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# 江西都昌茶链格孢叶霉病病原鉴定

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**摘要:**【目的】明确江西省都昌县“黄金叶”茶树链格孢叶霉病病原菌种类。【方法】2021年4月从江西省都昌县朱恋春茶场采集链格孢叶霉病叶片,按常规组织分离法进行病菌分离和纯化,通过观察病菌的培养性状和分生孢子、分生孢子链的形态特征,结合*rDNA-ITS*、*Alt α1*和*GAPDH*基因的序列分析,对病菌进行种类鉴定,并依据柯赫氏法则验证其致病性。【结果】从病叶中共分离到19株培养性状和形态特征一致的链格孢属菌株,菌落圆形,中央墨绿色,边缘灰白色,菌丝浓密,背面深褐色,有不明显的轮纹,平均生长速率8 mm/d。分生孢子梗褐色,单生,直立或弯曲,其上链状着生分生孢子。分生孢子椭圆形、卵形或倒棍棒形,深褐色,有3~5个横隔膜,0~3个纵隔膜,分隔处不缢缩或略缢缩,大小(17.4~36.3)μm×(8.3~14.4)μm(*n*=50)。分生孢子顶端有喙或无喙,喙短柱状或锥状,有的喙可转变为分生孢子梗继续产孢,使分生孢子链出现分支,主链一般不超过10个孢子,支链一般有1~5个孢子。代表性菌株JXAA1、JXAA2、JXAA3具有完全相同的*rDNA-ITS*、*Alt α1*和*GAPDH*基因序列,且与GenBank中链格孢(*Alternaria alternata*)对应序列的相似性为100%。按*rDNA-ITS*、*Alt α1*和*GAPDH*基因顺序拼接构建多基因系统发育树,与链格孢(*A. alternata*)聚类成一个分支而其他种类菌株各构成独立分支。将上述3个菌株分别接种健康叶片,7 d后接种处均出现圆形黑褐色病斑,引起的症状与田间症状相同,并从病斑中再次成功分离到原病菌,对照接种无发病。【结论】确定江西都昌县“黄金叶”茶链格孢叶霉病的病原菌为链格孢(*A. alternata*),这是在江西首次发现链格孢引致茶叶病害。

**关键词:**江西茶;叶霉病;病原鉴定;链格孢
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# Pathogen Identification of *Alternaria* Leaf Mold Disease on Tea Plant in Duchang, Jiangxi Province

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**Abstract**: [**Objective**] The objective of this research is to identify the pathogen causing tea leaf mold on "Golden leaf" in Duchang County, Jiangxi Province. [**Method**] In April, 2021, leaf samples infected with leaf mold were collected from Zhu Lianchun Tea Farm in Duchang County, Jiangxi Province. The pathogen was isolated and purified by using the routine tissue isolation method. The cultural characteristics of the pathogen and the morphological characteristics of its conidia and conidia chains were observed, the sequences of *rDNA-ITS*, *Alt α1* and *GAPDH* gene of the pathogen were analyzed, and its pathogenicity was determined. [**Result**] Nineteen isolates of *Alternaria* with the same cultural characteristics were obtained from the diseased leaves. The colonies on PDA were round, fluffy, dark green with gray-white margin. The back side of the colonies were dark brown and had indistinct concentric rings. They grew at an average growth rate of 8.0 mm/d. Conidiophores were brown, solitary, erect or curved, on which the conidia were produced in chain form. The conidia were elliptic, ovate or obclavated, dark brown, and (17.4-36.3) μm×(8.3-14.4) μm ($n$=50) in size. Each conidium contained 3-5 transverse septa and 0-3 longitudinal septa, and the septa were not constrict or slightly constrict. Conidia had beaks or no beaks on the top, and the beaks were short columnar or conical. Some beaks transformed into conidiophores and continued to produce conidia, thus forming conidia chain branches. The main chain generally had no more than 10 conidia, and the branch chain generally had 1-5 conidia. The representative isolates JXAA1, JXAA2 and JXAA3 had identical *rDNA-ITS*, *Alt α1* and *GAPDH* sequences, and their sequences were 100% similar to those of *Alternaria alternata* in GeneBank. In the phylogenetic tree constructed based on the sequences of *rDNA-ITS*, *Alt α1* and *GAPDH* gene, the three isolates were clustered together with *A. alternata*, while the other isolates formed independent branches. The three isolates were inoculated on healthy leaves respectively, and the symptoms as those of natural infection appeared 7 days after inoculation. The pathogen was successfully re-isolated from the diseased leaves. However, no disease occurred in the control. [**Conclusion**] The pathogen isolated from the mold tea leaves on "Golden Leaf" in Duchang County was identified as *A. alternata*. This is the first report of *A. alternata* causing tea disease in Jiangxi Province.

**Keywords**: Jiangxi tea; leaf mold; identification of pathogen; *Alternaria alternata*

【研究意义】茶树[*Camellia sinensis*(L.)O.Ktze.]是我国最重要的经济作物之一[1]。近年来,江西茶园面积快速扩大,然而,茶树在生长过程中也受到各种病害困扰,尤其是叶部病害,直接危害茶树生长,造成茶叶减产,影响茶叶品质[2]。2020—2021年,笔者先后两次在江西都昌县一处茶场调查,发现一种典型叶霉病,严重影响茶树生长,经镜检病原初步确定为链格孢属(*Alternaria* Nees ex Wallr.)真菌病害。本文拟对其病原进行分离鉴定和致病性测定,以明确链格孢菌对茶树叶片的致病作用,这对促进链格孢属病原研究和有效防治江西茶区叶部病害具有重要的理论和实际意义。【前人研究进展】链格孢是茶树叶部病害的重要病原菌,肖强等以茶链格孢叶霉病(病原:*Alternaria tenuis*)编入名录[3],但有关茶链格孢叶霉病系统报道较少。2005年印度北孟加拉首次报道链格孢(*A. alternata*)引起茶叶斑病,其病叶率达70%以上[4]。2014年湖北罗田县首次发现链格孢(*A. alternata*)引起茶树叶斑病,嫩叶为害率超20%[5]。随着茶树病害调查与研究深入,2019年周园园等[6]在安徽、福建、湖北茶区采样发现由链格孢(*A. alternata*)引起的茶叶斑病。2021年贵州发现长柄链格孢(*A. longipes*)为茶树链格孢新病原[7]。【本研究切入点】鉴于茶链格孢叶霉病系统研究较少,且多种链格孢菌均能引起茶树叶部病害,江西茶区叶霉病在发生品种和环境条件等方面有其独特性,究竟以何种链格孢菌危害尚不明确。本文从田间调查发现的典型茶树叶霉病叶中分离病原菌,选择代表性菌株对其进行培养性状和形态学特征观察,采用多基因序列进行分子生物学鉴定,同时进行致病性测定。【拟解决的关键问题】明确该病原菌的种类归属,为防控该病害提供理论依据。

# 1　材料与方法

## 1.1　田间调查与样品采集

发病茶园位于江西省都昌县朱恋春茶场(29.50°N,116.19°E),调查时间2020年9月中旬和2021年4月上旬,品种为黄金叶,为5年生壮龄茶树,面积30 $hm^2$。选择集中成片的茶园(1 $hm^2$以上),按对角线取样点5个(去掉头尾边角5 m,5行),每点随机取10~20个茶枝(每隔5~10行取1行,定距10~20步,从左右行随机各取1个茶枝),清数各枝总叶数和有病叶数,计算病叶率。病叶率=发病叶片数/调查叶片数×100%。随机摘取典型发病叶片和健康叶片若干,分开小包装,自封袋带回。

## 1.2　病原菌的分离纯化

取新鲜典型病叶按常规组织分离法[8]分离病菌。在病健交界处,剪取边长约3 mm的小块组织,放入75%酒精中浸泡10 s,然后移入0.1%升汞中处理20 s,再用无菌水漂洗3次,最后置于乳酸酸化的PDA平板上,于28 ℃下黑暗培养。待菌落长出后,挑取菌落边缘菌丝体进行纯化培养。

## 1.3　病原菌的培养特征及形态学观察

将纯化后的菌株转接新的PDA平板,逐日观察记载菌落形态、颜色等培养特性,采用十字交叉法测量菌落直径,3次重复。在产孢旺盛期,镜检分生孢子梗和分生孢子的形态特征,随机取50个成熟分生孢子测量其大小。采用带孔滤纸加载玻片法培养分生孢子链[9],即取PDA边缘生长旺盛菌丝涂抹于中间凿有方孔的无菌滤纸片上(滤纸湿润贴于载玻片),28 ℃下黑暗培养5~7 d,观察方孔内缘的分生孢子链。

## 1.4　分子生物学鉴定

1.4.1　*病原菌基因组DNA提取*　选择3株代表性菌株,培养7~10 d,用灭菌枪头刮取新鲜菌丝30~100 mg,液氮中迅速研磨至粉末状,采用Ezup柱氏真菌基因组DNA提取试剂盒(生工生物工程上海股份有限公司)提取DNA。

1.4.2　*PCR扩增及测序*　以病原菌基因组DNA为模板,分别进行核糖体RNA内转录间隔区基因(Internal Transcribed Spacer,*rDNA-ITS*)、链格孢过敏原基因(Alternaria major allergen gene,*Alt α1*)和甘油醛-3-磷酸脱氢酶基因(glyceraldehyde-3-phosphate dehydrogenase,*GAPDH*)进行扩增。所用引物对如表1[12-14]。25μL PCR扩增反应体系包含2×*Taq* PCR Master Mix 12.5 μL,引物对各1 μL(10 μmol/L),基因组DNA 2 μL,$ddH_2O$ 8.5 μL。PCR反应程序:94 ℃预变性4 min;94 ℃变性30 s,60 ℃退火30 s,72 ℃延伸1 min,35个循环;最后于72 ℃补平10 min,4 ℃保存。取扩增产物5 μL,12 g/L琼脂糖凝胶电泳检测,送生工生物工程(上海)股份有限公司纯化测序。

表1　试验中所用引物对

Tab.1　Primers used in the experiment

| 基因 Gene | 引物对 Primer | 引物序列 Sequence(5′—3′) | 参考文献 Reference |
|---|---|---|---|
| *rDNA-ITS* | ITS1 | TCCGTAGGTGAACCTGCGG | [10] |
| | ITS4 | CCTCCGCTTATTGATATGC | |
| *Alt α1* | Altα1-F | ATGCAGTTCACCACCATCGC | [11] |
| | Altα1-R | ACGAGGGTGAYGTAGGCGTC | |
| *GAPDH* | GDF-F | GCCGTCAACGACCCCTTCATTGA | [12] |
| | GDR-R | GGGTGGAGTCGTACTTGAGCATGT | |

1.4.3　*系统发育树构建*　使用DNA Star(Madison Wisconsin)软件对测序原始序列进行拼接,并提交至GenBank获取登录号。在NCBI中BLAST搜索同源序列,参照序列如表2。使用MEGA5.1软件构建系统发育树,采用邻位加入法(neighbor-joining,NJ),重复1000次bootstrap检测,找出病菌序列相似性关系。

## 1.5　致病性测定

选择健康叶片,无菌水冲洗后晾干,75%酒精棉球轻擦接种处,用灭菌针头对称刺伤。取无菌水5 mL冲洗长满菌丝体平板,四层纱布过滤菌丝,血球计数板计数,以$1\times10^5$/mL孢子悬浮液接种。试验选代表性菌株3株,每株3次重复,设无菌水对照。

表 2 用于构建多基因系统发育树的菌株及 GenBank 登录号

Tab.2 Strains used to construct multigene phylogenetic tree and their GenBank accession numbers

| 种名 Species | 菌株编号 Isolate code | 基因登录号 GenBank accession no. | | |
|---|---|---|---|---|
| | | *rDNA-ITS* | *Alt α1* | *GAPDH* |
| *Alternaria* sp. | JXAA1 | ON540390 | ON560940 | ON561100 |
| *Alternaria* sp. | JXAA2 | ON509653 | ON513384 | ON513385 |
| *Alternaria* sp. | JXAA3 | ON540391 | ON560941 | ON561101 |
| *A. alternata* | B2c | MZ823462 | MZ802960 | MZ835386 |
| *A. alternata* | B5a | MZ823469 | MZ802967 | MZ835393 |
| *A. alternata* | RG3 | MG250608 | MG250644 | MG250620 |
| *A. gaisen* | CBS 118488 | KP124427 | KP123975 | KP124278 |
| *A. gaisen* | CPC 25268 | KP124428 | KP123976 | KP124279 |
| *A. longipes* | 20NL02 | OK426388 | OK469304 | OK469302 |
| *A. longipes* | 20NL05 | OK426389 | OK469305 | OK469303 |
| *A. tenuissima* | SY-4 | MK560480 | MK593137 | MK571538 |
| *A. tenuissima* | SN6-2 | LC621622 | LC631828 | LC621656 |
| *A. brassicae* | AC62 | LC440628 | LC481640 | LC482049 |
| *A. brassicae* | CCPY2 | MG250600 | MG250636 | MG250612 |
| *A. cucumerina* | CBS 116114 | KJ718153 | KJ718668 | KJ718000 |
| *A. cucumerina* | CBS 117226 | KJ718155 | KJ718670 | KJ718002 |
| *A. solani-nigri* | CBS 116447 | KJ718246 | KJ718752 | KJ718074 |
| *A. solani-nigri* | CBS 117101 | KJ718247 | KJ718753 | KJ718075 |
| *Embellisia telluster* | EGS 33.026 | FJ357316 | AY563325 | FJ357304 |

## 2 结果与分析

### 2.1 病叶田间症状及致病性测定

田间调查发现，病株在田间呈集中分布，有较典型扩散趋势，病叶率约 30%，病菌以危害芽下第 4~5 片成叶为主，发病部位为叶边缘或叶尖处，病斑多为半圆形或不规则形，黑褐色，早期病斑病健交界分明，后期模糊不清，潮湿时病部呈灰黑色霉状（图 1A，图 1B）。用 3 株代表性菌株孢子悬浮液分别接种茶树健康叶片，5 d 后开始发病，7 d 后接种处均出现圆形黑褐色病斑，后期扩展有黑褐色霉状物出现（图 1C），发病率 100%。无菌水接种处无发病。对接种处黑褐色病斑进行再分离，得到的病菌培养性状和形态特征与原接种菌一致，完成了柯赫氏法则验证。

### 2.2 形态学观察

从病叶中共分离到 19 株培养性状和形态特征一致的链格孢属菌株，菌落圆形，中央墨绿色，边缘灰白色，菌丝浓密，背面深褐色，有不明显的轮纹，平均生长速率 8 mm/d（图 2A，图 2B）。分生孢子梗褐色，单生，直立或弯曲，其上链状着生分生孢子。分生孢子椭圆形、卵形或倒棍棒形，深褐色，有 3~5 个横隔膜，0~3 个纵隔膜，分隔处不缢缩或略缢缩，大小（17.4~36.3）μm×（8.3~14.4）μm（$n$=50）（图 2C，图 2D）。分生孢子顶端有喙或无喙，喙短柱状或锥状，有的喙可转变为分生孢子梗继续产孢，作合轴式延伸，形成简单的矮树状有分支的分生孢子链，主链一般不超过 10 个孢子，侧面与基部易萌生次生分生孢子梗而产孢并形成支链，一般长有 1~5 个孢子（图 2E~图 2G）。根据病原菌的培养性状和形态特征，查阅文献《中国真菌志-链格孢属》[13]，初步鉴定该病原菌为链格孢属真菌（*Alternaria* sp.），与链格孢（*A. alternata*）基本相符。

### 2.3 分子生物学鉴定

对代表性菌株 JXAA1、JXAA2、JXAA3 分别进行 *rDNA-ITS*、*Alt α1* 和 *GAPDH* 基因 PCR 扩增，电泳后均获得 3 条特异性目的条带，大小分别为 570，517，177 bp（图 3），测序后具有完全相同的 *rDNA-ITS*、*Alt α1* 和 *GAPDH* 基因序列，且与 GenBank 中链格孢（*Alternaria alternata*）对应序列的相似性为 100%，（登录号见

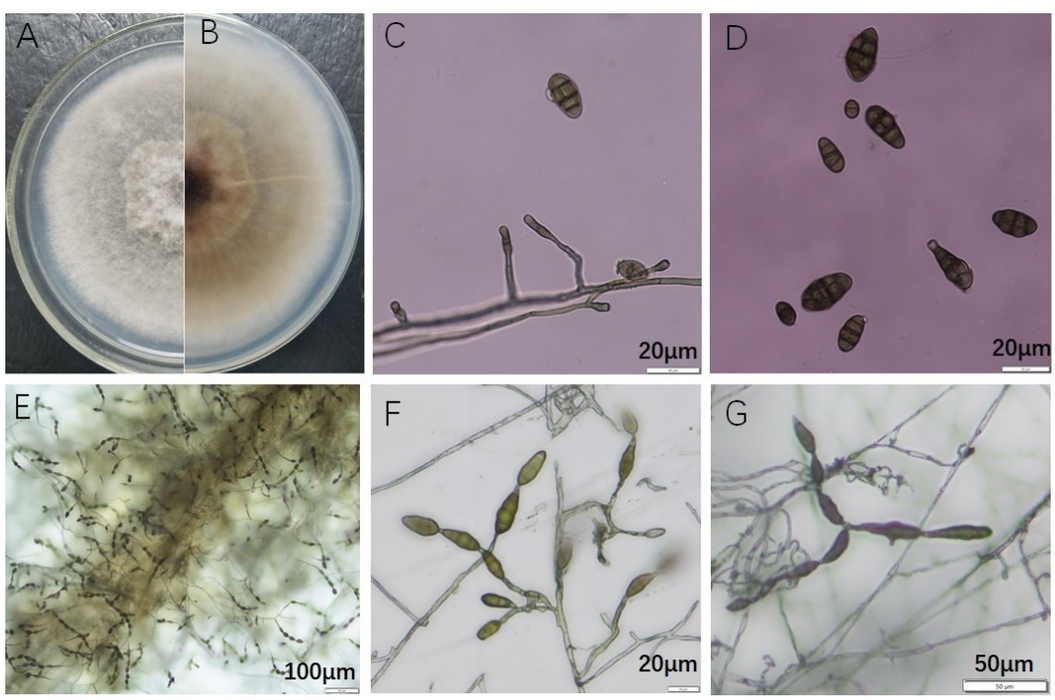
表 2)。按 *rDNA-ITS*、*Alt α1* 和 *GAPDH* 基因顺序拼接构建多基因系统发育树,选用埃里格孢(*Embellisia telluster*)作外群,与链格孢(*A. alternata*)聚类成一个分支而其他种类菌株各构成独立分支(图 3)。根据亲缘发生关系,将代表菌株鉴定为链格孢(*A. alternata*)(图 4)。


A:田间病株;B:病叶;C:接种后 7 d 症状。

A:Diseased plants in the field;B:Symptoms on the natural infected leaves;C:Symptoms on the artificial inoculated leaf(7 d).

图 1　茶树链格孢叶霉病自然及人工接种发病症状

Fig.1　Natural and artificial inoculation symptoms of Alternaria alternata leaf mold in tea plants

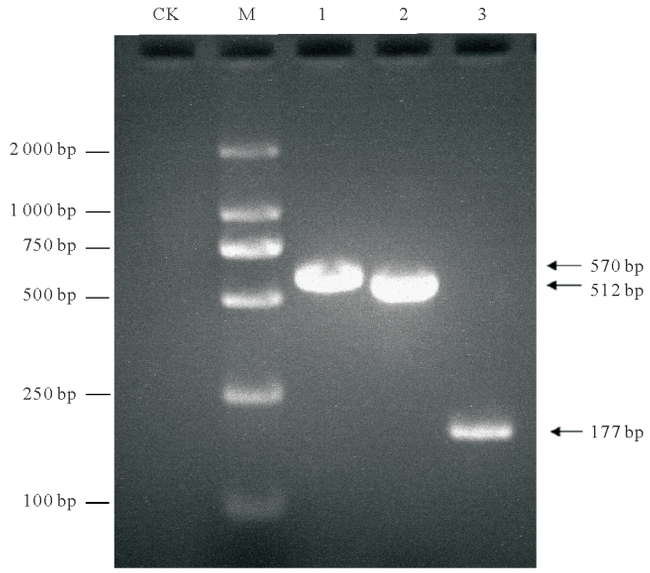



A,B:7 天菌落(正反面);C,D:分生孢子梗及分生孢子;E~G:分生孢子链及支链。

A,B:Colony cultured for 7 days (Front and back);C,D:Conidiophores and Conidia;E-G:Conidial chains and branches.

图 2　茶树叶霉病病菌 JXAA2 菌株形态学特征

Fig.2　Morphological characteristics of strain JXAA2 of tea leaf mold




CK:阴性对照;M:DL2000 Marker;1:*rDNA-ITS*;2:*Alt α1*;3:*GAPDH*

CK:Negative control;M:DL2000Marker;1:*rDNA-ITS*;2:*Alt α1*;3:*GAPDH*

图3 菌株JXAA2的不同基因PCR扩增产物电泳结果

Fig.3 Electrophoresis of PCR products amplified from different genes of strain JXAA2

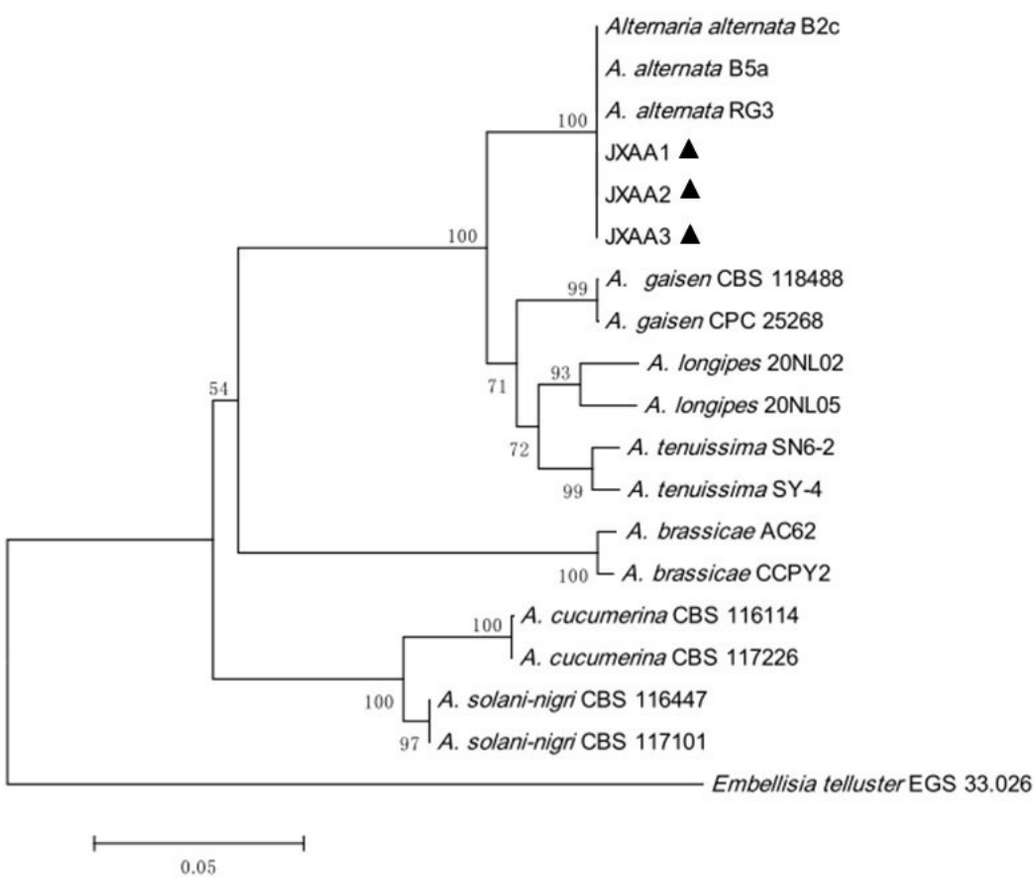



图4 基于*rDNA-ITS*、*Alt α1*和*GAPDH*串联基因序列构建的链格孢属真菌的系统发育树

Fig.4 Phylogenetic tree of *Alternaria* spp. based on the concatenated sequences of gene *rDNA-ITS*, *Alt α1* and *GAPDH*

## 3 结论与讨论

本文对江西都昌县茶树“黄金叶”品种上发生的一种叶霉病进行了病菌分离,获得19株一致性链格

孢属(*Alternaria*)菌株,各菌株在PDA上的菌落形态、生长速率、分生孢子梗、分生孢子形态和大小、以及分生孢子链特征等相同,符合文献对链格孢(*A. alternata*)的描述。代表菌株的*rDNA-ITS*、*Alt α1*和*GAPDH*等基因序列与链格孢对应序列均具有100%的相似性,构建的多基因系统发育树与链格孢聚为一个分支。鉴于以上结果,将这种典型叶片黑斑或霉变病定为茶链格孢叶霉病,病原鉴定为链格孢菌(*A. alternata* Fr. Keissl)[14]。该病原菌在茶树上虽有记载但系统报道较少,在江西茶区属首次发现,是典型的茶链格孢叶霉病。这与安徽等地已报道的病原基本一致,但田间症状明显不同,其报道的病原与刺盘孢属(*Colletotrichum* spp.)引起的症状相似,常常形成复合侵染。

链格孢属(*Alternaria* Nees)分生孢子具有多型现象,形态特征变化较大[13]。因此,在病菌鉴定过程中仅依靠分生孢子梗和分生孢子的形态差异显然不够,相比较而言根据产孢表型,观察分生孢子链更为直观可靠。链格孢(*A. alternata*)形成简单的树状分支的分生孢子链[15],分生孢子的孢身分隔处不缢缩或略缢缩,主链一般不超过10个孢子,易萌生次生分生孢子梗而产孢并形成支链,一般长有1~5个孢子。这与细极链格孢(*A. tenuissima*)具有明显区别,其分生孢子一般呈长链状排列达10个分生孢子以上,不分支或稀少分支。本文据此鉴定的病原与*A.alternata*基本相符。随着分子生物学的飞速发展,越来越多的研究采用多基因构建系统发育树的方法对链格孢属真菌进行区分和鉴定,采用的基因有*ITS*、*SSU*、*LSU*、*GAPDH*、*RPB2*、*TEF1-α*、*Alt a1*、*endoPG*和*OPA*等[16-17]。本文采用*rDNA-ITS*、*Alt α1*和*GAPDH* 3个基因序列对分离病菌进行分子鉴定,构建的多基因系统发育树与链格孢(*A.alternata*)聚为一支,而与同属形态上相似的梨黑斑链格孢(*A. gaisen*)、细极链格孢(*A. tenuissima*)、长柄链格孢(*A. longips*)、芸薹链格孢(*A. brassicae*)、瓜链格孢(*A. cucumerina*)、龙葵链格孢(*A. solani-nigri*)等明显区分开来。这表明,采用*rDNA-ITS*、*Alt α1*和*GAPDH*多基因可区分*Alternaria* Nees相似种,鉴定结果具有可靠性。

链格孢是一种重要的植物病原真菌,该菌不仅为害茶树,而且还可为害西兰花[18]、西瓜[19]、梨树[20]等多种作物,同时,该菌可产生细交链孢酮酸、交链孢酚、交链孢单甲醚等多种毒素[21],威胁食品安全,因此,需重视对链格孢及所致病害的研究和防控。“黄金叶”为湖南省高氨基酸品种,引入江西后,受赣鄱地区红壤和气候条件影响较大,其生长势、抗逆性和产量性状等均出现不同程度下降[22],病虫害发生也相对较重,引种时需注意植物检疫,引种后应加强栽培管理,充分发挥品种增产提质潜力。

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