# 基于时珍法的镰刀菌属近缘物种鉴定研究

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**摘要**: 镰刀菌属真菌是药材常见的污染真菌, 对中药材的有效成分和用药安全构成双重威胁, 同时也是农业和临床常见的病原体。该属近缘物种的准确鉴定一直是难点。本研究利用时珍法对该属173个物种共304个基因组进行生物信息学分析, 成功构建173个物种的特异靶标序列库, 特异靶标序列数量主要集中在10 001～50 000条。为验证特异靶标序列的可靠性, 选择关注度高的药材污染或农业致病的7个代表性物种: 尖孢镰刀菌 (*Fusarium oxysporum*)、腐皮镰刀菌 (*F. solani*)、藤仓镰刀菌 (*F. fujikuroi*)、接骨木镰刀菌 (*F. sambucinum*)、黄色镰刀菌 (*F. culmorum*)、肉红镰刀菌 (*F. incarnatum*) 和木贼镰刀菌 (*F. equiseti*), 选取特异靶标序列, 利用Sanger测序和CRISPR-Cas12a技术进行检测。结果表明7个物种的特异靶标序列具有高特异性, 解决高度近缘物种*F. incarnatum*和*F. equiseti*的鉴定难题, 且实现鉴定结果的可视化, 具备现场检测优势。本研究证明时珍法可用于镰刀菌属近缘物种的准确鉴定, 为中药材的安全监控和品质保障提供技术支撑。

**关键词**: 全基因组分析; 镰刀菌属; 近缘物种; 鉴定

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# Analysis of whole-GEnome for closely related species identification of *Fusarium*

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**Abstract**: The genus *Fusarium* is a prevalent contaminant in medicinal herbs, posing a dual threat to both the effective components and safety of traditional Chinese medicine (TCM), as well as a common pathogen in agriculture and clinical settings. Accurate identification of closely related species within this genus has always been a key challenge. In this study, we employed analysis of whole-GEnome (AGE) to conduct bioinformatics analysis on 304 genomes from 173 species of *Fusarium*, successfully constructing a species-specific target sequence library. The number of specific target sequences predominantly ranges from 10 001 to 50 000. To validate the reliability of the species-specific target sequences, we selected seven representative species of high concern in contaminated medicinal herbs and agricultural pathogens: *Fusarium oxysporum*, *F. solani*, *F. fujikuroi*, *F. sambucinum*, *F. culmorum*, *F. incarnatum*, and *F. equiseti*. Species-specific target sequence was selected from each species and

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subjected to detection using Sanger sequencing and CRISPR-Cas12a technology. The results showed that the species-specific target sequences exhibited high specificity, solving the identification challenge of closely related species *F. incarnatum* and *F. equiseti*. Furthermore, AGE enabled the visualization of identification results, offering an advantage for field detection. This study demonstrates that AGE can accurately identify closely related species of the genus *Fusarium*, providing technical support for the safety monitoring and quality assurance of medicinal herbs.

**Key words**: analysis of whole-GEnome; *Fusarium*; closely related species; identification

镰刀菌属 (*Fusarium*) 真菌是药材中污染率较高的真菌之一[1-3], 能够引起药材霉变和品质下降, 并产生多种毒素 (如玉米赤霉烯酮和脱氧雪腐镰刀菌烯醇), 对中药材的有效成分和用药安全构成双重威胁[4-8], 同时是公认的全球十大植物病原真菌之一[9], 也是临床上常见的病原体[10-12]。常见的镰刀菌属病原菌众多, 其中尖孢镰刀菌 (*F. oxysporum*) 是甘草等药材的常见污染菌[13,14], 同时是最具破坏力的农业病原体之一, 可侵染120余种经济作物, 引发维管枯萎病或根腐病, 造成重大经济损失[15,16]; 腐皮镰刀菌 (*F. solani*) 和木贼镰刀菌 (*F. equiseti*) 则分别污染何首乌、巴戟天及益智仁等药材[17,18]; 黄色镰孢菌 (*F. culmorum*) 是小麦赤霉病的主要病原菌, 其产生的脱氧镰刀烯醇和戊烯醇等霉菌毒素严重危害食品安全[19,20]; 此外, 藤仓镰刀菌 (*F. fujikuroi*) 引发的稻瘟病作为种子传播疾病, 可导致水稻减产高达90%, 对全球粮食生产构成持续性威胁[21-23]; 接骨木镰孢菌 (*F. sambucinum*) 是引起植物干腐病的常见病原菌, 该病害会显著降低作物产量, 是国内外马铃薯干腐病的主要致病菌[24,25]; 肉红镰刀菌 (*F. incarnatum)* 会导致水稻穗腐病[26]。因此, 快速准确鉴定镰刀菌属物种对于防治和检疫具有重要意义。

该属存在显著的种内遗传分化和生理分化特性, 且常出现过渡类型, 使其成为真菌类群中最难以通过形态分类的属之一[27-29]。基质辅助激光解吸/电离飞行时间质谱法 (MALDI-TOF) 已被证实能够较为准确地识别真菌分离株至种水平[30], 但其结果会受到多种因素的影响, 包括培养基类型、有无孢子的样本类型以及样本的处理方法等[31,32]。目前, DNA条形码是物种鉴定的常用分子鉴定方法[33-36], 但DNA条形码 (如ITS、EF-1$\alpha$) 在区分近缘物种时仍存在局限性[37,38], 如难以准确鉴定近缘物种 *F. incarnatum* 与 *F. equiseti*[39]。因此, 准确鉴定镰刀菌属物种仍然是一项具有挑战性的任务, 亟需新的鉴定方法。本研究团队根据不同物种的全基因组序列必然存在差异的原理, 提出物种鉴定新方法—时珍法, 即全基因组分析法 (analysis of whole-GEnome, AGE) 为物种鉴定提供新思路[40]。时珍法的操作流程为: 通过生物信息学分析从目标物种全基因组中筛选特异靶标序列, 利用实验技术检测特异靶标序列, 从而实现物种鉴定[40]。该方法已成功应用于不同植物[41]、动物[42]和真菌近缘物种的准确鉴定[43-46]。

NCBI数据库包含丰富的镰刀菌属物种基因组数据, 本研究采用镰刀菌属173个物种共304个基因组数据用于分析。为解决镰刀菌属近缘物种鉴定难题, 本研究拟用时珍法构建该属173个近缘物种特异靶标序列库, 重点选取镰刀菌属7个关注度高的药材污染或农业致病的物种 *F. oxysporum*、*F. solani*、*F. fujikuroi*、*F. sambucinum*、*F. culmorum*、*F. incarnatum* 和 *F. equiseti* 进行特异靶标序列的实验验证, 解决近缘物种 *F. incarnatum* 与 *F. equiseti* 鉴定难题, 为中药材的质量控制、药品安全保障和农业防治提供技术支撑。

## 材料与方法

**仪器** PL203型电子天平 [梅特勒–托利多仪器 (上海) 有限公司]; Pad3-H型加热制冷型金属浴 [卡尤迪生物科技 (北京) 有限公司]; 5424R型台式高速冷冻离心机和5430R型台式高速冷冻离心机 (德国Eppendorf公司); Veriti 96 Well Thermal Cycler型PCR仪 (ABIVeriti, 美国赛默飞公司); Tanon 2500B型凝胶成像仪 (上海天能科技有限公司); DYY-8C型电泳仪 (北京市六一仪器厂)。

**试剂** DP305型天根植物DNA提取试剂盒 [天根生化科技 (北京) 有限公司]; DNA提取液、50×TAE缓冲液和Tris-HCl缓冲液 (1 mol·L$^{-1}$, pH 8.0, 北京索莱宝科技有限公司); 2×Taq PCR Master Mix (北京艾德莱生物科技有限公司); 异丙醇 (天津市福晨化学试剂厂); $\beta$-巯基乙醇 (北京博奥拓达科技有限公司); 无水乙醇 (北京化工厂); BIOWEST琼脂糖Agarose G-10 (北京艾普立邦科技有限公司); DNA DL1000 marker [宝日医生物技术 (北京) 有限公司]; 先达基因ERA试剂盒 (KS101, 苏州先达基因科技有限公司); PCR引物于苏州金唯智生物科技有限公司合成; 成簇规律间隔短回文重复序列RNA (clustered regularly interspaced short palindromic repeats RNA, crRNA) 和单链DNA (single strand DNA, ssDNA) 均由金斯瑞 (江苏) 有限责

任公司合成。

**真菌样品及保藏编号** 尖孢镰刀菌 (*F. oxysporum*, SHBCC D25188)、腐皮镰刀菌 (*F. solani*, SHBCC D10312)、藤仓镰刀菌 (*F. fujikuroi*, SHBCC D19354)、肉红镰刀菌 (*F. incarnatum*, SHBCC D68686) 和木贼镰刀菌 (*F. equiseti*, SHBCC D82189) 购自上海微生物菌种保藏中心 (SHBCC); 接骨木镰刀菌 (*F. sambucinum*, CGMCC 3.17693) 和黄色镰刀菌 (*F. culmorum*, CGMCC 3.4283) 从中国菌种保藏中心 (CGMCC) 购买。

**镰刀菌属真菌基因组DNA提取** 菌种活化和培养提取均委托某生物科技有限公司 (具备质量管理体系认证证书; 证书编号: 16422Q30560ROS) 进行操作。公司收到菌种按照菌种保藏中心提供的培养要求在对应培养基平板活化, 并在合适培养温度条件下黑暗倒置培养5～7天。培养后的菌种继代至新培养基平板, 待生长至合适大小, 采菌饼至50%甘油中保存于−80 ℃。刮取适量菌丝于2 mL离心管中用于DNA提取。DNA提取采用常规的CTAB法抽提, 步骤: 离心管液氮冷冻, 充分研磨。趁冷加入600 μL CTAB DNA提取Buffer, 充分振荡混匀; 将离心管放入65 ℃加热器上放置30～60 min, 期间颠倒混匀几次, 并敞开盖放气; 加入等体积 (600 μL) Tris-酚仿, 充分混匀, 12 000 r·min$^{-1}$离心15 min, 吸取500 μL上清液; 加入等体积 (500 μL) 氯仿/异戊醇 (24∶1), 12 000 r·min$^{-1}$离心15 min, 吸取上清400 μL; 加入等体积异丙醇 (400 μL), 混匀后−20 ℃沉淀半小时以上 (此时肉眼可见絮状沉淀) 12 000 r·min$^{-1}$离心15 min; 倒掉上层溶液, 在吸水纸吸干, 加入600 μL 70%乙醇洗1次; 100%乙醇洗一次, 超净台吹干; 加入50 μL TE溶液, 37 ℃加热溶解, 收集DNA样品; 用NanoDrop 2000紫外分光光度计检测DNA样品的$OD_{260/280}$与$OD_{260/230}$的值, 并记录总DNA的浓度。提取的DNA于−40 ℃冰箱保存、备用。

**DNA条形码鉴定** 利用真菌ITS通用引物[47] (ITS5F: GGAAGTAAAAGTCGTAACAAGG; ITS4R: TCCTCCGCTTATTGATATGC) 对上述镰刀菌属真菌样品进行PCR扩增。ITS引物PCR扩增反应体系均为20 μL, 包括2×*Taq* Master Mix 10 μL, 上下游引物 (10 μmol·L$^{-1}$) 各1 μL, 基因组DNA 2 μL (10 ng·μL$^{-1}$), 最后加灭菌双蒸水6 μL。其中ITS的PCR扩增程序为94 ℃, 3 min; 94 ℃, 30 s, 55 ℃, 30 s, 72 ℃, 30 s, 共30个循环; 72 ℃, 7 min。配置1%琼脂糖凝胶检测PCR扩增结果, 电泳条件为100 V、120 mA、30 min, 并与DL1000 marker对比, 确定扩增目的条带的有无和序列长度范围, 将扩增成功的PCR产物利用ABI3730XL测序仪进行双向测序。使用CodonCode Aligner (CodonCode Co., 美国, 10.0.3版) 进行测序峰图的校对和一致性序列组装。

**物种基因组数据信息** 从NCBI基因组数据库中下载镰刀菌属181个物种共1 802个基因组数据 (2024年5月19日)。为最大程度保证用于生物信息学分析的物种基因组的正确性, 根据以下原则进行基因组筛选: 菌种来源是否为权威的标准菌株 (如编号为CBS、CGMCC、ATCC和NRRL等)、发表菌株基因组文章的质量和数量、提交菌株基因组单位权威性、文献已报道经建树分析的菌株基因组, 最终筛选173个物种共304个基因组数据 (表1)。

**Table 1** Genome information of 173 *Fusarium* species analyzed by bioinformatics

| Species | GenBank | Species | GenBank |
|---|---|---|---|
| *Fusarium abutilonis* | GCA_021655885.1 | *F. irregulare* | GCA_004367085.1 |
| *F. acaciae-mearnsii* | GCA_014822065.1 | *F. irregulare* | GCA_027945235.1 |
| *F. acaciae-mearnsii* | GCA_017657105.1 | *F. irregulare* | GCA_027945245.1 |
| *F. acaciae-mearnsii* | GCA_017657115.1 | *F. keratoplasticum* | GCA_025433545.1 |
| *F. acaciae-mearnsii* | GCA_017657135.1 | *F. keratoplasticum* | GCA_025433555.1 |
| *F. acuminatum* | GCA_013363215.1 | *F. kyushuense* | GCA_013184315.1 |
| *F. acutatum* | GCA_012932015.1 | *F. langsethiae* | GCA_001292635.1 |
| *F. aethiopicum* | GCA_017657045.1 | *F. langsethiae* | GCA_018104275.1 |
| *F. agapanthi* | GCA_001654545.1 | *F. lateritium* | GCA_014898835.1 |
| *F. agapanthi* | GCA_001654555.2 | *F. liriodendri* | GCA_023509735.1 |
| *F. albidum* | GCA_013618265.1 | *F. longipes* | GCA_003012285.1 |
| *F. albosuccineum* | GCA_012931995.1 | *F. longipes* | GCA_013618485.1 |
| *F. algeriense* | GCA_002982035.1 | *F. longipes* | GCA_013618495.1 |
| *F. algeriense* | GCA_002982055.1 | *F. longipes* | GCA_013623335.1 |
| *F. ambrosium* | GCA_003947045.1 | *F. louisianense* | GCA_017656775.1 |
| *F. anguioides* | GCA_012977745.1 | *F. louisianense* | GCA_017656825.1 |
| *F. annulatum* | GCA_019189775.1 | *F. luffae* | GCA_013184325.1 |
| *F. anthophilum* | GCA_013364935.1 | *F. mangiferae* | GCA_013758935.1 |
| *F. armeniacum* | GCA_013618295.1 | *F. marasasianum* | GCA_022833035.1 |
| *F. asiaticum* | GCA_001717835.1 | *F. meridionale* | GCA_001717825.1 |

Continued

| Species | GenBank | Species | GenBank |
|---|---|---|---|
| *F. asiaticum* | GCA_001717845.1 | *F. meridionale* | GCA_001717855.1 |
| *F. asiaticum* | GCA_013618305.1 | *F. meridionale* | GCA_009617515.1 |
| *F. asiaticum* | GCA_017657095.1 | *F. meridionale* | GCA_017656785.1 |
| *F. austroafricanum* | GCA_012932025.1 | *F. mesoamericanum* | GCA_017656745.1 |
| *F. austroamericanum* | GCA_009617505.1 | *F. mexicanum* | GCA_013396015.1 |
| *F. austroamericanum* | GCA_009617525.1 | *F. miscanthi* | GCA_014898875.1 |
| *F. austroamericanum* | GCA_013364965.1 | *F. mundagurra* | GCA_013396205.1 |
| *F. austroamericanum* | GCA_017657025.1 | *F. musae* | GCA_013623345.1 |
| *F. austroamericanum* | GCA_017657035.1 | *F. musae* | GCA_019915245.1 |
| *F. avenaceum* | GCA_000769295.1 | *F. nanum* | GCA_004367095.1 |
| *F. avenaceum* | GCA_013753855.1 | *F. napiforme* | GCA_013396005.1 |
| *F. avenaceum* | GCA_018282135.1 | *F. nelsonii* | GCA_014898925.1 |
| *F. avenaceum* | GCA_020744115.1 | *F. nematophilum* | GCA_013623595.1 |
| *F. avenaceum* | GCA_025948275.1 | *F. neocosmosporiellum* | GCA_006518225.1 |
| *F. aywerte* | GCA_013186375.1 | *F. neocosmosporiellum* | GCA_021655985.1 |
| *F. azukiicola* | GCA_001680625.1 | *F. nepalense* | GCA_017656675.1 |
| *F. babinda* | GCA_012977765.1 | *F. nepalense* | GCA_017656735.1 |
| *F. babinda* | GCA_013184435.1 | *F. newnesense* | GCA_013184375.1 |
| *F. bactridioides* | GCA_013623355.1 | *F. nisikadoi* | GCA_013623555.1 |
| *F. begoniae* | GCA_013186755.1 | *F. nodosum* | GCA_014898975.1 |
| *F. beomiforme* | GCA_002980475.2 | *F. nurragi* | GCA_012977755.1 |
| *F. boothii* | GCA_017656945.1 | *F. nygamai* | GCA_001262555.1 |
| *F. boothii* | GCA_017656985.1 | *F. nygamai* | GCA_013186815.1 |
| *F. boothii* | GCA_017656995.1 | *F. nygamai* | GCA_022813395.1 |
| *F. brasilicum* | GCA_013184295.1 | *F. odoratissimum* | GCA_000260195.2 |
| *F. brasilicum* | GCA_017656955.1 | *F. odoratissimum* | GCA_000350365.1 |
| *F. brasiliense* | GCA_001680685.1 | *F. oxysporum* | GCA_000149955.2 |
| *F. brevicatenulatum* | GCA_013363135.1 | *F. oxysporum* | GCA_019843925.1 |
| *F. buharicum* | GCA_014822075.1 | *F. oxysporum* | GCA_013363075.1 |
| *F. bulbicola* | GCA_013186765.1 | *F. oxysporum* | GCA_012610785.1 |
| *F. bulbicola* | GCA_013758895.1 | *F. oxysporum* | GCA_012610835.1 |
| *F. burgessii* | GCA_002980515.1 | *F. oxysporum* | GCA_020975975.1 |
| *F. buxicola* | GCA_014899095.1 | *F. oxysporum* | GCA_020975995.1 |
| *F. caatingaense* | GCA_013624355.1 | *F. oxysporum* | GCA_020975875.1 |
| *F. camptoceras* | GCA_004367475.1 | *F. oxysporum* | GCA_014843565.1 |
| *F. cerealis* | GCA_019055205.1 | *F. oxysporum* | GCA_002233865.1 |
| *F. chaquense* | GCA_020137375.1 | *F. palustre* | GCA_014899045.1 |
| *F. chaquense* | GCA_020137435.1 | *F. papillatum* | GCA_013186395.1 |
| *F. chaquense* | GCA_020137885.1 | *F. penzigii* | GCA_013623535.1 |
| *F. chlamydosporum* | GCA_014898915.1 | *F. phaseoli* | GCA_001680515.1 |
| *F. chuoi* | GCA_022627105.1 | *F. phyllophilum* | GCA_013396025.1 |
| *F. chuoi* | GCA_022627125.1 | *F. pilosicola* | GCA_020615335.1 |
| *F. cicatricum* | GCA_021730345.1 | *F. pininemorale* | GCA_002165215.1 |
| *F. circinatum* | GCA_000497325.4 | *F. piperis* | GCA_027946385.1 |
| *F. circinatum* | GCA_013396185.1 | *F. poae* | GCA_001675295.1 |
| *F. circinatum* | GCA_024514935.1 | *F. poae* | GCA_013623615.1 |
| *F. citri* | GCA_004367485.1 | *F. poae* | GCA_019609905.1 |
| *F. clavum* | GCA_004367155.1 | *F. praegraminearum* | GCA_002093855.1 |
| *F. coffeatum* | GCA_003316985.1 | *F. proliferatum* | GCA_003290285.1 |
| *F. coffeatum* | GCA_004367465.1 | *F. proliferatum* | GCA_003615215.1 |
| *F. coicis* | GCA_013781345.1 | *F. proliferatum* | GCA_036288945.1 |
| *F. commune* | GCA_013618355.1 | *F. proliferatum* | GCA_900029915.1 |
| *F. concentricum* | GCA_014824425.1 | *F. proliferatum* | GCA_900067095.1 |
| *F. concolor* | GCA_013184415.1 | *F. protoensiforme* | GCA_011320165.1 |
| *F. continuum* | GCA_013184455.1 | *F. pseudoanthophilum* | GCA_013395995.1 |
| *F. cortaderiae* | GCA_009617495.1 | *F. pseudocircinatum* | GCA_013396035.1 |
| *F. cortaderiae* | GCA_013184305.1 | *F. pseudograminearum* | GCA_000303195.2 |
| *F. cortaderiae* | GCA_017656885.1 | *F. pseudograminearum* | GCA_000974265.2 |
| *F. cortaderiae* | GCA_017656915.1 | *F. pseudograminearum* | GCA_001703955.2 |
| *F. cortaderiae* | GCA_017656935.1 | *F. pseudonygamai* | GCA_013186785.1 |

Continued

| Species | GenBank | Species | GenBank |
|---|---|---|---|
| *F. culmorum* | GCA_013618375.1 | *F. ramigenum* | GCA_013186855.1 |
| *F. culmorum* | GCA_019055245.1 | *F. redolens* | GCA_014899085.1 |
| *F. culmorum* | GCA_900074845.1 | *F. redolens* | GCA_019843785.1 |
| *F. cuneirostrum* | GCA_001680505.1 | *F. redolens* | GCA_020744475.1 |
| *F. cyanostomum* | GCA_014824385.1 | *F. robinianum* | GCA_013623725.1 |
| *F. decemcellulare* | GCA_013266205.1 | *F. robinianum* | GCA_024115165.1 |
| *F. denticulatum* | GCA_013396175.1 | *F. sacchari* | GCA_013759005.1 |
| *F. devonianum* | GCA_017140155.1 | *F. sacchari* | GCA_017165645.1 |
| *F. dimerum* | GCA_013623525.1 | *F. sambucinum* | GCA_001567575.1 |
| *F. dlaminii* | GCA_013186775.1 | *F. sambucinum* | GCA_014899025.1 |
| *F. domesticum* | GCA_013618395.1 | *F. sarcochroum* | GCA_013266185.1 |
| *F. drepaniforme* | GCA_012978555.1 | *F. scirpi* | GCA_004367495.1 |
| *F. duplospermum* | GCA_003946985.1 | *F. secorum* | GCA_013363185.1 |
| *F. equiseti* | GCA_003313175.1 | *F. setosum* | GCA_013623625.1 |
| *F. equiseti* | GCA_004367125.1 | *F. sibiricum* | GCA_014898995.1 |
| *F. equiseti* | GCA_019054785.1 | *F. solani* | GCA_002215905.1 |
| *F. equiseti* | GCA_019054855.1 | *F. solani* | GCA_023522795.1 |
| *F. equiseti* | GCA_019054885.1 | *F. solani* | GCA_027945525.1 |
| *F. equiseti* | GCA_019055045.1 | *F. solani-melongenae* | GCA_023101225.1 |
| *F. equiseti* | GCA_019055055.1 | *F. sororula* | GCA_017579625.1 |
| *F. equiseti* | GCA_019055165.1 | *F. sporotrichioides* | GCA_003012315.1 |
| *F. falciforme* | GCA_013363125.1 | *F. sporotrichioides* | GCA_019054645.1 |
| *F. falciforme* | GCA_026873545.1 | *F. staphyleae* | GCA_017140175.1 |
| *F. falciforme* | GCA_027945395.1 | *F. sterilihyphosum* | GCA_013186845.1 |
| *F. falciforme* | GCA_027945495.1 | *F. stilboides* | GCA_014822085.1 |
| *F. falciforme* | GCA_027945505.1 | *F. subglutinans* | GCA_013396075.1 |
| *F. falciforme* | GCA_027945515.1 | *F. sublunatum* | GCA_013623665.1 |
| *F. flagelliforme* | GCA_003012295.1 | *F. subtropicale* | GCA_003670145.1 |
| *F. flagelliforme* | GCA_004367175.1 | *F. succisae* | GCA_013186925.1 |
| *F. floridanum* | GCA_003947005.1 | *F. tanahbumbuense* | GCA_012977735.1 |
| *F. foetens* | GCA_013623845.1 | *F. temperatum* | GCA_001513835.1 |
| *F. fracticaudum* | GCA_003353625.1 | *F. thapsinum* | GCA_013186935.1 |
| *F. fujikuroi* | GCA_013759025.1 | *F. tjaetaba* | GCA_013396195.1 |
| *F. fujikuroi* | GCA_900079805.1 | *F. torreyae* | GCA_014824505.1 |
| *F. fujikuroi* | GCA_900096505.1 | *F. torreyae* | GCA_027945595.1 |
| *F. fujikuroi* | GCA_900096615.1 | *F. torulosum* | GCA_013623875.1 |
| *F. fujikuroi* | GCA_900096625.1 | *F. transvaalense* | GCA_013623685.1 |
| *F. fujikuroi* | GCA_900096635.1 | *F. tricinctum* | GCA_012977725.1 |
| *F. fujikuroi* | GCA_900096645.1 | *F. tricinctum* | GCA_020744515.1 |
| *F. fujikuroi* | GCA_900096685.1 | *F. tuaranense* | GCA_013363205.1 |
| *F. fujikuroi* | GCA_900096705.1 | *F. tucumaniae* | GCA_001680535.1 |
| *F. gaditjirri* | GCA_013266175.1 | *F. tucumaniae* | GCA_001680595.1 |
| *F. gerlachii* | GCA_017656835.1 | *F. tucumaniae* | GCA_001680725.1 |
| *F. gerlachii* | GCA_017656845.1 | *F. tucumaniae* | GCA_021730365.1 |
| *F. globosum* | GCA_013396165.1 | *F. tupiense* | GCA_013364945.1 |
| *F. goolgardi* | GCA_014899075.1 | *F. udum* | GCA_002194535.1 |
| *F. graminearum* | GCA_000240135.3 | *F. udum* | GCA_013186905.1 |
| *F. graminearum* | GCA_018345295.1 | *F. ussurianum* | GCA_017656685.1 |
| *F. graminearum* | GCA_018345365.1 | *F. vanettenii* | GCA_000151355.1 |
| *F. graminearum* | GCA_018345335.1 | *F. venenatum* | GCA_013623635.1 |
| *F. graminearum* | GCA_018346575.1 | *F. venenatum* | GCA_020744135.1 |
| *F. graminearum* | GCA_018346815.1 | *F. verrucosum* | GCA_013623715.1 |
| *F. graminearum* | GCA_018345325.1 | *F. verticillioides* | GCA_000149555.1 |
| *F. graminearum* | GCA_001717905.1 | *F. verticillioides* | GCA_003316995.2 |
| *F. graminearum* | GCA_018219475.1 | *F. verticillioides* | GCA_003317015.2 |
| *F. graminearum* | GCA_018219485.1 | *F. verticillioides* | GCA_020882315.1 |
| *F. graminearum* | GCA_018345315.1 | *F. verticillioides* | GCA_027571605.1 |
| *F. graminearum* | GCA_018345835.1 | *F. virguliforme* | GCA_000585705.1 |
| *F. graminearum* | GCA_018346795.1 | *F. virguliforme* | GCA_001680525.1 |
| *F. graminum* | GCA_013266165.1 | *F. virguliforme* | GCA_013363175.1 |

Continued

| Species | GenBank | Species | GenBank |
|---|---|---|---|
| *F. guadeloupense* | GCA_021655865.1 | *F. vorosii* | GCA_017656575.1 |
| *F. guadeloupense* | GCA_021655875.1 | *F. vorosii* | GCA_017656615.1 |
| *F. guttiforme* | GCA_013186795.1 | *F. xylarioides* | GCA_004329255.1 |
| *F. haematococcum* | GCA_004026335.1 | *F. xylarioides* | GCA_013183765.1 |
| *F. haematococcum* | GCA_004026385.1 | *F. xylarioides* | GCA_013623735.1 |
| *F. hainanense* | GCA_013618405.1 | *F. xylarioides* | GCA_018296325.1 |
| *F. heterosporum* | GCA_013396295.1 | *F. xyrophilum* | GCA_008711575.1 |
| *F. hostae* | GCA_013184365.1 | *F. xyrophilum* | GCA_008711595.1 |
| *F. humuli* | GCA_004366955.1 | *F. xyrophilum* | GCA_008711615.1 |
| *F. humuli* | GCA_013753835.1 | *F. zanthoxyli* | GCA_013623745.1 |
| *F. illudens* | GCA_013623515.1 | *F. zealandicum* | GCA_013266195.1 |
| *F. incarnatum* | GCA_004367075.1 | *F. parabolicum* | GCA_013623825.1 |

**生物信息学分析构建物种特异靶标序列库** 使用Jellyfish (v1.1.12) 将镰刀菌属173个物种共304个基因组切割成25-kmer的片段。随后, 提取所有包含TTTV开头 (V代表G、C或A) 或VAAA结尾 (V代表C、G、T) 的25-kmer原间隔序列相邻基序 (protospacer adjacent motif, PAM)。使用shell脚本和perl脚本对同一物种多个基因组的25-kmer进行频率计算 (在基因组中出现的频率, f ≥ 50%), 保证25-kmer具有种内保守性。对于每个物种, 将所有含PAM的25-kmer序列都与镰刀菌属其他172个物种的全基因组含PAM的25-kmer进行比对, 选择除PAM序列外21-kmer与其他物种序列存在有三个核苷酸以上差异或1个插入缺失的序列, 从而构建物种特异靶标序列库。

**基于BLAST的物种特异靶标序列的特异性验证** 从镰刀菌属7个物种: *F oxysporum*、*F. solani*、*F. fujikuroi*、*F. sambucinum*、*F. culmorum*、*F. incarnatum* 和 *F. equiseti* 的特异靶标序列库中为镰刀菌属7个物种各随机选择4～20条特异靶标序列在NCBI数据库中进行BLAST比对 (https://blast.ncbi.nlm.nih.gov/Blast.cgi), 确保特异靶标序列与其他物种相比没有PAM序列或者除PAM序列外的21-kmer与其他物种序列有三个核苷酸以上差异或1个插入缺失。使用Primer Premier 6.0软件根据经过NCBI特异性验证的7个物种的特异靶标序列上下游250 bp范围设计扩增特异靶标序列的引物, 对特异靶标序列进行简单PCR、琼脂糖凝胶电泳以及Sanger测序, 筛选能在目标物种扩增并在目标物种中成功检测的特异靶标序列。最后, 从中为每个物种随机各选取一条用于后续实验检测的特异靶标序列。此外, 为了解特异靶标序列的注释信息, 将每个物种的特异靶标序列与目标物种基因组进行比对。若存在于基因组注释区域进一步通过文献进行检索该区域的功能及在物种鉴定的情况; 若存在于基因组未注释区域亦需进一步检索该序列是否有用于物种鉴定的报道。

**基于Sanger测序验证物种特异靶标序列** 取镰刀菌属7个物种的DNA作为样本, 分别以每个物种的特异靶标序列引物扩增目标物种和其他物种。PCR扩增总体系为20 μL, 其中包括10 μL 2×*Taq* Master Mix、上下游引物 (10 μmol·L$^{-1}$) 各1 μL、2 μL DNA模板 (10 ng·μL$^{-1}$) 和6 μL双蒸水。样品在PCR仪器中进行扩增, PCR程序设定如下: 94 ℃, 3 min; 94 ℃, 30 s, 55 ℃, 30 s, 72 ℃, 30 s, 共30个循环; 72 ℃, 7 min。分别吸取6 μL PCR产物和5 μL DL1000 marker点样于1.5%的琼脂糖凝胶点样孔中, 在110 V、120 mA条件下电泳1 h, 检查目标物种的特异靶标序列扩增序列是否在250 bp范围出现条带, 若出现目的条带, 将对应的PCR产物在ABI 3730 XL分析仪上进行双向测序。使用Codon-Code Aligner软件 (10.0.3版) 进行序列拼接组装和一致序列生成, 并删除低质量的序列。拼接后的序列使用MEGA 7.0进行特异靶标序列比对。

**基于CRISPR-Cas12a系统检测物种特异靶标序列** 采用ERA室温扩增试剂盒与基因组编辑技术相结合的快速鉴定系统对镰刀菌属7个物种的特异靶标序列进行检测, 包括扩增和检测两个步骤。扩增反应体系的总体积为50 μL, 包括43 μL ERA扩增混合物、上下游引物 (10 μmol·L$^{-1}$) 各2.5 μL和2 μL DNA (10 ng·μL$^{-1}$)。检测系统的总体积也为50 μL, 包括10 μL 10×NEBuffer 2.1、2 μL Cas12a (20 nmol·L$^{-1}$)、3.3 μL crRNA (300 nmol·L$^{-1}$)、4 μL ssDNA (400 nmol·L$^{-1}$): [5′-FAM-CCCCCCCCCC-BHQ-1-3′,6-羧基荧光素 (6-carboxyfluorescein, FAM), 黑洞淬灭剂 (black hole quencher, BHQ)], 或4 μL FAM-Biotin荧光信号分子 (5′-FAM-TTATTATT-Biotin-3′, 80 nmol·L$^{-1}$), 以及30.7 μL无酶水。ERA扩增试剂、DNA和引物在37 ℃下孵育20 min。随后加入NEBuffer 2.1、Cas12a、crRNA和无酶水, 并将混合物进一步在37 ℃下孵育10 min。最后, 加入ssDNA在蓝光照射器下直接观察荧光。当用试纸条

进行特异靶标序列的特异性检测时, 使用 80 nmol·L$^{-1}$ 的 FAM-Biotin 荧光信号分子替代 ssDNA, 并直接将试纸条放进试剂管中进行检测。检测过程中, 扩增产物被固定在检测线上, 通过生物素配体和抗 FAM 单克隆抗体识别。试纸条上同时出现测试线和对照线, 或仅出现测试线都表示阳性结果。

## 结果

### 1 镰刀菌属 7 个物种的 DNA 条形码鉴定

利用 ITS 条形码在镰刀菌属 7 个近缘物种鉴定中的应用效果进行评估。结果表明, 作为真菌通用 DNA 条形码的 ITS 区域获得 100% 的扩增成功率, 具有明显的单一明亮条带。将条带进行 Sanger 测序后于 NCBI 进行 BLAST 比对, 发现 *F. oxysporum*、*F. sambucinum* 和 *F. solani* 的 ITS 序列仅分别与目标物种具有 100% 的相似性, 说明 ITS 能够对这三个物种进行准确区分; 而 *F. culmorum*、*F. equiseti*、*F. fujikuroi* 及 *F. incarnatum* 的 ITS 序列比对分析显示同时 100% 比对到目标物种与同属其他近缘物种。这也证明了 ITS 在真菌近缘物种鉴定中的局限性。

### 2 镰刀菌属 173 个物种特异靶标序列库构建

通过对镰刀菌属物种基因组进行生物信息学分析, 成功构建镰刀菌属 173 个物种的特异靶标序列库, 统计结果显示, 物种含 PAM 的靶标序列及频率 ≥ 50% 的含 PAM 的靶标序列去重后的数量主要在 860 001～1 260 000 条, 特异靶标序列及频率 ≥ 50% 的特异靶标序列去重后数量主要集中在 10 001～50 000 条 (图 1)。其中, 镰刀菌属 7 个物种特异靶标序列数量为 *F. oxysporum* 196 335、*F. solani* 132 328、*F. fujikuroi* 42 086、*F. sambucinum* 76 804、*F. culmorum* 6 155、*F. incarnatum* 26 604 和 *F. equiseti* 86 797 (表 2)。对于高度近缘物种 *F. incarnatum* 和 *F. equiseti* 在过滤掉非特异靶标序列之后, 两个近缘物种仍有丰富的特异靶标序列, 充分说明利用时珍法能够为近缘物种鉴定筛选丰富的特异靶标序列。

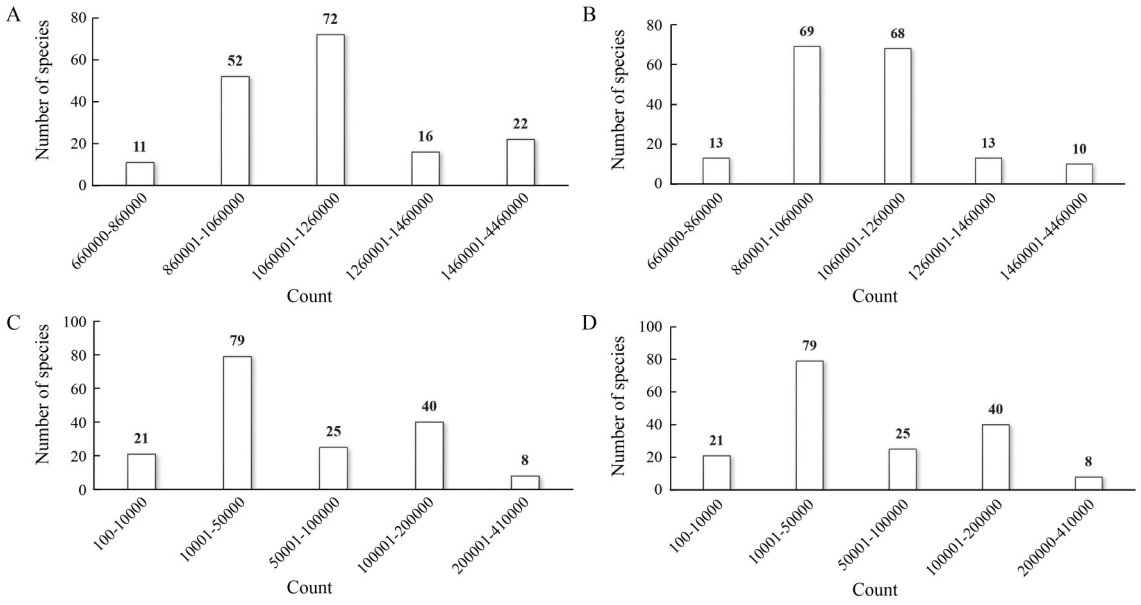



**Figure 1** Distribution of 173 species of *Fusarium* and target sequence counts. A and B: Distribution counts of 173 species based on the number of PAM target sequences (deduplicated) and PAM target sequences (frequency ≥ 50%, deduplicated) across different intervals, respectively; C and D: Distribution of 173 species based on the number of specific target sequences (deduplicated) and specific target sequences (frequency ≥ 50%, deduplicated) across different intervals, respectively

**Table 2** The bioinformatics analysis results of specific target sequences for 7 *Fusarium* species. f: Frequency

| Species | 25-bp unique sequence with PAM (count) | High-frequency shared unique target with PAM (f ≥ 0.5) (count) | Specific target sequence (count) | High-frequency specific target sequence (f ≥ 0.5) (count) |
|---|---|---|---|---|
| *Fusarium culmorum* | 1 180 380 | 975 727 | 6 155 | 3 115 |
| *F. fujikuroi* | 1 819 655 | 1 103 386 | 42 086 | 15 120 |
| *F. equiseti* | 1 574 243 | 1 010 242 | 86 797 | 40 926 |
| *F. incarnatum* | 952 560 | 952 560 | 26 604 | 26 604 |
| *F. oxysporum* | 4 161 493 | 1 057 351 | 196 335 | 7 922 |
| *F. sambucinum* | 1 101 755 | 1 101 755 | 76 804 | 76 804 |
| *F. solani* | 2 497 904 | 904 987 | 132 328 | 32 503 |

从特异靶标序列库中为7个物种: *F. oxysporum*、*F. solani*、*F. fujikuroi*、*F. sambucinum*、*F. culmorum*、*F. incarnatum*和*F. equiseti*各随机选择4～20条具有物种特异性的靶标序列, 并根据各特异靶标序列上下游250 bp范围内设计扩增引物, 进行简单PCR、琼脂糖凝胶电泳, 结果显示, 上述7个物种分别获得3条、1条、7条、6条、3条、3条和2条能在对应目标物种中扩增到目的条带的特异靶标序列 (表3、图2); 随后通过Sanger测序技术验证上述7个物种的特异靶标序列均存在于对应目标物种基因组中; 从测序验证后的物种特异靶标序列中为7个物种随机选取一条用于后续特异性实验验证的特异靶标序列 (表4)。

**Table 3** Validated specific target sequences for seven *Fusarium* species in the target species

| Species | Validated specific target sequences in the target species | Primer (5′→3′) |
|---|---|---|
| *Fusarium culmorum* | Fcu-1: TTTCGAGATGAGTTGCGGAAACTTG | Fcu-1-F: TTGGTGAATGAATCAGCCCATGTAG |
| | Fcu-2: TTTACATGCTGATCAATCATGGATG | Fcu-1-R: AATGTTCATGTCGGAGAATGATACG |
| | Fcu-3: AGGTACAGGTCCAGAATCAGGTAAA | Fcu-2-F: GCTGAACATGCCTGAGATTCTGATC |
| | | Fcu-2-R: AATAGGTACAGCAGGACACCAAGAA |
| | | Fcu-3-F: TGAAGTTGATGGCAAATAAGGACTC |
| | | Fcu-3-R: CTGTGAATCTGGGCAAATATGAATC |
| *F. fujikuroi* | Ffu-1: TGTACCTCCGTAGGTGCGCAGCAAA | Ffu-1-F: AAAGACAATTCCTCCCGCTAGACTT |
| | Ffu-2: GTGCACACCCTTCGAAGTGGGCAAA | Ffu-1-R: AGTTAGAACGAATGGTAATGCTGTC |
| | Ffu-3: AATTGCTAGAAATCGGGGGGTTAAA | Ffu-2-F: GCAGGTTGTATCCCATCCAATATTC |
| | Ffu-4: TTTCCTTGAGAGCAACTCGCTAGCA | Ffu-2-R: TGAAGATGAGTCTACGGTCCATGAT |
| | Ffu-5: TTTGCCCTTGTATAGTTATGGTTCA | Ffu-3-F: GAACCAGTTTGGCGATCTCAGAAGA |
| | Ffu-6: TTTCCTCCCATATTTCGAGCCTTAA | Ffu-3-R: TTGTCTCCCTCCTTACCGTATCATT |
| | Ffu-7: TTTACCAAGGAGCGACAGACTCCGA | Ffu-4-F: GAAACTTGAGAATATGCTTGGCTAC |
| | | Ffu-4-R: TGTGTATAATTGTGAGGGCAGTATC |
| | | Ffu-5-F: TGTACCTCTACACTGAGAACAAACT |
| | | Ffu-5-R: TATTGCTTGCCAAGTCATCGGACAA |
| | | Ffu-6-F: AGCTCTACCTATAGATGCCATACTT |
| | | Ffu-6-R: AGACCGTGTTTGGAATTTGGATTTG |
| | | Ffu-7-F: ATGCCAGATTCGATCAGAACATAAC |
| | | Ffu-7-R: ATGCCAGATTCGATCAGAACATAAC |
| *F. equiseti* | Fe-1: GCACGGCAAGAGATATCAATCTAAA | Fe-1-F: TTGAAACCCAGTTCTGGAGATTATC |
| | Fe-2: GGGCACTTGGCCGAATTATACGAAA | Fe-1-R: AACTTTGGCACACTACGCAATATAC |
| | | Fe-2-F: TATTGAGGACATATGCTCTCGGAAG |
| | | Fe-2-R: ATAACGCCTCCTGAAGCTGAGATAG |
| *F. incarnatum* | Fi-1: TTTAACATATTAGCGGTATTCAAAA | Fi-1-F: GTGCGCGAACATTATAGTCAACATA |
| | Fi-2: TTTCATACACTACTTGATCGATAAA | Fi-1-R: CATGTACCACAGTCAGAGCATAACA |
| | Fi-3: TTGACGGTACCTCCGCTTTTACAAA | Fi-2-F: TGATTGGAAGTAAGGATCGAATGAG |
| | | Fi-2-R: GCGAAGTTGTAGATATTGCTGTTTC |
| | | Fi-3-F: AAAGCATATTTGACGAGCATGAGAG |
| | | Fi-3-R: AGAGATAGGTCAAGGATGTGAGATG |
| *F. oxysporum* | Fo-1: TTTCCATGAGTGAAGACTCCTCGCA | Fo-1-F: AATGCCGACAATCGATCCATTACTC |
| | Fo-2: TTTGACTTCACCTCACTCACGATAA | Fo-1-R: CTGCCTGAACGAGATATTCTTGACT |
| | Fo-3: TTTCTACCTTAAGGATGTGGACGAA | Fo-2-F: TACGTACCTAGCTTCCATTGGAGCA |
| | | Fo-2-R: CGCCCATTATGAATGTTTGCTTGTG |
| | | Fo-3-F: AAAGAGAGGAACAACGTTACAGGAC |
| | | Fo-3-R: GCTACATACCTTACAGTCACAAGTC |
| *F. sambucinum* | Fsam-1: TTTCACAAGCCTCAATATGTTCGGT | Fsam-1-F: ATCATCATTACTCTCACTCGCAACA |
| | Fsam-2: TTTGTTGTCGTCAAGGTTATTAAAA | Fsam-1-R: CATAGCTAACGACTTCTTTCACCTC |
| | Fsam-3: TTTTTCGGATATGCATGCCGAGAAA | Fsam-2-F: GTAATAACGTCCAGTCGAAGAAGGT |
| | Fsam-4: TTGGTCATGCAATGAGCACCACAAA | Fsam-2-R: CTGTGAACCATTCCCGTTGTCTTAC |
| | Fsam-5: TTGACTGCTTTTAAAACTGAATAAA | Fsam-3-F: CTATCAAATATTCCGAAGAGGTGAG |
| | Fsam-6: TTTAGAGGATCTACAGATACAATAA | Fsam-3-R: GTATTGGTTTCTAGGTGGAAGTTGA |
| | | Fsam-4-F: GAGCATGGAAGTTGTATCAATTCAG |
| | | Fsam-4-R: TCCATCCGTAGCCATAACTACATAC |
| | | Fsam-5-F: GTACAGCAACTTGATTAGGAACTAG |
| | | Fsam-5-R: GCTGGGATAAATACATGGAACTCAT |
| | | Fsam-6-F: ATTCCCTGCTTATCAACAAACTCTC |
| | | Fsam-6-R: TGAGGTGATGAGATTGACAATGAAG |
| *F. solani* | Fso-1: CCTTCTATAAGTCCGACCCGGTAAA | Fso-1-F: CTTACTGGTCGAAGAAGCTCAGGAC |
| | | Fso-1-R: TGTCTTGAAAGTCTCGAAGCATCTC |

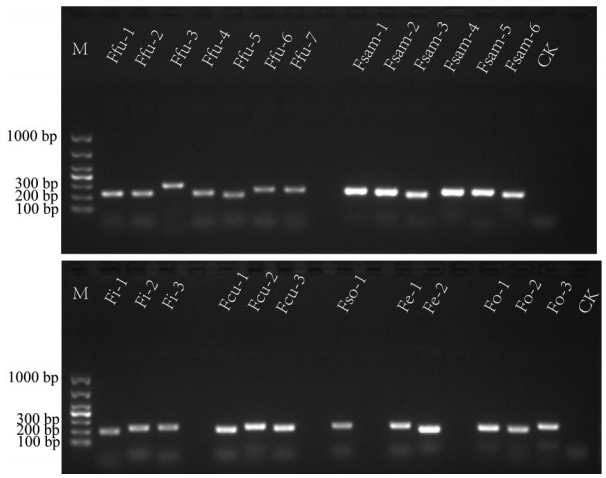



**Figure 2** Agarose gel electrophoresis showing the amplified target bands of specific target sequences for seven *Fusarium* species in the target species. M: DL1000 marker; CK: Blank control

对于7条进行实验验证的特异靶标序列通过NCBI核酸数据库进行BLAST比对均具有物种特异性, 尤其是对于用条形码序列难以准确鉴定的物种*F. culmorum*、*F. incarnatum*、*F. equiseti*和*F. fujikuroi*。此外, 通过将7个物种的特异靶标序列于目标物种基因组中查询, 发现*F. solani*的特异靶标序列位于基因组编码未知蛋白的区域, 数据库检索中未发现该序列用于物种鉴定, 这表明位于未知功能的基因序列上的特异靶标序列具备物种鉴定的潜力; 而其余物种特异靶标序列均位于基因组未注释区域 (表4), 且在物种鉴定中表现出良好的分辨能力, 为镰刀菌属物种的准确鉴定提供新的视角, 不局限于基因区域。

## 3 Sanger测序成功检测物种特异靶标序列

对镰刀菌属7个物种的特异靶标序列进行特异性实验验证, 通过PCR扩增和琼脂糖凝胶电泳分析, 结果显示7个物种的特异靶标序列扩增引物均表现出良好的特异性, 只有目标菌种在250 bp左右产生单一的明亮条带, 其他物种未出现明显条带 (图3A～G)。随后, 采用Sanger测序技术对7个物种的特异靶标序列进行检测。结果表明, 这些特异靶标序列的测序质量高且能够在目标物种中完全匹配 (图3H～N), 说明这

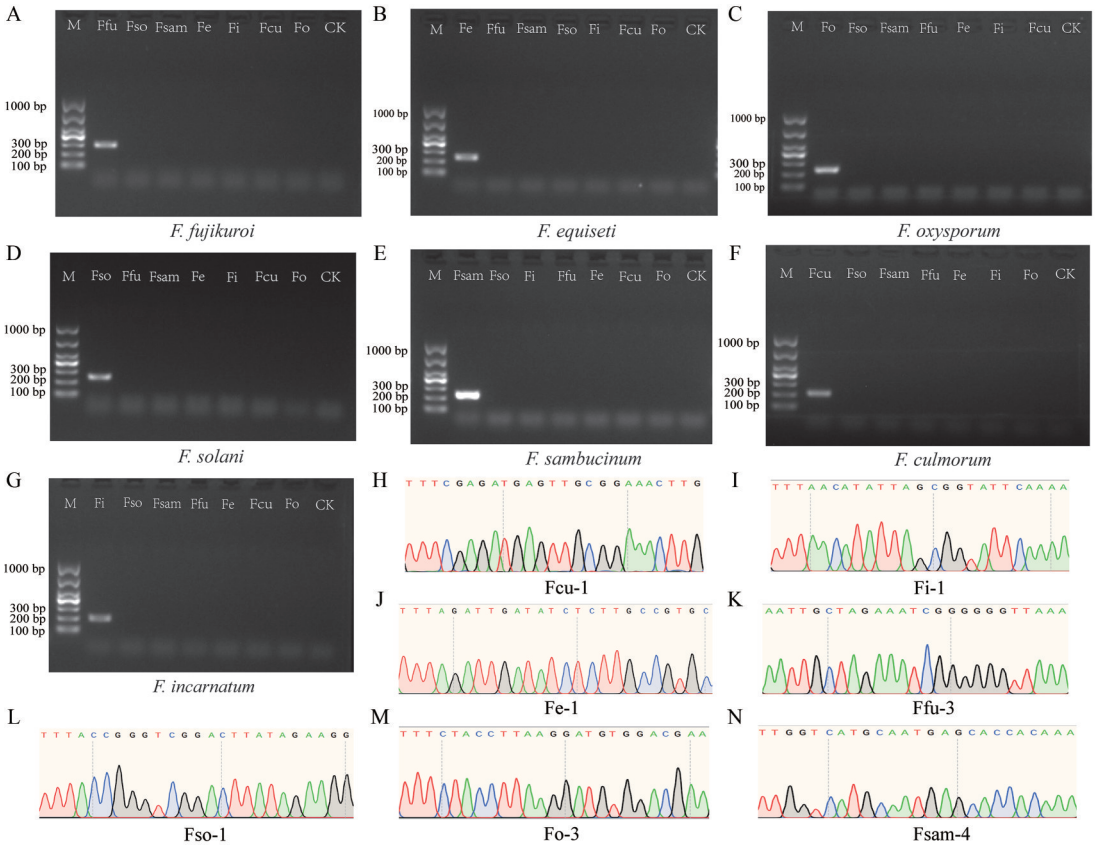



**Figure 3** Agarose gel electrophoresis and sequencing peak graphs results of specific target sequences for seven *Fusarium* species. A−G: Specific target sequences for *F. fujikuroi*, *F. equiseti*, *F. oxysporum*, *F. solani*, *F. sambucinum*, *F. culmorum* and *F. incarnatum*; M: DL1000 marker; CK: Blank control. H-N: The sequencing peak graphs results of species-specific target sequences for *F. culmorum*, *F. incarnatum*, *F. equiseti*, *F. fujikuroi*, *F. solani*, *F. oxysporum*, *F. sambucinum*, respectively. Ffu: *F. fujikuroi*; Fso: *F. solani*; Fsam: *F. sambucinum*; Fe: *F. equiseti*; Fi: *F. incarnatum*; Fcu: *F. culmorum*; Fo: *F. oxysporum*

7个物种的特异靶标序列能够准确鉴定目标物种。

## 4 CRISPR-Cas12a系统快速检测物种特异靶标序列

为避免分子鉴定对测序技术的依赖, 设计每个物种特异靶标序列对应的crRNA (表4), 利用CRISPR-Cas12a技术对筛选的物种特异靶标序列进行检测。结果显示, 只有目标物种能够产生明显的荧光信号和试纸条阳性条带, 非目标物种未出现阳性结果 (图4), 表明CRISPR-Cas12a系统能够有效区分目标物种与其近缘物种, 特异靶标序列具有高度特异性。此外, 通过CRISPR-Cas12a技术在30 min内将鉴定结果以可视荧光和试纸条的可视化形式呈现, 缩短物种鉴定的时限, 具有现场检测的优势。

**Table 4** Seven species-specific target sequences in the genus *Fusarium* and their corresponding genomic locations, and crRNA

| Species | Species-specific target sequence | Genomic location | Annotation | crRNA (5′→3′) |
|---|---|---|---|---|
| *Fusarium oxysporum* | Fo-3 | Contig_0001134 | Unannotated | UAAUUUCUACUAAGUGUAGAUUACCUUAAGGAUGUGGACGAA |
| *F. solani* | Fso-1 | Unknown | Uncharacterized protein | UAAUUUCUACUAAGUGUAGAUCCGGGUCGGACUUAUAGAAGG |
| *F. fujikuroi* | Ffu-3 | Chromosome I | Unannotated | UAAUUUCUACUAAGUGUAGAUACCCCCCGAUUUCUAGCAAUU |
| *F. sambucinum* | Fsam-4 | Contig_0000243 | Unannotated | UAAUUUCUACUAAGUGUAGAUUGGUGCUCAUUGCAUGACCAA |
| *F. culmorum* | Fcu-1 | C0000028 | Unannotated | UAAUUUCUACUAAGUGUAGAUCAUGCUGAUCAAUCAUGGAUG |
| *F. incarnatum* | Fi-1 | Contig_0000048 | Unannotated | UAAUUUCUACUAAGUGUAGAUACAUAUUAGCGGUAUUCAAAA |
| *F. equiseti* | Fe-1 | Scaffold3 | Unannotated | UAAUUUCUACUAAGUGUAGAUGAUUGAUAUCUCUUGCCGUGC |

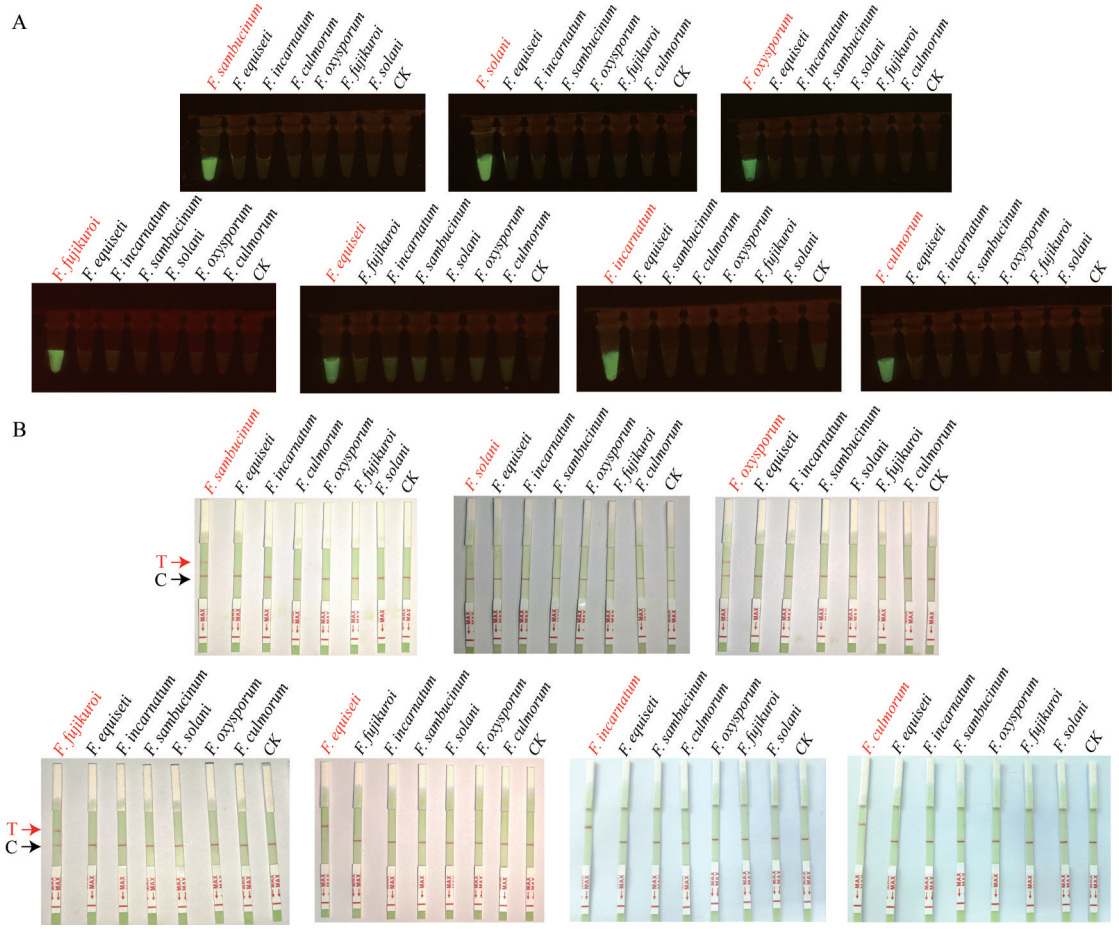



**Figure 4** Precise species identification of seven *Fusarium* species using the CRISPR-Cas12a technology. A and B: Representing two forms of presentation, visible fluorescence, and lateral flow strip. CK: Nuclease-free water. Each group contains DNA from different species as the substrate. T: Text line; C: Control line. The presence of T indicates a positive result, whereas the presence of only C indicates a negative result

## 讨论

镰刀菌属真菌一直是物种鉴定的难点, 传统形态鉴定和现有的分子鉴定方法均难以准确区分近缘物种[27,38,39,48,49]。本研究首次利用时珍法构建了镰刀菌属173个近缘物种的特异靶标序列库, 并通过实验证明筛选的特异靶标序列具备物种鉴定能力, 解决了条形码难以区分的近缘物种*F. incarnatum*和*F. equiseti*的鉴定难题, 凸显了时珍法在近缘物种鉴定中的优势。课题组前期运用时珍法在动物、植物和真菌物种鉴定领域取得一定进展, 在植物鉴定方面, 该方法成功区分了番红花 (*Crocus sativus*) 及其混伪品, 同时可有效鉴别不同类群的药用植物[41]; 在动物鉴定中, 精确鉴定了梅花鹿 (*Cervus nippon*) 及其近缘种马鹿 (*C. elaphus*)[42]; 对于真菌类群, 该方法不仅能够准确识别13种远缘真菌[43], 还可区分青霉属 (*Penicillium*) 7个近缘物种[44], 并成功解决了传统方法难以鉴别的链格孢属 (*Alternaria*) 与曲霉属 (*Aspergillus*) 近缘物种的鉴定难题[45,46]。本研究进一步证实, 时珍法可用于镰刀菌属近缘物种的准确鉴定。

本研究从NCBI基因组数据库下载了镰刀菌属181个物种的1 802个基因组数据, 鉴于该属部分近缘物种分类学鉴定困难, 作者对这些基因组数据进行了全面评估, 发现存在菌株基因组提交信息不完整、文献引用信息参考价值有限等问题。为确保生物信息学分析的准确性, 制定了严格的筛选标准: 优先选择国内外知名菌种保藏中心 (CBS、CGMCC、NRRL、ATCC等) 上传的标准菌株基因组; 要求基因组数据具有高质量或数量的文献支持; 基因组数据提交机构需为微生物研究的权威机构; 优先选用经系统发育分析验证的基因组。通过严格筛选, 最终获得173个物种304个高质量基因组数据。然而, 筛选标准严格, 部分正确的物种基因组数据可能被排除, 导致分析范围相对缩小。因此, 未来研究计划进一步扩展至现有具备基因组数据的镰刀菌属物种, 纳入更多菌株基因组数据进行生物信息学分析; 筛选高保守性的物种特异靶标序列, 减少种内变异对鉴定结果的影响; 纳入更多存在鉴定难题的近缘物种和具有重要经济影响的物种进行实验验证, 以实现物种的准确鉴定。综上所述, 本研究为镰刀菌属真菌的精准鉴定提供了新思路, 在药材污染和农业病害防治及真菌分类学研究中具有重要应用价值。

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**利益冲突**: 本文的研究内容无任何利益冲突。

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