella*、*Microbispora*、*Nocardia*、*Nonomuraea*、*Rhodococcus*、*Streptomyces*、*Streptosporangium*和*Tsukamurella*, 其中*Streptomyces* (42.58%)和*Micromonospora* (42.19%)是优势属(表1)。本研究获得的可培养放线菌多样性指数为1.32, 用同样方法对其他地点的红树林土壤可培养放线菌多样性进行了评估, 其中在海南三亚沿海红树林土壤(冯玲玲等, 2018)、海南西海岸真红树根系土壤(候师师等, 2020)、广西茅尾海红树林植物根际土壤(叶景静等, 2018)、印度洋红树林沉积物(何洁等, 2012)、马来西亚红树林土壤(Lee *et al*, 2014)和印度南部沿海红树林土壤(Arumugam *et al*, 2017)中可培养放线菌多样性指数分别为1.74、1.75、1.61、2.16、1.44、0.63, 通过比较可知从印度洋红树林沉积物分离得到的放线菌多样性较高, 可能与选用的培养基种类多有关, 在该研究中选用了24种唯一碳源分离培养基, 可见选用多种不同的培养基对于获得多样的菌株是很重要的, 本研究后续将继续选用其他培养基分离放线菌, 以期获得更多样的种类。通过与非培养水平放线菌多样性对比分析, 利用扩增子高通量测序能检测到更高的多样性(通过放线菌相关引物和细菌通用引物得到的放线菌多样性指数分别为2.96和2.41), 表明与传统培养法相比, 分子方法在发现微生物多样性方面更有效(Li *et al*, 2014)。并非所有培养的细菌都在土壤环境DNA样本中被检测到, 例如属于*Actinomadura*、*Micromonospora*、*Nocardia*和*Streptomyces*的可培养菌株在DNA样本中存在, 但其他10个属, 包括*Allostreptomyces*、*Amycolatopsis*、*Blastococcus*、*Jishengella*、*Krasilnikoviella*、*Microbispora*、*Nonomuraea*、*Rhodococcus*、*Streptosporangium*和*Tsukamurella*, 没有被检测到。在环境DNA中未检测到, 可能与这10个属的丰度低有关系, 导致提取DNA的难度较大。

以往研究表明*Streptomyces*、*Micromonospora*和*Rhodococcus*是海洋沉积物中常被分离到的放线菌(Maldonado *et al*, 2005; Bredholdt *et al*, 2007; Duncan *et al*, 2015; Huang *et al*, 2018)。郑志成等(1989)从红树林根际土壤中分离到的放线菌中*Streptomyces*占75.7%。Eccleston等(2008)从澳大利亚Sunshine Coast红树林土壤中分离到放线菌主要为*Micromonospora*。在本研究中分离到最多的是*Streptomyces*, 其中有菌株(HA161045、HA161164、HA161166、HA161001)与模式菌株的相似性较低(表1), 是潜在新种。本研究还分到大量的稀有放线菌, 如*Micromonospora*、*Actinomadura*、*Allostreptomyces*、*Nocardia*、*Nonomuraea*等。稀有放线菌的分离通常需要预处理或者复杂的富集培养过程(Janssen *et al*, 2002; Jensen *et al*, 2005; Pathom-Aree *et al*, 2006; Bredholdt *et al*, 2007; Solano *et al*, 2009)。例如本研究中大量的*Micromonospora*菌株就是通过将土壤样品进行55 °C水浴后在腐殖酸维生素培养基分离到, 其中包括一些潜在新种(如菌株HA13702、HA13639、HA13643)(表1)。近年来已经发表许多新种, 例如*Streptomyces xiamenensis* (Xu *et al*, 2009)、*Streptomyces colonosanans* (Law *et al*, 2017)、*Streptomyces malaysiense* (Ser *et al*, 2016a)、*Micromonospora rifamycinica* (Huang *et al*, 2008)、*Pseudonocardia nematodicida* (Liu *et al*, 2015)、*Nocardiopsis mangrovei* (Huang *et al*, 2015)。此外, 本研究在非培养水平放线菌多样性分析中得到大量的未分类或者未培养放线菌, 所有以上研究结果表明红树林湿地中分布着大量未被挖掘的放线菌资源, 是发现新的放线菌的关键区域。

2.3　拮抗病原菌活性测定

放线菌因其可产生许多重要的具有生物活性的天然产物, 因此, 仍是生物技术领域最有用的微生物之一(Sharon *et al*, 2014; Xu *et al*, 2014)。本研究对123株代表性菌株进行了病原微生物拮抗活性测定, 其中来自9个属(*Actinomadura*、*Amycolatopsis*、*Blastococcus*、*Krasilnikoviella*、*Microbispora*、*Micromonospora*、*Nocardia*、*Rhodococcus*、*Streptomyces*)的92株被测菌株(占总菌株数的74.8%)

具有病原微生物拮抗活性, 其中对1种病原微生物有拮抗活性的菌株数量是57, 2种的菌株数量是32, 3种的菌株数量是3(分别是菌株HA161225/ HA161279/ HA1362)(表1)。来自*Streptomyces*和*Micromonospora*两个属的大部分菌株对至少1种病原菌是有拮抗活性的, 活性菌株数量分别是32和50。所有被测菌株中有71株(占总菌株数的57.7%)对*Colletotrichum gloeosporioides*有拮抗活性, 25株(占总菌株数的20.3%)对*Fusarium oxysporum*有拮抗活性, 6株(占总菌株数的4.9%)对*Escherichia coli*有拮抗活性, 9株(占总菌株数的7.3%)对*Staphylococcus aureus*有拮抗活性, 19株(占总菌株数的15.4%)对*Vibrio neocaledonicus*有拮抗活性。

来自*Streptomyces*的成员可产生天然抗生素(Kinkel *et al*, 2014; Ser *et al*, 2016a)和多种具有抗菌、抗癌、抗氧化和免疫抑制活性的化合物(Rashad *et al*, 2015; Ser *et al*, 2016b; Law *et al*, 2017;候师师等, 2020)。已有研究报道, 红树林放线菌中*Streptomyces*是天然产物的最主要来源, 其次是*Micromonospora* (Xu *et al*, 2014), 本研究也得到相似的结果, 作为天然产物主要来源的红树林放线菌已成为天然药物研究的热点。此外, 来自*Amycolatopsis*和*Nocardia*两个属的菌株具有拮抗*Colletotrichum gloeosporioides*和*Fusarium oxysporum*的活性, 类似结果在以前的研究中也有报道(Singh *et al*, 2007; Zhang *et al*, 2008; 奚逢源等, 2014)。来自以下属*Actinomadura*、*Blastococcus*、*Krasilnikoviella*和*Microbispora*的抗菌活性以往未见报道, 在本研究中检测到了病原菌拮抗活性, 可用于后续菌株的开发利用。本研究中来自*Allostreptomyces*、*Jishengella*、*Nonomuraea*、*Streptosporangium*和*Tsukamurella*的菌株没有检测到抗病原菌活性。由于抗菌活性物质的产生受到培养基、pH值和培养温度等因素的影响, 因此, 下一步将研究不同培养条件下菌株的抗菌活性检测, 以便为菌株开发利用提供前期基础数据支撑。

3 结论

本研究在培养水平和非培养水平分别对八门湾红树林湿地土壤中放线菌的多样性及其抗病原菌活性进行了分析。对于非培养水平多样性, 与细菌通用引物相比, 利用放线菌相关引物可以提高放线菌丰度的检测水平, 可以检测到放线菌门更多的目、科和属; 优势类群在两对不同引物得到的结果中的百分含量差异较大; 放线菌相关引物更适合环境样品中放线菌多样性的分析。本研究获得的可培养放线菌多样性指数为1.32, *Streptomyces* (42.58%)和*Micromonospora* (42.19%)是优势属; 与模式菌株的相似性小于98.5%有13株, 是潜在新种。与传统培养法相比, 基于分子生物学的方法可以检测到更高的微生物多样性。来自9个属的92株(74.8%)代表性菌株具有抗病原菌活性, 其中对*Colletotrichum gloeosporioides*、*Fusarium oxysporum*、*Escherichia coli*、*Staphylococcus aureus*和*Vibrio neocaledonicus*有拮抗活性的菌株数量分别为71、25、6、9和19株。

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DIVERSITY AND ANTIMICROBIAL ACTIVITY OF ACTINOBACTERIA IN THE SOIL OF THE BAMENWAN MANGROVE IN HAINAN, CHINA

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Abstract　Mangrove is recognized a rich source of new Actinobacteria strains. To obtain more Actinobacteria for development of new bioactive compounds, it is important and necessary to understand their diversity. The diversity at culture-independent and culture-dependent level of Actinobacteria in the Bamenwan mangrove soil (Hainan, South China) was investigated by using amplicon high-throughput sequencing and traditional culture methods. The antimicrobial activities of representative isolates were characterized. For the diversity at culture-independent level, compared with bacterial universal primers, the detection level of actinobacterial abundance can be improved using Actinobacteria-related primers by 2.47 times in percentage. More orders, families, and genera of actinomycetes can be detected. Acidimicrobiales, Corynebacteriales, Gaiellales, Kineosporiales, Solirubrobacterales are the dominant orders, but their percentage contents are quite different in the results obtained by two pairs of different primers. Actinobacteria-related primers are more suitable for the analysis of actinobacterial diversity in environmental samples. For the diversity at culture-dependent level, 256 strains of actinobacterial strains were isolated, being affiliated to 7 orders, 9 families, and 14 genera. The Shannon-Wiener diversity index was 1.32. *Streptomyces* (42.58%) and *Micromonospora* (42.19%) were the dominant genera. The similarities of 13 strains with the type-strain were below 98.5%. Among them, strains HA161004 and HA161010 were the closest to *Amycolatopsis thermoflava* N1165^{T} with similarities of 95.56% and 96.80%, respectively, indicating that both were potentially novel strains of species. The determination of antagonistic activity of pathogenic microorganisms showed that 92 representative strains from 9 genera had antimicrobial activity, among which 71, 25, 6, 9, and 19 strains had antagonistic activity against *Colletotrichum gloeosporoides*, *Fusarium oxysporum*, *Escherichia coli*, *Staphylococcus aureus*, and *Vibrio neocaldonicus*, respectively. This study provides data support for selecting more appropriate primers for the actinobacterial diversity, and supplies strain resources for the collection, activity evaluation, and utilization of Actinobacteria in mangrove.

Key words　Actinobacteria;　diversity;　antimicrobial activity;　mangrove

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附表 1、附表 2 见 http://dx.doi.org/10.11693/hyhz20210800176