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研究报告

三种杓兰根相关真菌多样性和生态功能

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摘　要：【背景】除了菌根真菌(Orchid mycorrhizal fungi，OrMF)外，兰科植物根中还有其它内生真菌，称为根相关真菌(Root-associated fungi，RAF)。【目的】采用分离培养的方法获得同一栖息地针叶林和灌木林两种不同生境西藏杓兰、黄花杓兰和无苞杓兰的 RAF 菌株，研究其真菌谱系、多样性和生态功能结构。【方法】从杓兰根碎屑中分离 RAF，通过总 DNA 提取、PCR 扩增及测序得到 ITS (Internal transcribed spacer)序列；进行系统发育和多样性分析，并通过 NCBI 数据库比对得到相似性最高序列的注释信息来分析 RAF 生态学特性。【结果】共分离得到 278 株 RAF，25 种 OTU 类型，包括 23 个子囊菌门 OTU，2 个毛霉菌门 OTU。RAF 物种丰富度分析发现西藏杓兰的较黄花杓兰高，不同生境没有显著差异；不同杓兰物种较不同生境的 RAF 群落分化程度高。生态功能分析显示 25 个 OTU 包括共生型、腐生型和致病型 3 种营养型，以及外生菌根菌群、植物病原菌群、内生真菌群、动物病原菌群、真菌寄生菌群、杜鹃花类菌根群、未定义的腐生菌群和不确定型 8 种共位群。【结论】阐明不同生境采集的不同杓兰中 RAF 的分布特点和生态功能，为未来研究 RAF 与杓兰属植物的共生关系奠定基础。

关键词：杓兰属，内转录间隔(ITS)区，营养型，共位群

Diversity and ecological function of root-associated fungi in three *Cypripedium* species

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Abstract: [Background] In addition to orchid mycorrhizal fungi, the roots of orchids harbor plant fungal endophytes termed root-associated fungi. **[Objective]** Three endangered photosynthetic *Cypripedium* species distributed in coniferous forest and shrub habitats were screened for root-associated fungi using culture-dependent (isolations from root fragments) techniques. The species richness and the degree of

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root-associated fungi community differentiation of examined *Cypripedium* species and two different habitats were determined. Ecological function analysis of root-associated fungi was also estimated. **[Methods]** RAF were isolated from surface sterilized root fragments of orchids. Total DNA were extracted from isolated root-associated fungi, and internal transcribed spacer (ITS) regions were amplified. The ITS-PCR products were sequenced. Phylogenetic analysis was applied. Species richness and diversity of fungal communities of studied orchid species and collecting sites were estimated. After blasting the ITS sequences of root-associated fungi in NCBI database, the annotation of the closest matched sequences were used to analyse the ecological function. **[Results]** 278 root-associated fungi isolates, corresponding to 25 operational taxonomic units (OTUs), were identified, including 23 *Ascomycota* OTUs and 2 *Mucoromycota* OTUs. The root-associated fungi species richness of *Cypripedium tibeticum* was higher than that of *Cypripedium flavum*. The degree of root-associated fungi community differentiation of different *Cypripedium* species is bigger than that of different habitats. Fungal taxa in the roots of the three *Cypripedium* species could be assigned to 3 trophic modes, which were symbiotroph, saprotroph and pathotroph, respectively; and 8 guilds, which were ectomycorrhizal, plant pathogen, endophyte, animal pathogen, fungal parasite, ericoid mycorrhizal, undefined saprotroph and uncertain, respectively. **[Conclusion]** This study revealed the distribution characteristics and ecological function of RAF in roots of three *Cypripedium* species sampled from two different habitats, and also laid a foundation for the symbiotic relationship study of root-associated fungi and *Cypripedium* species in the future.

Keywords: *Cypripedium*, Internal transcribed spacer (ITS) region, Trophic mode, Guild

兰科植物(*Orchidaceae*)是被子植物中种类最丰富的一个科，是植物中较为进化的类群之一[1]。兰科植物根中普遍存在着内生真菌,参与兰科植物种子萌发和植物生长发育的整个生活史[2-3]。兰科植物根中的内生菌包括参与菌根形成的菌根真菌(Orchid mycorrhizal fungi，OrMF)[4]和非 OrMF 的内生菌，又称为根相关真菌(Root-associated fungi，RAF)[5]。RAF 在兰科植物中不形成典型解剖学特征，也不引起任何疾病症状[6-7]，与已知 OrMF 没有系统发育关系[5,8-10]且数量远大于 OrMF[8,11]，总类群超过 110 属，其中大约 76 属属于子囊菌[12]。在温带地生兰研究中，RAF 谱主要由柔膜菌目(*Helotiales*)相关真菌主导[5,8-10,13]，附生兰的根也被不同 RAF 定殖[6,14-16]。RAF 主要存在于根被(Velamen)中[14,17-18]，可以促进兰科植物吸收根际土壤养分[19-20]，还对次级代谢物的产量或变化有影响[21]，也被认为是保护宿主免受土壤病原体侵害的生物活性化合物资源[12]。

杓兰属(*Cypripedium*)植物是多年生地生型兰科植物，中国是杓兰属植物的分布中心。目前已经报道杓兰属植物 RAF 有球囊霉属(*Glomus*)、光柄菇属(*Pluteus*)、层孔菌属(*Fomes*)、伏革菌属(*Corticium*)等类群[22-23]。随着高通量测序技术在兰科植物内生真菌研究中的应用，柔膜菌目(*Helotiales*)、肉座菌目(*Hypocreales*)和炭角菌目(*Xylariales*)[24]的一些类群也在杓兰属植物中发现。杓兰属植物因为分布的海拔相对较高，难以实现迁地保护，这就给保育工作带来了挑战。因此，研究不同生境不同杓兰 RAF 多样性，尤其是获得该菌株对进一步了解杓兰属植物与 RAF 互作机制非常重要，更能为杓兰属植物的保育工作奠定基础。但目前还缺少不同生境和不同杓兰中 RAF 分布规律以及生态功能的研究。

随着对 RAF 研究和认识的深入，海量核酸数据大量产生，如何鉴定与宿主互作真菌分类单元的生态学特征与功能成为研究热点[25]。目前，FUNGuild 软件[26]被广泛应用于 RAF 群落功能的注释，有助于 RAF 群落功能在营养型(Trophic mode)和共位群(Guild)层次上的多样性研究。

四川黄龙自然保护区地处四川省松潘县境内，其主要景区是黄龙沟。黄龙沟拥有 1.2 km 长的钙华滩流，属典型的高原温带-亚寒带季风气候，非常适合兰科植物生长[27]。本研究采用分离培养的方法从黄龙沟两种生境采集的 3 种杓兰根中分离

到根相关真菌(RAF)，分析不同杓兰和不同生境RAF物种丰富度(α多样性)和群落结构分化程度(β多样性)以及RAF生态功能，以期有利于了解杓兰属植物RAF区系和生态学意义，为今后开展RAF与杓兰属植物互作机制研究和保育工作奠定基础。

1 材料与方法

1.1 样品采集

采集的西藏杓兰(*Cypripedium tibeticum*)、黄花杓兰(*Cypripedium flavum*)和无苞杓兰(*Cypripedium bardolphianum*)分布在黄龙沟海拔3 170 m–3 400 m。该采样地区根据主要植被不同分为两种生境，分别是海拔3 170 m–3 260 m针叶林和海拔3 260 m–3 400 m的灌木林。杓兰主要生长于针叶林下苔藓上和灌木丛周围。在针叶林和灌木林分别随机设置9和12个大小为10 m×10 m的样方。西藏杓兰、黄花杓兰和无苞杓兰分别在21、10和19个样方中有分布(表1)。于2015年6月3种杓兰都处于花期时采集根。每个样方根据3种杓兰的分布情况采集每种杓兰各5株的根，每个植株采集3个根。因为采集的根数没有超过杓兰根总数的1/3，所以不会影响杓兰生长。就地采集苔藓包裹的根，回到住地后于4 °C冰箱中保存，10 d内带回实验室进行RAF分离实验。

1.2 主要试剂和仪器及培养基

基因组提取试剂盒和PCR反应试剂盒，天根生化科技(北京)有限公司；无水乙醇、NaClO、硫酸链霉素和青霉素钾，天津市致远化学试剂有限公司；PDA培养基，BD Difco公司。样品组织研磨仪，Qiagen公司；PCR扩增仪和电泳仪，Bio-Rad公司；紫外凝胶成像系统，UVP公司。

1.3 RAF分离

将采集的根用自来水冲洗干净，用70%酒精浸泡1 min，20% NaClO (0.5%有效氯)浸泡4 min，无菌水冲洗4–5次。在无菌培养皿中加少量无菌水，用无菌镊子夹取一小段冲洗好的根放入培养皿中，用解剖刀将根粉碎。用镊子夹取植物根碎屑，无菌水漂洗3次后接种到准备好的含双抗(50 mg/L硫酸链霉素和50 mg/L青霉素钾)的1/4 PDA培养基上，每个培养基接种6–10个根的碎屑，用封口膜封好培养皿倒置于恒温培养箱中25 °C恒温暗培养两个月。每隔2 d观察是否有菌丝长出。用接种针挑取菌落边缘部分转接到PDA培养基培养，转接3–4次至菌丝形成纯培养物，再转接到铺有灭菌玻璃纸的培养基上培养，用于后续DNA提取。

1.4 DNA提取、PCR扩增及测序

DNA提取后进行PCR扩增，引物采用ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′)和ITS4 (5′-TCCTCCGCTTATTGATATGC-3′)。25 μL PCR反应体系：模板DNA 1 μL，ITS5和ITS4引物(10 μmol/L)各1 μL，2×*Taq* PCR MasterMix 12.5 μL，ddH$_2$O 9.5 μL。PCR反应条件：94 °C 3 min；95 °C 40 s，56 °C 40 s，72 °C 1 min，35个循环；72 °C 10 min。将PCR产物送生工生物工程(上海)股份有限公司测序，根据扩增引物的类型采用ITS5作为测序引物。代表性序列提交NCBI GenBank数据库获得登录号，在NCBI数据库中进行比对，相似性最高的序列作为代表性序列注释信息(表2)。

表1 不同杓兰在两种生境中的分布

Table 1 The distribution of different *Cypripedium* species in two habitats

Orchid species	Coniferous forest		Shrub	
	Number of quadrat with orchid distribution	Number of roots	Number of quadrat with orchid distribution	Number of roots
Cypripedium tibeticum	9	135	12	180
Cypripedium flavum	8	105	2	30
Cypripedium bardolphianum	9	135	10	150

表 2 **RAF ITS 序列分析、真菌生态型和宿主来源**

Table 2 ITS sequence identity, ecological type and host plant species of RAF

OTU	Representative sequences	Closest match in GenBank (accession number)	Sequence identity (%)	Order	Ecological characteristic			Number of fungi isolated from different hosts		
					Trophic mode	Guild	Confidence ranking	*Cypripedium tibeticum*	*Cypripedium flavum*	*Cypripedium bardolphianum*
1	HLIO15_21a_35^{A} (MH730191)	*Truncatella angustata* (MG098315.1)	100	*Xylariales*	Pathotroph-Symbiotroph	Ectomycorrhizal-Endophyte-Plant pathogen	Highly probable	141	0	31
2	HLIO15_16c_14 (MH730182)	*Leptodontidium orchidicola* (JX630667.1)	99	*Helotiales*	Symbiotroph	Endophyte	Highly probable	19	0	35
3	HLIO15_1c_12 (MH730170)	*Ciboria americana* (DQ431172.1)	100	*Helotiales*	Pathotroph	Plant pathogen	Probable	0	0	1
4	HLIO15_17a_35 (MH730185)	*Botrytis cinerea* (KT898761.1)	99	*Helotiales*	Pathotroph-Saprotroph	–	Probable	1	0	0
5	HLIO15_1a_12 (MH730167)	*Helotiales* sp. (HG764575.1)	99	*Helotiales*	–	–	–	2	0	0
6	HLIO15_2c_16 (MH730172)	*Helotiales* sp. (JN859265.1)	99	*Helotiales*	–	–	–	1	0	2
7	HLIO15_1b_6 (MH730169)	*Cryptosporiopsis* sp. (KX377690.1)	99	*Helotiales*	Saprotroph	Undefined saprotroph	Probable	0	1	0
8	HLIO15_12c_1 (MH730178)	*Cryptosporiopsis* sp. (LT821484.1)	100	*Helotiales*	Saprotroph	Undefined saprotroph	Probable	1	1	1
9	HLIO15_16c_15 (MH730183)	*Leptosphaeria* sp. (KU516518.1)	99	*Pleosporales*	Pathotroph	Plant pathogen	Probable	0	0	2
10	HLIO15_16c_12 (MH730181)	*Leptosphaeria* sp. (GU288818.1)	100	*Pleosporales*	Pathotroph	Plant pathogen	Probable	1	2	3
11	HLIO15_21a_30 (MH730190)	*Alternaria tenuissima* (MH594736.1)	100	*Pleosporales*	Pathotroph-Saprotroph-Symbiotroph	Animal pathogen-Endophyte-Plant pathogen-Wood saprotroph	Possible	1	0	2
12	HLIO15_20a_2 (MH730187)	*Alternaria* sp. (MH473886.1)	100	*Pleosporales*	Pathotroph-Saprotroph-Symbiotroph	Animal pathogen-Endophyte-Plant pathogen-Wood saprotroph	Possible	1	0	0
13	HLIO15_20a_12 (MH730188)	*Didymella glomerata* (MG832567)	99	*Pleosporales*	Pathotroph-Saprotroph	Animal pathogen-Plant pathogen-Undefined saprotroph	Probable	2	0	0

(待续)

(续表 2)

14	HLIO15_6b_12 (MH730175)	*Peyronellaea prosopidis* (MH866094.1)	99	*Pleosporales*	Saprotroph	Undefined saprotroph	Probable	0	1	0
15	HLIO15_17a_12 (MH730184)	*Epicoccum nigrum* (KX074010.1)	100	*Pleosporales*	Pathotroph	Fungal parasite-Plant pathogen	Probable	1	0	0
16	HLIO15_8b_16 (MH730177)	*Penicillium granulatum* (MH393382.1)	100	*Eurotiales*	Saprotroph	Undefined saprotroph	Possible	2	1	0
17	HLIO15_14c_16 (MH730179)	*Oidiodendron griseum* (KX440150.1)	99	*Helotiales*	Pathotroph-Symbiotroph	Ericoid mycorrhizal	Probable	0	0	1
18	HLIO15_17c_20 (MH730186)	*Podospora* sp. (HQ829072.1)	100	*Sordariales*	Saprotroph-Symbiotroph	Dung saprotroph-Endophyte-Litter saprotroph-Undefined saprotroph	Possible	0	0	1
19	HLIO15_15a_49 (MH730180)	*Mucor hiemalis* (MF186043.1)	100	*Mucorales*	Saprotroph	Undefined saprotroph	Probable	1	0	0
20	HLIO15_3a_1 (MH730173)	*Mucor hiemalis* (KM668138.1)	100	*Mucorales*	Saprotroph	Undefined saprotroph	Probable	13	0	0
21	HLIO15_4a_51 (MH730174)	*Pleurostomophora richardsiae* (KF525814.1)	91	*Calosphaeriales*	Saprotroph	Undefined saprotroph	Probable	1	0	0
22	HLIO15_8a_11 (MH730176)	*Diaporthe arctii* (NR_147544.1)	98	*Diaporthales*	Pathotroph-Symbiotroph	Endophyte-Plant pathogen	Possible	1	0	0
23	HLIO15_1a_31 (MH730168)	*Cladosporium herbarum* (KY039306.1)	100	*Capnodiales*	Pathotroph-Saprotroph-Symbiotroph	Animal pathogen-Endophyte-Lichen Parasite-Plant Pathogen-Wood saprotroph	Possible	2	0	0
24	HLIO15_2a_1 (MH730171)	*Elaphocordyceps* sp. (KF753860.1)	99	*Hypocreales*	Pathotroph	Fungal parasite	Highly probable	1	0	0
25	HLIO15_20a_21 (MH730189)	*Fusarium oxysporum* (MF162321.1)	100	*Hypocreales*	Pathotroph-Saprotroph	Plant pathogen-Soil Saprotroph-Wood saprotroph	Possible	1	0	0

注：[A]：该菌株已经鉴定到种(待发表). 通过收集该物种报道过的相关文献[36-39]确定了其营养型和共位群，不同于其它 OTU 是通过 FUNGuild 进行生态功能分析得到结果.

Note: [A] refers to the taxon of this OTU was identified as *Truncatella angustata* (unpublished). The trophic mode and guild of *Truncatella angustata* were determined by directly referring to related papers [36-39]. However, ecological characteristics of the other OTU were annotated by FUNGuild.

1.5 OTU 的划分

在微生物生态学研究中，常用 97%的相似度划分可操作分类单元(Operational taxonomic unit，OTU)表示物种信息，该方法在兰科植物 RAF 的研究中得到了广泛应用[28-29]。本研究使用 Uparse 软件将序列按照 97%的一致性进行聚类划分 OTU，基于划分后的 OTU 统计得到每个样本中的 OTU 丰度。

1.6 系统发育树的构建

用 Modeltest 3.7 中的 modelblockPAUPb10 模块进行最佳模型检验。MrBayes 3.1.2 构建贝叶斯系统发育树。参数如下：Nst=6，Rates=invgamma，显示的代数(Printfreq)为 1 000，取样代数(Sample freq)为 1 000，马尔可夫链运行链数(Nchains)为 4，舍弃(Burnin)掉前 3 棵树(前 2 000 代)。用软件 Tracer Version 1.4 打开 MrBayes 运行结果中的 P 文件来评价各参数是否合理以及结果是否达到稳态。

1.7 不同生境和不同杓兰 RAF 物种丰富度、群落分化程度分析

用 3 个非参数估计量(Non-parametric estimators)：Chao1、First-order Jacknife 1 (Jack 1)和 Bootstrap[30-33]对不同生境和不同杓兰 RAF 物种丰富度(α 多样性)进行估算。用 SPSS 19 软件进行卡方检验(χ^2)比较不同生境和不同杓兰中 RAF 群落丰富度差异是否显著。用 Jaccard 和 Sørensen 指数[34-35]比较不同生境和不同杓兰 RAF 群落分化程度(β 多样性)。两个指数都是基于两个群落共享的 OTU 物种数量除以每个群落独特的 OTU 物种数量。Jaccard 和 Sørensen 指数值在 0−1 之间，数值越大群落相似度越高，越接近 0 差异越大。

1.8 生态功能群分析

用 FUNGuild 数据库[26]解析杓兰根相关真菌的生态营养型(Trophic mode)和共位群(Guild)。置信水平仅选用可能(Probable)和很可能(Highly probable)的 OTU 及其类别。因为采用的是分离培养的方法，所以将所有的 OTU 都进行了分析。OTU1 已经鉴定为截盘多毛孢(*Truncatella angustada*) (待发表)，因此通过查阅有关截盘多毛孢的文献来确定其生态营养型和共位群[36-39]。

2 结果与分析

2.1 RAF 谱系分析

黄龙沟采集的 3 种杓兰属植物根中共分离出 RAF 菌株 278 株，划分为 25 个 OTU。涉及 2 个门 5 个纲 10 个目。门的水平包括了子囊菌门(*Ascomycota*)和毛霉菌门(*Mucoromycota*)。其中，子囊菌门包括 23 个 OTU，分别是粪壳菌纲(*Sordariomycetes*)的炭角菌目(*Xylariales*)、美球菌目(*Calosphaeriales*)、间座壳菌目(*Diaporthales*)、粪壳菌目(*Sordariales*)各 1 个 OTU，肉座菌目(*Hypocreales*) 2 个 OTU；锤舌菌纲(*Leotiomycetes*)只有柔膜菌目(*Helotiales*)，但有 8 个 OTU；散囊菌纲(*Eurotiomycetes*)散囊菌目(*Eurotiales*) 1 个 OTU；座囊菌纲(*Dothideomycetes*)煤炱目(*Capnodiales*) 1 个 OTU，格孢腔菌目(*Pleosporales*) 7 个 OTU。毛霉菌门包括毛霉纲(*Mucoromycetes*)毛霉目(*Mucorales*) 2 个 OTU。在纲的水平上，锤舌菌纲和座囊菌纲为 OTU 种类的最多类群，各占 32%，粪壳菌纲占 24%，毛霉纲为 8%，散囊菌纲为 4%。在目的水平上柔膜菌目(*Helotiales*)为 OTU 种类的优势类群，占 32%；其次是格孢腔菌目，占 28%；毛霉目和肉座菌目均为 8%，炭角菌目、美球菌目、间座壳菌目、粪壳菌目、散囊菌目和煤炱目均为 4% (表 2)。

OTU1 截盘多毛孢(*Truncatella angustada*)为优势 OTU，占总菌株数的 61.9%；次优势 OTU 为(*Leptodontidium*)属真菌，占总菌株数的 19.4% (表 2)。在西藏杓兰、黄花杓兰和无苞杓兰中 RAF 的 OTU 种类分别为 19、5 和 10 个，菌株数分别为 193、6 和 79 株。基于真菌 ITS 序列构建的贝叶斯系统发育树(图 1) (最佳模型为 GTR+I+G)表明，24 个 OTU 与 NCBI 数据库中相似性最高的代表性序列的一致性≥97%，分别隶属于截盘多毛孢属(*Truncatella*)、*Leptodontidium*、杯盘菌属(*Ciboria*)、*Botrytis*、*Cryptosporiopsis*、小球腔菌属

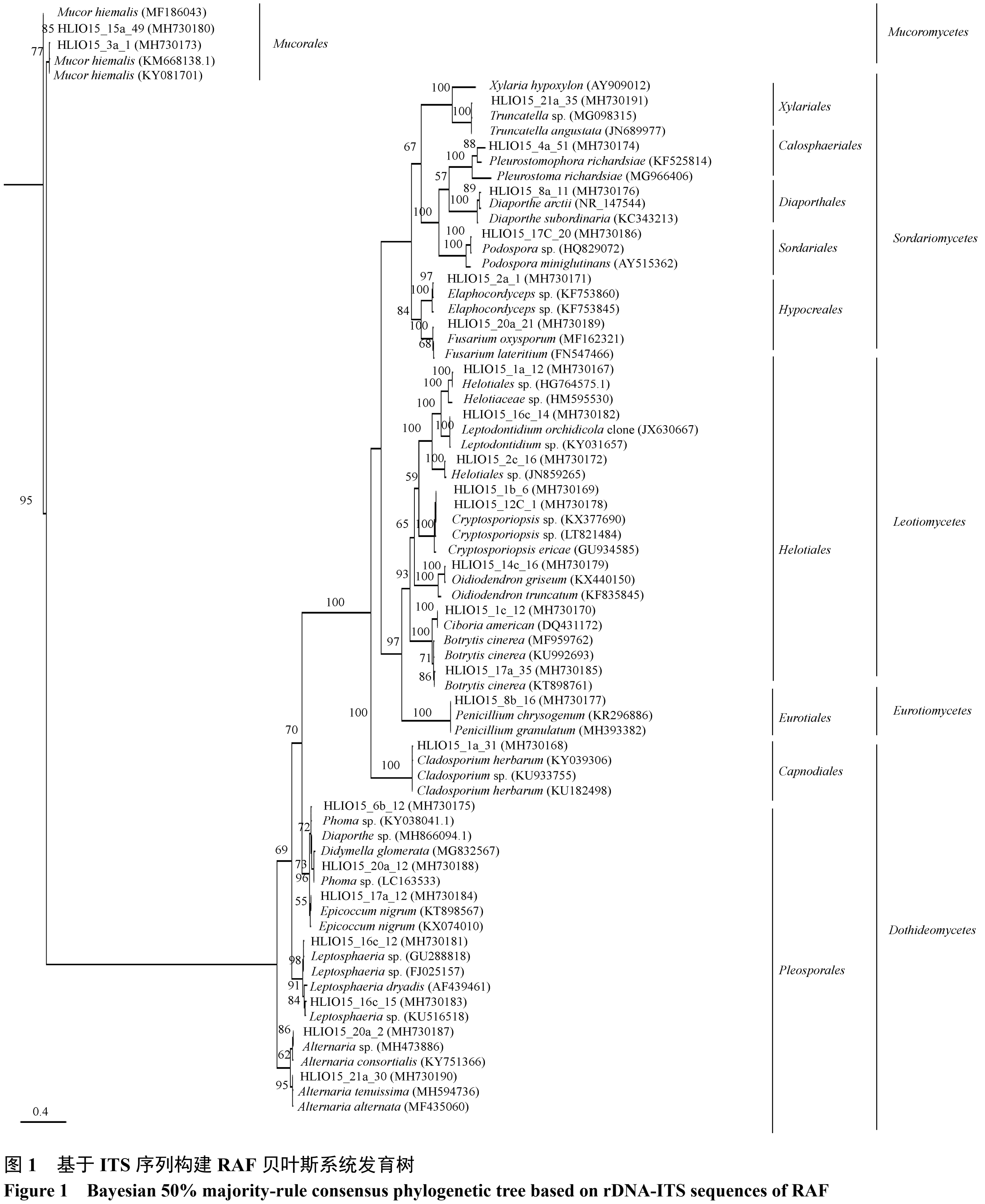

图 1 基于 ITS 序列构建 RAF 贝叶斯系统发育树

Figure 1 Bayesian 50% majority-rule consensus phylogenetic tree based on rDNA-ITS sequences of RAF

注：*Mucor hiemalis* 作为外群；节点处标注的是>50%的后验概率值；比例尺代表替换率为 0.4.

Note: *Mucor hiemaliss* were used as outgroup; Posterior probability values>50% were shown above nodes; The scale bar represents 0.4 substitutions/site.

(*Leptosphaeria*)、链格孢属(*Alternaria*)、*Didymella*、派伦霉属(*Peyronellaea*)、附球菌属(*Epicoccum*)、青霉属(*Penicillium*)、树粉孢属(*Oidiodendron*)、柄孢壳属(*Podospora*)、毛霉属(*Mucor*)、间座壳属(*Diaporthe*)、枝孢属(*Cladosporium*)、*Elaphocordyceps* 和镰刀菌属(*Fusarium*)；OTU5 和 OTU6 代表性序列只能确定为柔膜菌目真菌(图 2，表 2)。只有 OTU21 与代表性序列 *Pleurostomophora richardsiae* (KF525814.1)相似性<97%，为 91%，由于其在系统发育树中聚为同一支系，推断 OTU21 可能为 *Pleurostomophora* 属真菌，还需要进一步研究。

2.2 不同生境和不同杓兰 RAF 物种丰富度、群落分化程度

针叶林和灌木林两种不同生境相比，Chao1、Jack1 和 Bootstrap 都显示灌木林的 RAF 物种丰富度较针叶林高(表 3)，但两者之间差异不显著($P<0.05$，表 4)。两种生境之间群落差异比较结果表明 Jaccard 和 Sørensen 指数分别为 0.36 和 0.53 (表 5)，说明两者 RAF 群落有一定程度的分化但不明显。

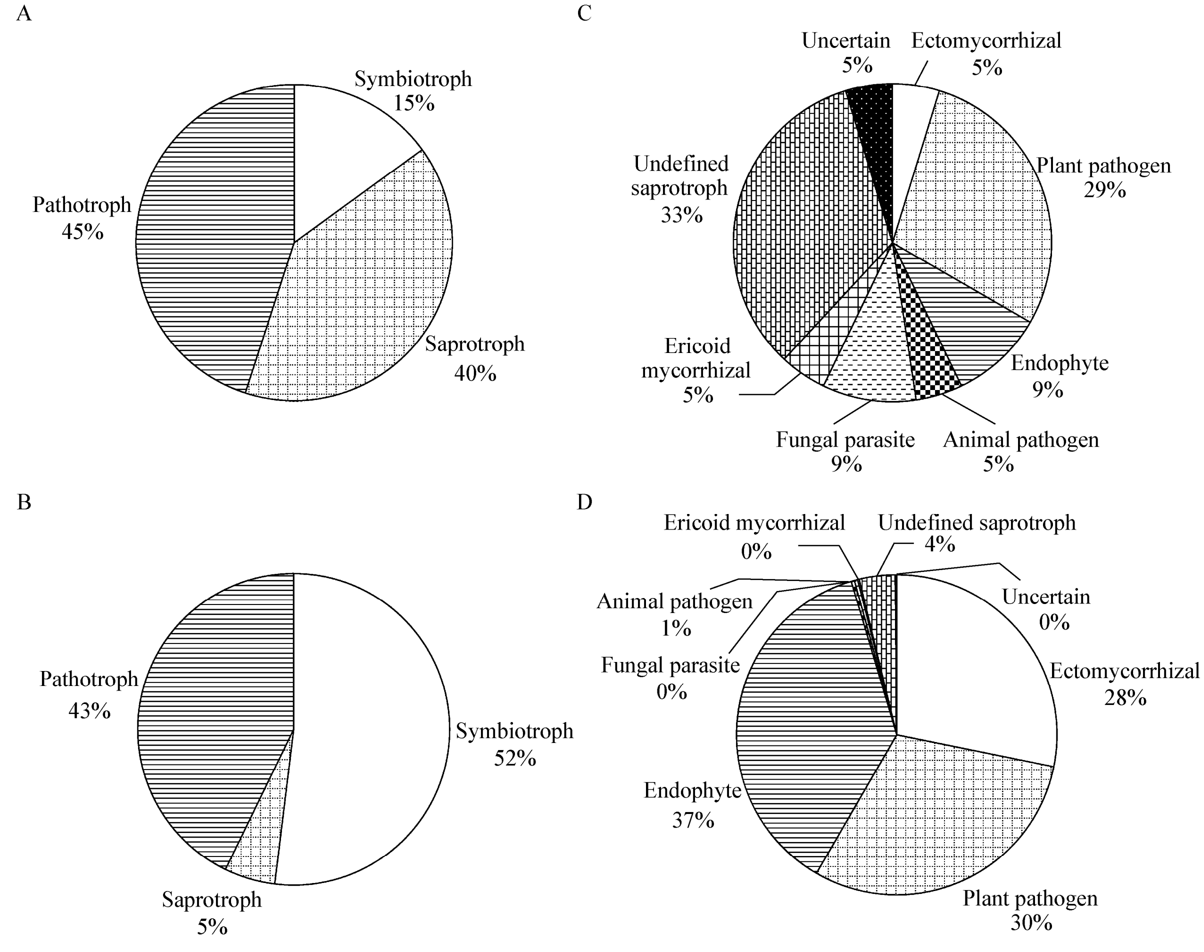

图 2 RAF 群落中不同营养型、共位群真菌 OTU 数百分比与菌株数百分比

Figure 2 Percentage of species and isolates in different trophic mode and guild of RAF

注：A：不同营养型真菌 OTU 数百分比；B：不同营养型真菌菌株数百分比；C：不同共位群真菌 OTU 数百分比；D：不同共位群真菌菌株数百分比. 0%表示占比<0.5%.

Note: A: Percentage number of OTU grouped by trophic mode; B: Percentage number of fungal isolates grouped by trophic mode; C: Percentage number of OTU grouped by guild; D: Percentage number of fungal isolates grouped by guild. 0% means proportion<0.5%.

表 3　不同生境和不同杓兰 RAF 物种丰富度

Table 3　Estimators of RAF species richness in two different habitats and studied orchid species

Category	Number of fungal OTUs	Number of isolates	Chao1	Jack1	Bootstrap
Coniferous forest	16	267	23.85±7.35	23.85±2.78	19.45±1.43
Shrub	18	258	33.04±12.40	28.94±3.30	22.58±1.70
Cypripedium tibeticum	19	193	36.90±14.31	30.93±3.44	23.94±1.76
Cypripedium flavum	5	6	11.67±9.78	8.33±1.67	6.43±0.75
Cypripedium bardolphianum	10	79	12.62±3.43	13.93±1.96	11.89±1.10

表 4　卡方检验比较 RAF OTU 数量

Table 4　Comparison of numbers of RAF OTU using chi-square test (χ^2)

Pairwise comparison	Number of OTUs			
	N	DF	χ^2	*P*-value
Coniferous forest vs. shrub	16 vs. 18	1	0.118	0.732
Cypripedium tibeticum vs. *Cypripedium flavum*	19 vs. 5	1	8.167	0.004
Cypripedium tibeticum vs. *Cypripedium bardolphianum*	19 vs. 10	1	2.793	0.095
Cypripedium flavum vs. *Cypripedium bardolphianum*	5 vs. 10	1	1.667	0.197

表 5　不同生境和不同杓兰 RAF 群落相似性指数

Table 5　Indices of similarity among RAF communities of different habitats and *Cypripedium* species

Pairwise comparison	Number of shared OTUs	Jclas	Sclas
Coniferous forest vs. shrub	9	0.36	0.53
Cypripedium tibeticum vs. *Cypripedium flavum*	3	0.14	0.25
Cypripedium tibeticum vs. *Cypripedium bardolphianum*	6	0.26	0.41
Cypripedium flavum vs. *Cypripedium bardolphianum*	2	0.15	0.27

Chao1、Jack1和Bootstrap都显示3种杓兰RAF物种丰富度由高到低分别为西藏杓兰(19个OTU)、无苞杓兰(10个OTU)和黄花杓兰(5个OTU) (表3)。但只有西藏杓兰和黄花杓兰之间物种丰富度差异显著($P<0.05$，表 4)。3 种杓兰两两之间群落差异比较结果表明 Jaccard 指数和 Sørensen 指数都不高(表 5)，其中 Jaccard 指数在 0.14−0.26，Sørensen 指数在 0.25−0.41，说明两两杓兰之间的 RAF 群落差异较大，分化程度较高。

2.3　杓兰 RAF 生态学特征

用 FUNGulid 数据库进行 OTU 生态功能注释显示，25 种 RAF OTU 中，置信水平为可能(Probable)和很可能(Highly probable)的有 4 个纲 6 个目的 16 个 OTU (表 2)，分别是粪壳菌纲炭角菌目 1 个 OTU，煤炱目 1 个 OTU，肉座菌目 1 个 OTU；锤舌菌纲柔膜菌目 6 个 OTU；座囊菌纲格孢腔菌目 5 个 OTU；毛霉纲毛霉目 2 个 OTU (表 2)。这 16 种 OTU 属于 3 个营养型，分别是共生型(Symbiotroph)、腐生型(Saprotroph)和致病型(Pathotroph) (表 2)，OTU 种类占比分别为 15%、40%和 45% (图 2A)，菌株数所占比例分别为 52%、5%和 43% (图 2B)；8 种共位群，分别是外生菌根菌群(Ectomycorrhizal)、植物病原菌群(Plant pathogen)、内生真菌群(Endophyte)、动物病原菌群

(Animal pathogen)、真菌寄生菌群(Fungal parasite)、杜鹃花类菌根群(Ericoid mycorrhizal)、未定义的腐生菌群(Undefined saprotroph)和不确定型(Uncertain) (表 2)，OTU 种类占比分别为 5%、29%、9%、5%、9%、5%、33%和 5% (图 2C)，菌株数所占比例分别为 28.2%、30.2%、37%、0.3%、0.3%、0.2%、3.6%和 0.2% (图 2D)。

3 讨论与结论

本研究从四川黄龙沟采集的 3 种杓兰属植物根中直接分离可培养的 RAF，尽管采用的是分离培养的方法，但得到的 RAF OTU 种类和生态功能类型较为丰富。这与已报道的兰科植物 RAF 不仅谱系多样性高，而且涉及多种生态类型真菌[5,8-10,13]结果一致。子囊菌是本研究 RAF 的优势类群，也与已有研究报道一致[5,8,14,16]。粪壳菌纲的炭角菌目是杓兰(*Cypripedium calceolus*[24])和其它兰科植物[5-6,8,15]内生菌的常见类群。本研究中只分离到一种炭角菌目真菌，即截盘多毛孢(*Truncatella angustata*)，在所有类型中占绝对优势，该物种多为植物内生菌或病原菌[36-37,39]，也有报道为外生菌根真菌[38]。虽然截盘多毛孢在兰科植物中未见报道，但其另外两个近缘种 *Pestalotiopsis bicilliata* 和 *Neopestalotiopsis clavispora* 在其它兰科植物根中有被分离到[8]，也是常见的植物内生真菌或病原菌[40-43]。

柔膜菌目的 OTU 种类最多，为 8 个，菌株数量仅次于截盘多毛孢，共计 66 株(23.7%)，具有 3 种营养型和多种共位群。柔膜菌目成员是温带或地中海地区光合地生兰中 RAF 的优势类群[13]，共生型[44]、腐生型[13]和致病型[45]的类群都有，可以栖息在土壤、植物甚至是水中[46-47]。本研究柔膜菌目成员包括了 *Leptodontidium*、*Ciboria*、*Botrytis*、*Cryptosporiopsis* 和 *Oidiodendron* 5 个属的 OTU。其中，*Leptodontidium*、*Cryptosporiopsis* 和 *Botrytis* 3 个属的内生真菌在手参 *Gymnadenia conopsea* 中有过报道[48-49]。*Oidiodendron* 属的 OTU12 为杜鹃花类菌根真菌，在 *Pseudorchis albida* (*Orchidaceae*)中也有杜鹃花类菌根真菌的报道[5]。在黄龙沟 3 种杓兰分布区就有大量杜鹃花科植物分布，说明同一生境的兰科和杜鹃花科植物有可能通过 RAF 和菌根真菌形成真菌网络发生联系。

美球菌目、格孢腔菌目、肉座菌目和毛霉菌目的菌株都只有腐生或寄生型，没有共生型。这 4 个目分离出来的真菌菌株比较少，很可能是因为没有共生营养型，与 3 种杓兰缺少明显的互利关系造成的。肉座菌目菌株(*Hypocrea* sp.)和冻土毛霉(*Mucor hiemalis*)对 *Thelymitra longifolia* 的种子萌发有抑制作用[50]。冻土毛霉和尖孢镰刀菌(*Fusarium oxysporum*)是一种兰属植物(*Cymbidium* sp.)假鳞茎腐烂病的病原[51]。肉座菌目在 *Cyrtochilum myanthum*、*Scaphyglottis punctulata* 和 *Stelis superbiens* 3 种兰科植物根内生真菌的研究中是优势类群[8]，该结果说明虽然在本研究中没有发现共生营养型，但在其它兰科植物也有共生型存在的可能性。

西藏杓兰 RAF 的物种丰富度(α 多样性)较黄花杓兰高，而针叶林和灌木林两种不同生境对 RAF 物种丰富度影响不显著。群落分化程度(β 多样性)显示不同杓兰物种之间的菌落差异较两种不同生境大。说明两种生境虽然不同，但在同一栖息地(黄龙沟)的不同海拔距离较近。虽然植被有所不同，但土壤中真菌类群可能类似。因为土壤的特性和微生物多样性会影响植物的分布[52]，所以这可能是造成两种生境中 RAF 物种丰富度和群落分化程度没有不同杓兰高的原因。

综上所述，通过分离培养的方法，发现 3 种杓兰属植物中 RAF 的多样性较高且存在多种生态功能类型。培养方法可以获得 RAF 的菌株，为未来进行 RAF 与兰科植物在种子萌发、幼苗生长和环境适应等方面的研究打下基础。但分离培养方法会低估 RAF 真菌的种类和数量，因此未来还需要用高通量测序等方法对杓兰属植物 RAF 多样性和群落结构进行系统研究。

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