

# 广州地区番石榴根结线虫鉴定与14-3-3基因的克隆

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**摘要:**【目的】摸清广州地区番石榴根结线虫的种类、分布及致病机制。【方法】观察番石榴根结线虫的雄虫、二龄幼虫、雌虫及其会阴花纹的形态学特征, 根据根结线虫的通用引物#C2F3和#1108对线虫mtDNA的CO II和1rRNA基因间序列进行PCR扩增, 获得750 bp的特异扩增产物, 将扩增片段在GenBank上进行Blast比对。通过南方根结线虫与北方根结线虫的14-3-3序列设计简并引物, 进一步扩增已鉴定的象耳豆根结线虫的14-3-3基因。【结果】广州地区番石榴根结线虫的形态与象耳豆根结线虫(*Meloidogyne enterolobii*)相似; 其扩增片段与象耳豆根结线虫(*M. enterolobii*)比对, 序列相似性在99%以上。扩增了象耳豆根结线虫的14-3-3基因, 结果显示该基因的开放阅读框包含783 bp的片段, 编码261个氨基酸, 命名为*Me-14-3-3*。【结论】广州地区番石榴根结线虫为象耳豆根结线虫(*M. enterolobii*), 14-3-3蛋白基因被成功克隆, 扩增结果为进一步研究14-3-3蛋白基因在象耳豆根结线虫生长发育中的功能、摸清致病机制等方面奠定良好的基础。

**关键词:** 番石榴; 根结线虫; 种类鉴定; *Me-14-3-3*; 克隆

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## Identification of guava root-knot nematodes in Guangzhou and the cloning of 14-3-3 gene

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**Abstract:** 【Objective】To make sure what species the root knot nematode was that affected the guava in Guangzhou, six guava cultivation areas affected by root knot nematodes were investigated. In addition, to study the pathogenic factor, the 14-3-3 gene was cloned from the identified *Meloidogyne enterolobii*. 【Methods】The females were separated from the diseased roots of guava, and the second instar larvae were incubated from the eggs separated from the roots. The perineal were cut manually under an anatomical microscope, and then the perineal patterns were observed under microscope. The males were separated from the soils of diseased guava using the Baermann method. Then, the female, male and the second instar larvae were observed under optical microscope using the morphological method to identify the root knot nematode species they belonged to. Molecular biology method was also applied for further verification of the molecular results. The primer set #C2F3 and #1108 were used for mtDNA PCR amplification. Then, the degenerate primers were designed according to the 14-3-3 gene of *M. incongnita* and *M. hapla*. RT-PCR was performed to amplify the 14-3-3 gene using the designed primers. The PCR product was run with electrophoresis. The fragment was cloned and sequenced. The sequence was analysis by bioinformatics method. Sequence analysis of genes and proteins was carried out using Blast for sequence similarity analysis at NCBI. Open reading frame (ORF) was analyzed with ORF Finder (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>). The physicochemical properties were predicted by EXPASY (<http://expasy.org/tools/>

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protparam.html), transmembrane analysis using TMPRED forecast ([http://www.ch.embnet.org/software/TMPRED\\_form.html](http://www.ch.embnet.org/software/TMPRED_form.html)). Protein translation and motif analysis were performed using proteomic on-line ExPASy.【Results】The morphological and molecular characteristics of guava root-knot nematodes from diseased root tissue of guava in Xintianer village and Lianan village of Huadu district, Guangzhou and Shixia village of Zengcheng district, Guangzhou, were monitored. The root knot nematodes including female, male and the second instar larvae were observed under optical microscope. The second instar larvae were worm shaped, body ring was small and clear. Side lips were large triangular with lip slightly higher than the middle lip. The mouth needle was slender, conical whole slender, blunt head, crown obvious, sharp tail. The slender needles of the two instar larvae were able to be clearly seen. The base of the stylet was clear, large and round, the middle esophagus line and middle esophagus ball are clear, and the middle esophagus ball was oval. The female body was pear shaped with a prominent neck. Lips was discoid, with slightly rose. The mouth needle was slender, with a large baseball. The perineum was ovoid or oval in shape, the lines were fine and smooth, the tip of the tail was in irregular ring shape, and the lateral line was not obvious. The male was worm shaped, with a clear head ring. The head was high round, slightly constricted, apex cone. The middle esophagus ball was oval. The tail was short and round. Transition was curve. The morphological characteristics showed that they were *M. enterolobii*. With molecular biological techniques, primer #C2F3 and #1108 were used for mtDNA PCR amplification. The amplification product was near 750 bp. Both the morphological and molecular results showed that the guava root-knot nematode was *M. enterolobii*. Degenerate primers were designed according to the 14-3-3 gene of *M. incongnita* and *M. hapla*. The results showed that the open reading frame of the *M. enterolobii* 14-3-3 gene contained 783 bp fragments, encoding a protein of 261 amino acids. It was named as *Me-14-3-3*. The resulting sequence was compared to blastx on NCBI, and the results showed that the 14-3-3 gene was the same as expected. The top two genes with the highest similarity were the 14-3-3 genes from *M. incongnita* (accession number: AAL40719) and *Aphelenchoides besseyi* (accession number: AJE60959), with the similarity of 98% and 80%, respectively. The molecular mass of 14-3-3 protein was predicted with Protparam tools to be 29.6 ku. The theoretical isoelectric point was 4.67, and the molecular formula was  $C_{1296}H_{2065}N_{337}O_{424}S_{13}$ , with a total of 4 135 atoms. The stability coefficient was 46.60, suggesting that the protein was not stable. It had the unique domain of 14-3-3 gene through analysis. The 14-3-3 sequences of *M. incongnita* (AAL40719 and AAR85527) were downloaded. Two *M. hapla* 14-3-3 sequences (2475 and 1597) were obtained by comparison with the transcriptome database of northern root knot nematodes. The 14-3-3 gene cloned from *M. enterolobii* was the same group as the AAL40719 from *M. incongnita* and the 1 597 gene from *M. hapla*. By comparison, most of the three 14-3-3 genes with the same group were conserved, but there were still 8.92% variations.【Conclusion】All the root-knot nematode that separated from the diseased guava in Guangzhou were *M. enterolobii*. The *M. enterolobii* 14-3-3 gene was successfully cloned, and the *M. enterolobii* sequence was analyzed with some tools on internet.

**Key words:** Guava (*Psidium guajava* L.); Root-knot nematode; Species identification; *Me-14-3-3*; Cloning

番石榴(*Psidium guajava* L.)别名鸡矢果、拔子, 桃金娘科番石榴属。属于常绿灌木或小乔木, 是一种重要的热带水果。我国广东、广西、台湾、福建、海南等地区均有引种栽培。由于番石榴营养丰富, 受到越来越多人重视, 在海南、广东、广西、福建、台湾等地已被大面积种植<sup>[1]</sup>。广州地区的从化、萝岗, 南沙、番禺、增城等地也相继大面积种植。然而近年来

由于番石榴根结线虫在广州地区番石榴园的危害越来越严重, 造成成片果树长势弱, 产量骤减, 最后枯黄死去, 挖出病树后病树根部全是瘤状凸起, 老根呈黑褐色, 使广州地区的番石榴生产受到了严重的威胁。为此开展了广州地区番石榴根结线虫普查及鉴定的研究, 明确广州地区番石榴根结线虫的种类与分布, 为开展防治工作提供重要的理论依据。

根结线虫的分类、鉴定方法具有多元化的特点,目前鉴定线虫的主要方法是形态学鉴定方法和分子生物学鉴定方法2种。还有其它方法如:鉴别寄主辅助鉴别、细胞遗传学方法鉴别、同工电泳技术等<sup>[2]</sup>。本文以分子生物学鉴定为主,形态学鉴定为辅的方法来鉴定番石榴根结线虫种类。

14-3-3蛋白是一种广泛存在于植物、动物和酵母菌等真核生物中的一种可溶性酸性蛋白。它是一种具有高度保守性,但又是功能繁多的同源或异源二聚体分子<sup>[3-4]</sup>。14-3-3蛋白参与许多生理过程,它可以通过调节信号转导来调节大量蛋白质的活性,在信号传递、蛋白跨膜转运、细胞周期和凋亡等重要生命活动过程中起到重要作用。研究表明,14-3-3蛋白在线虫生长发育、适应性和致病性起到了很大的作用<sup>[5]</sup>。

笔者采用形态学和分子生物学的方法鉴定了广州从化、南沙等6个地区侵染番石榴的根结线虫的种类,并进一步对鉴定的线虫进行了14-3-3蛋白基因的克隆。研究结果为明确危害番石榴的根结线虫种类,研究14-3-3蛋白基因在其生长发育中的功能,为弄清致病机制等方面奠定良好的基础。

## 1 材料和方法

### 1.1 材料获取

1.1.1 线虫种群的采集与保存 从广州的从化鳌头镇、南沙万顷沙镇十五涌、番禺东涌镇、黄岗区九龙镇、花都区花东镇、增城石滩镇6个地点采集新鲜的被根结线虫侵染的有明显根结线虫症状的番石榴树根部组织以及根附近土壤,标记好采集时间地点后放置于10℃冰箱中保存备用。

1.1.2 各虫态的获取 在体视镜下用解剖针和镊子从获取的根结组织内解剖雌虫、雄虫和卵囊,卵囊放置在双蒸水中在25℃下孵化2~3d获取2龄幼虫。雄虫的获取还可采用贝曼漏斗法<sup>[6]</sup>分离,漏斗铺垫2~3层纱布(漏斗下接软管和去盖离心管),先加入双蒸水然后加入适量土壤进行24h以上的过滤获取雄虫。按以上方法获取各虫态用于以下观察及测量。

### 1.2 线虫的形态鉴定

1.2.1 雌虫形态测量 (1)雌虫会阴花纹制作和观察。在解剖镜下,将成熟的雌虫放置在45%的乳酸中,先切除根结线虫雌虫虫体后部角质膜,用毛刷刷除线虫内含物后修正和清洗角质膜,切割包含会阴

花纹的角质膜转移到载玻片上在显微镜下观察拍照。

(2)雌虫形态观测。挑选雌虫放置在滴水的凹玻片上盖上盖玻片进行观察,主要观测最大体长、最大体宽、口针基部至背食道腺开口的距离/口针长、口针锥部长/口针长。每个数据重复测量10条虫取平均值( $n=10$ ),下同。

1.2.2 2龄幼虫形态测量 挑取各取样点2龄幼虫,放置在滴有无菌水的载玻片上,过酒精灯杀死2龄幼虫,压上盖玻片放置在光学显微镜下观察线虫的口针,口针基部球,尾透明区的数量特征。通过形态特征观察,将主要的形态特征结合其质量特征和数量特征,运用比较形态学的方法进行鉴定。

1.2.3 雄虫形态测量 雄虫制片类似于2龄幼虫制片,主要观测雄虫最大体长、最大体宽、尾长。

### 1.3 线虫的分子鉴定

1.3.1 DNA提取 用挑针挑选各取样点卵囊孵化培养皿中的2龄幼虫各10条放入离心管中,离心管放置在水浴板上在65℃水浴锅中加热60min,而后转入95℃水浴锅水浴加热15min。将水浴加热后的离心管放置在离心机中以8000 r·min<sup>-1</sup>的转速离心1min后,取出离心管放置在-20℃的冰箱中保存备用。

1.3.2 线粒体DNA鉴定引物 选用根结线虫鉴定引物#C2F3(5'-GGTCAATGTCAGAAATTTGTGG-3')和#1108(5'-TACCTTTGACCAATCACGCT-3')进行扩增mtDNA(Mitochondrial DNA)的CO II(cytochrome oxidase II)和1rRNA(1r ribo nucleic acid)基因间序列。

1.3.3 PCR扩增 以#C2F3和#1108作为引物进行PCR扩增,按照以下反应体系进行:模板DNA 1.5 μL, 2×PCR buffer for KOD FX 12.5 μL, dNTPs(2 mmol·L<sup>-1</sup>)5 μL,引物各1 μL, KOD FX 0.5 μL,加H<sub>2</sub>O至25 μL。反应参数:94℃预变性2min;98℃变性10s,55℃退火30s,68℃延伸2min,共35个循环;68℃延伸5min。退火后将PCR管取出。放置于-20℃的冰箱中保存备用或进行电泳。

1.3.4 电泳 分别取上述5点的样本的PCR扩展液5 μL用1.5%(ω)的琼脂糖进行电泳,用凝胶成像系统观察、拍照。

### 1.4 14-3-3基因的克隆

1.4.1 RNA提取 将鉴定后的线虫,选取其卵囊进行

孵化,选取其2龄幼虫进行RNA提取。提取RNA使用北京全式金生物科技有限公司生产的 Easy-Pure<sup>®</sup>RNA Kit试剂盒进行,具体步骤按照说明书进行。

1.4.2 反转录 合成cDNA由北京全式金生物科技有限公司生产的 *TransScript<sup>®</sup> One-Step gDNA Removal and cDNA Synthesis Super Mix* 试剂盒。反应体系如下: Total RNA/mRNA 7  $\mu\text{L}$ , Anchored Oligo (dT)<sub>18</sub> Primer(0.5  $\text{g}\cdot\text{L}^{-1}$ ) 1  $\mu\text{L}$ , 2 $\times$ TS Reaction Mix 10  $\mu\text{L}$ , *TransScript<sup>®</sup> RT/RI Enzyme Mix* 1  $\mu\text{L}$ , gDNA Remover 1  $\mu\text{L}$ , 加入 RNase-free Water 至 40  $\mu\text{L}$ 。按照以下程序进行: 42  $^{\circ}\text{C}$  30 min, 85  $^{\circ}\text{C}$  5 min, 随后将反转录的cDNA取出进行PCR扩增。

1.4.3 引物设计 根据NCBI基因库中的南方根结线虫(*M. incognita*)的14-3-3基因的序列,搜索北方根结线虫转录组数据库中的14-3-3基因序列,进行比对分析后,设计包含开放阅读框的1对简并引物来扩增象耳豆根结线虫的14-3-3基因的cDNA全长序列。引物如下:14-3-3F: ATGACKGAGACAATGTTGGA; 14-3-3R: TTAATTTGTGCCTTCGGCATCA。

1.4.4 cDNA ORF 全长序列的扩增 将2.4.3合成的cDNA用于进一步的扩增,具体反应体系如下: 2 $\times$  PCR buffer 10  $\mu\text{L}$ , dNTPs(2  $\text{mmol}\cdot\text{L}^{-1}$ ) 5  $\mu\text{L}$ , 上下游引物各 1.5  $\mu\text{L}$ , 模板 cDNA 2  $\mu\text{L}$ , *rTaq* 酶 1  $\mu\text{L}$ , 加水至 50  $\mu\text{L}$ 。

PCR 按照以下反应程序进行: 94  $^{\circ}\text{C}$  预变性 3 min; 94  $^{\circ}\text{C}$  变性 30 s, 50  $^{\circ}\text{C}$  退火 30 s, 72  $^{\circ}\text{C}$  延伸 1 min, 共 35 个循环; 72  $^{\circ}\text{C}$  延伸 5 min。退火后将PCR管取出。

反应结束后用1%琼脂糖凝胶电泳检测, 剩余PCR产物-20  $^{\circ}\text{C}$ 保存。

1.4.5 克隆测序 将2.4.4的PCR产物送至 Invitrogen 公司进行克隆测序。

1.4.6 基因和蛋白的序列分析 基因和蛋白的序列分析利用 Blast 在 NCBI 网站进行序列相似性比对分析。ORF 用 ORF Finder (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>) 进行分析,理化性质用 EXPASY 进行在线预测 (<http://expasy.org/tools/protparam.html>), 跨膜分析运用 TMPRED 进行预测 ([http://www.ch.emblnet.org/software/TMPRED\\_form.html](http://www.ch.emblnet.org/software/TMPRED_form.html))。蛋白翻译和基序分析采用蛋白组学在线 ExPASy。

## 2 结果与分析

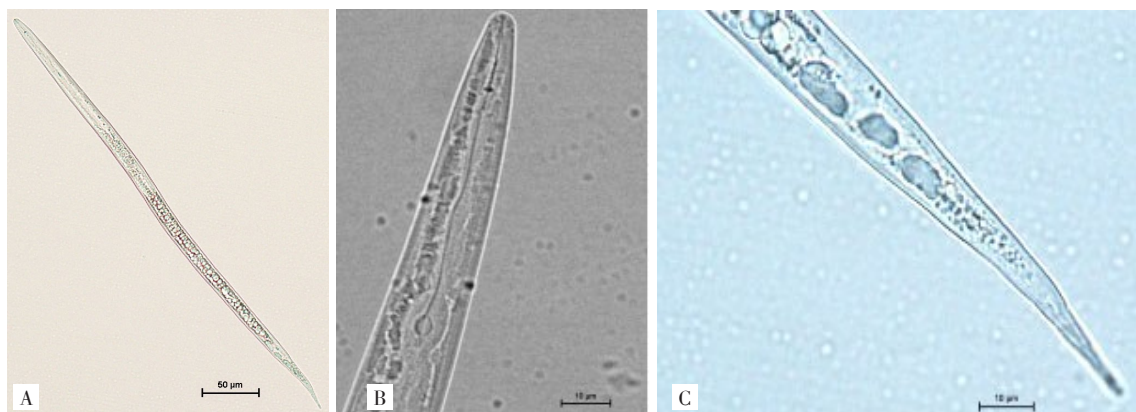
### 2.1 线虫的形态鉴定

2.1.1 根结线虫2龄幼虫 2龄幼虫特征: 2龄幼虫蠕虫形, 体环小而清晰、侧唇大呈三角形, 唇盘略高于中唇; 口针纤细, 锥体整体细长头部钝尖、头冠明显、尾部锐尖。能够清晰看到2龄幼虫纤细口针, 口针基部球清楚, 且大、圆, 中食道线及中食道球清晰, 中食道球呈椭圆形(图1)。

根据表1与对比数据<sup>[7]</sup>中2龄幼虫测量数据和形态描绘对比象耳豆根结线虫2龄测量值, 可以确定采自上述6个地点的根结线虫形态与象耳豆根结线虫的描述相一致。

2.1.2 根结线虫雌虫 显微镜观察其形态特征如下: 雌虫虫体膨大, 呈梨形有明显突出的颈, 唇盘圆盘状, 略突起; 口针细长, 口针基部球粗大; 会阴花纹整体卵圆形或椭圆形, 线纹细且较平滑, 尾尖区环纹不规则, 侧线不明显(图2)。

根据表2中雌虫测量数据和形态描绘对比象耳



A. 二龄幼虫侧面观; B. 二龄幼虫头部; C. 二龄幼虫尾部。

A. The side of J2; B. The head of J2; C. The tail of J2.

图1 象耳豆根结线虫二龄幼虫形态

Fig. 1 The second instar larvae of *Meloidogyne enterolobii*

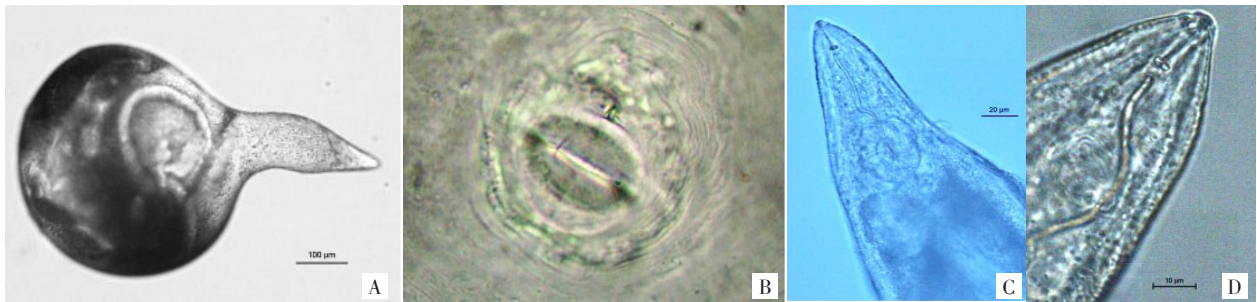
表1 根结线虫二龄幼虫测量值(n=10)

Table 1 The measured values of *Meloidogyne enterolobii* juvenile (J2) (n=10)

参数 Parameter	花都 Huadu	番禺 Panyu	增城 Zengceng	从化 Conghua	南沙 Nansha	黄埔 Huangpu	<i>M. enterolobii</i>
BL	415.9 (378.4~420.9)	417.4 (389.1~425.6)	419.9 (391.1~438.5)	417.1 (388.0~436.5)	414.2 (386.3~442.5)	416.8 (368.3~431.3)	388.6~436.9
GBW	15.8 (15.1~16.3)	15.6 (14.5~15.9)	16.1 (15.1~16.7)	14.8 (14.1~16.6)	14.8 (14.1~16.6)	15.3 (14.1~16.6)	14.0~16.6
M	11.5 (10.6~12.1)	11.8 (10.7~12.8)	12.1 (10.5~12.6)	11.3 (10.9~12.6)	11.2 (10.9~12.5)	10.9 (10.1~12.6)	11.6~12.9
h	19.6 (16.2~23.1)	18.9 (14.2~20.2)	20.8 (15.2~23.8)	16.3 (13.2~23.1)	15.9 (14.2~23.2)	16.2 (13.2~23.8)	12.7~22.1
TL	53.4 (41.6~59.7)	55.3 (42.7~60.1)	51.4 (42.3~58.6)	54.4 (41.8~59.7)	54.3 (41.6~60.1)	51.4 (41.3~59.4)	41.9~66.5

注:BL. 最大体长;GBW. 最大体宽;M. 口针锥部长/口针长;h. 尾后部透明区区长;TL. 尾长。括号内为数值范围。下同。

Note: BL. Maximum body length; GBW. Maximum body width; M. The length of the needle cone/Stylet length; h. The length of tail hyaline area; TL. tail length. The numbers in brackets are value range. The same below.



A. 雌虫整体;B. 会阴花纹;C. 雌虫头部;D. 雌虫口针。

A. The entirety of female; B. The perineal patten of female; C. The head of female; D. The stylet of female.

图2 象耳豆根结线虫雌虫形态

Fig. 2 The morphology of *Meloidogyne enterolobii* mature females

表2 根结线虫雌虫测量值(n=10)

Table 2 The measured values of *Meloidogyne enterolobii* mature females (n=10)

参数 Parameter	花都 Huadu	番禺 Panyu	增城 Zengceng	从化 Conghua	南沙 Nansha	黄埔 Huangpu	<i>M. enterolobii</i>
BL	598.6 (438.6~730.4)	682.3 (578.4~849.9)	730.8 (592.8~896.4)	417.1 (388.3~436.5)	698.0 (584.0~868.0)	711.0 (592.0~896.0)	552.0~995.0
GBW	389.8 (302.6~562.4)	462.5 (391.3~690.4)	541.8 (391.7~710.8)	14.8 (14.1~16.6)	435.0 (321.3~690.8)	405.0 (311.3~710.8)	315.0~702.3
M	12.1 12.3(10.4~13.1)	11.8 (9.4~12.7)	11.5 (9.1~13.2)	11.3 (10.9~12.6)	11.7 (10.9~13.8)	12.0 (9.2~13.5)	9.2~13.2
O	1.9 (1.7~2.3)	2.7 (2.1~2.9)	2.5 (2.2~2.9)	2.3 (1.9~2.8)	2.5 (2.2~2.9)	2.1 (1.8~2.6)	1.7~2.9

注:O. 口针基部至背食道腺开口的距离/口针长。

Note: O. The length between stylet base and gland opening of dorsal esophagus / stylet length.

豆根结线虫雌虫测量值,确定从化鳌头镇、南沙万顷沙镇十五涌、番禺东涌镇、黄埔区九龙镇、花都区花东镇、增城石滩镇的番石榴根结线虫与之前报道过的象耳豆根结线虫的描述相一致。

2.1.3 根结线虫雄虫 显微镜观察其形态特征如下:雄虫蠕虫形,体环清晰;头区高圆,略缢缩,锥体部尖;口针基部球大;中食道球卵圆形;尾短、圆;交

接刺略弯(图3)。根据表3与对比数据<sup>[8]</sup>中根结线虫雄虫测量数据和形态描绘对比象耳豆根结线虫雄虫的测量值,可以确定采自上述6个地点的根结线虫形态与象耳豆根结线虫的描述相一致。

### 2.2 线虫的分子鉴定

使用引物#C2F3和#1108对从化民乐镇、南沙十五涌、番禺东涌镇、萝岗广州市水果世界、广州花都



A. 雄虫整体; B. 雄虫头部; C. 雄虫交结刺; D. 雄虫口针。

A. The entirety of male; B. The head of male; C. The spicules of male; D. The stylet of male.

图 3 象耳豆根结线虫雄虫形态

Fig. 3 The morphology of *Meloidogyne enterolobii* mature males

表 3 根结线虫雄虫测量值(n=10)

Table 3 The measured values of *Meloidogyne enterolobii* mature males (n=10)

参数 Parameter	花都 Huadu	番禺 Panyu	增城 Zengceng	从化 Conghua	南沙 Nansha	黄埔 Huangpu	<i>M. enterolobii</i>
BL	1 860.6 (1 438.6~1 910.4)	1 682.2 (1 578.4~1 849.9)	1 730.8 (1 592.8~1 896.4)	1 678.0 (1 389.6~1 821.2)	1 596.3 (1 430.0~1 789.2)	1 820.4 (1 521.3~1 980.3)	1 348.6~1 913.3
GBW	43.6 (38.6~46.4)	44.2 (39.1~48.4)	44.8 (37.5~48.1)	42.3 (39.3~44.3)	44.8 (42.4~46.8)	43.6 (37.0~46.2)	37.0~48.3
TL	12.5 (11.2~13.1)	11.8 (10.4~12.7)	11.5 (9.1~13.2)	12.5 (10.3~16.5)	11.2 (9.8~15.2)	11.6 (10.6~13.7)	8.6~20.2

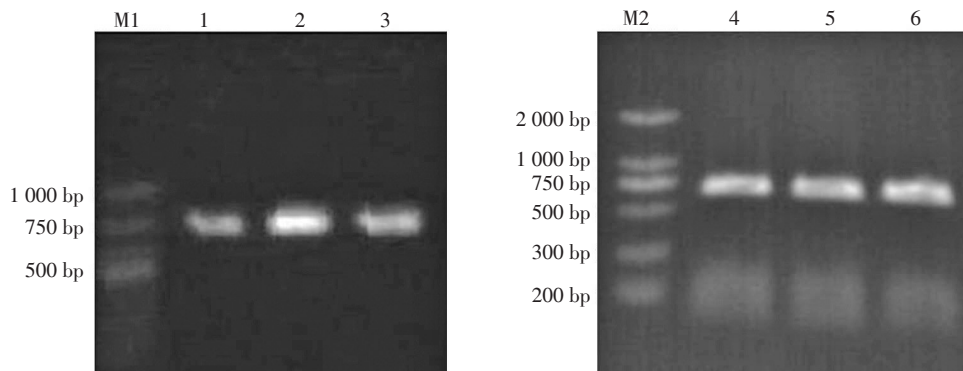
区莘田、增城石滩镇提取线虫的DNA扩增其mtDNA的CO II和1rRNA区,PCR扩增产物片段大小为750 bp(图4)。

结合形态鉴定与分子鉴定的结果,推断该根结线虫为象耳豆根结线虫(*M. enterolobii*)<sup>[8-9]</sup>。

2.3 14-3-3基因的克隆

2.3.1 PCR扩增 根据NCBI基因库中的南方根结线虫(*Meloidogyne incognita*)的14-3-3基因的序列,

搜索北方根结线虫转录组数据库中的14-3-3基因序列,进行比对后,设计包含开放阅读框的1对简并引物(14-3-3F和14-3-3R)来扩增象耳豆根结线虫的14-3-3基因的cDNA全长序列。PCR扩增大小在DL 1000的第1条带与第2条带之间(图5),与设计引物扩增的片段大小相一致,推断其为扩增目的片段。通过送样克隆测序后,获得783 bp大小的片段。



M1. DL 1000; M2. DL 2000.

图 4 引物#C2F3和#1108扩增电泳分析

Fig. 4 PCR amplification by primer #C2F3 and #1108

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1 atgacggagacaatgttgattgctctgacaaagtgactgagaccaaggaagaactgttcagcttgcctgacgacagctgag
M T E T M L D C S D K V T E T K E E L V Q L A K L A E Q A E
91 cgttatgatgacatggcagagtcaatgaagaaggttaccgaatttgagacgaactgtctaagaggagcgcaatctctcgttgc
R Y D D M A E S M K K V T E F G D E L S N E E R N L L S V A
181 tataagaatgttggggctcgtcgttcttggcgtgtgcttctctattgacaaaagaccgaaggggggaagaagacgatgagt
Y K N V V G A R R S S W R V L S S I E Q K T E G G K K T M S
271 aaggaataccgtgagaaggtgaatgtaattgcgtgacattgcaaaaggtgtatgaatctctggacaaattctattccgaaagct
K E Y R E K V E C E L R D I C K G V M N L L D K F L I P K A
361 ggaactcctgattctaaagtgtttacctaagatgaagggcgactactcgttatctggctgaggttctctggtgatgagtgact
G T P D S K V F Y L K M K G D Y Y R Y L A E V S S G D E L T
451 gatgttatcgacaaatcgaacagagttaccaagaggcttgcgatattgctaaggacaaaatgcgccaactatccaattcgttgg
D V I D K S Q Q S Y Q E A F D I A K D K M P P T H P I R L G
541 ctggctctcaacttttcggtcttactttgagattctcgacaataagggcaaggttgcagttggcttaagcagctttttgacgaggcc
L A L N F S V F Y F E I L D N K G K A C Q L A K Q S F D E A
631 gttccgagttggacactctgaagagaattgtacaaggactcgactctcattatgcaactcttcgcgacaattgactctgtggact
V A E L D T L E E N L Y K D S T L I M Q L L R D N L T L W T
721 tctgatggcggcgttgaggaaccggagacggctccaattgttgctgatccgaaggcacaat[aa]
S D G G V E E P E T A P I V A D A E G T N *

```

图 5 象耳豆根结线虫 *Me-14-3-3* 基因及其氨基酸序列

Fig. 5 *Meloidogyne enterolobii Me-14-3-3* gene and its amino acid sequence

2.3.2 *Me-14-3-3* 基因的 cDNA 序列及推测氨基酸序列 将所获得的序列在 NCBI 上进行 blastx 比对,结果显示与其比对上的为 *14-3-3* 基因,与预期相一致。排在前 2 位的分别是南方根结线虫与水稻干尖线虫的 *14-3-3* 基因,相似性分别为 98% (登录号: AAL40719.1) 和 80% (登录号: AJE60959.1),与南方根结线虫另外一个 *14-3-3* 基因的相似性为 80% (*14-3-3b*, 登录号: AAR85527.1)。通过 ORF Finder 软件(<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>)分析,所扩增片段包含了象耳豆根结线虫 *14-3-3* 基因的 ORF,将该基因命名为 *Me-14-3-3*。该基因的开放阅读框包含 783 bp 的片段,编码 261 个氨基酸(图

5)。用 ProtParam 工具对该基因编码的蛋白进行预测,其分子质量为 29.568 4 ku;理论等电点为 4.67;分子式为 C<sub>1296</sub>H<sub>2065</sub>N<sub>337</sub>O<sub>424</sub>S<sub>13</sub>,共有 4 135 个原子组成;稳定系数为 46.60,推测该蛋白是不稳定存在的蛋白。通过分析,其具有 *14-3-3* 基因的特有结构域(图 6)。

将象耳豆根结线虫 *14-3-3* 蛋白在 NCBI 上进行比对,将下载的序列用邻近法进行分析,结果表明克隆所得的象耳豆根结线虫的 *14-3-3* 基因与南方根结线虫的 *14-3-3* 基因(AAL40719)聚为一支,而与南方根结线虫另外一个 *14-3-3b* 基因(AAR85527)分属两支;水稻干尖线虫与松材线虫聚为一支,腐烂茎线虫与大豆孢囊线虫及南方根结线

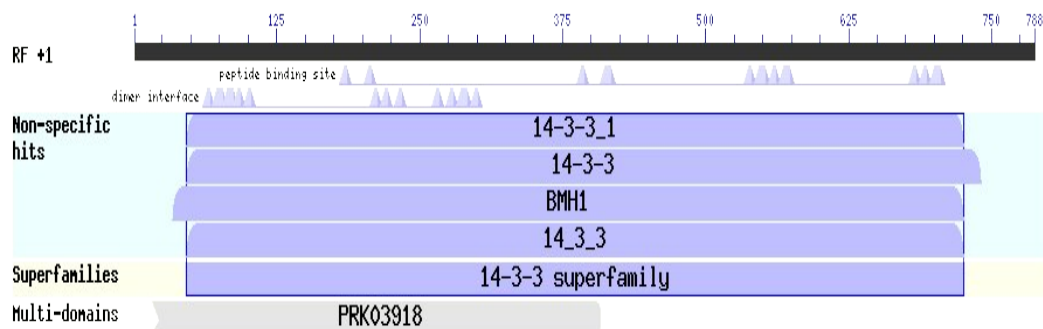


图 6 象耳豆根结线虫 *14-3-3* 基因结构域

Fig. 6 *Meloidogyne enterolobii* domain of *14-3-3* gene

虫的 14-3-3b 基因聚为一支,这与形态学分类的结果相一致。表明所克隆得到的 Me-14-3-3 基因与

南方根结线虫的来源于登录号 AAL40719 的 14-3-3 为同一支系,而与 14-3-3b 基因为不同支系(图 7)。

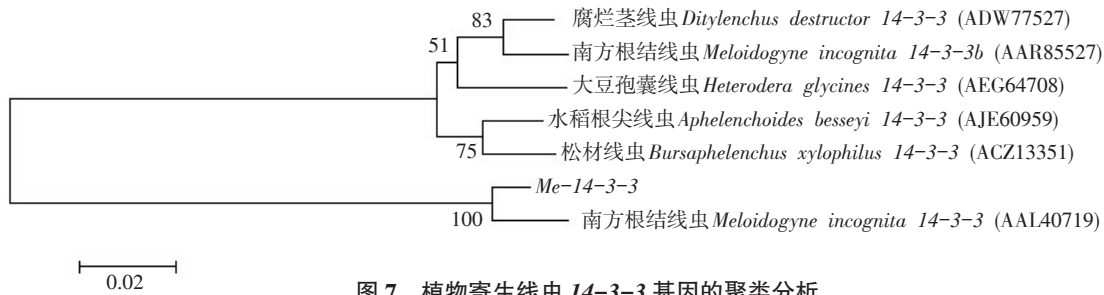


图 7 植物寄生线虫 14-3-3 基因的聚类分析

Fig. 7 The cluster analysis of root knot nematode 14-3-3 genes

下载南方根结线虫 14-3-3 基因的序列 (AAL40719 和 AAR85527),通过比对北方根结线虫转录组数据库,获得了 2 个 14-3-3 序列(2475 和 1597)。通过比对,发现所克隆的序列应该与南方根结线虫的 14-3-3 基因(AAL40719)和北方根结线虫的 2475 序列为同一类,而另外的 14-3-3b 与北方根结线虫的 1597 属于一类。将前 3 个基因进行比对分析,其大部

片段是保守的,但是仍然有 8.92% 的变异(图 8)。

### 3 讨论

象耳豆根结线虫有很强的致病力,尤其对中国许多经济作物致病性很强。加上它有寄主广泛、繁殖快、易传播的特点,它的传播扩散势必会造成巨大的经济损失<sup>[10]</sup>。目前,我国华南热区除海南省以外,

Me-14-3-3.seq	ATGACCGAGACAATGTTGGATGCTCGACAAGGTGACTGAGCCCAAGGAGAACTTGTTCAGCTTGCCAAACICGCCGA	80
Mn-14-3-3-2475.seq	ATGACCGAGACAATGTTGGATGCTCGACAAGGTGACTGAGCCCAAGGAGAACTTGTTCAGCTTGCCAAACICGCCGA	80
Mi-14-3-3.seq	ATGACCGAGACAATGTTGGATGCTCGACAAGGTGACTGAGCCCAAGGAGAACTTGTTCAGCTTGCCAAACICGCCGA	80
Consensus	atgac gagacaatgttggatgctc gacaaggtgactgag ccaagga gaacttgtt cagcttgccaaacicgccga	
Me-14-3-3.seq	ACAGGCGAGCGTTATGATGACATGGCAGTCAATGAAGAAGTTACCGAATTTGGACGCAATGTCCTAAATGGGAGC	160
Mn-14-3-3-2475.seq	ACAGGCGAGCGTTATGATGACATGGCAGTCAATGAAGAAGTTACCGAATTTGGACGCAATGTCCTAAATGGGAGC	160
Mi-14-3-3.seq	ACAGGCGAGCGTTATGATGACATGGCAGTCAATGAAGAAGTTACCGAATTTGGACGCAATGTCCTAAATGGGAGC	160
Consensus	acaggc gagcgttatgatgacatggcagtcaatgaagaagttaccgaatitggacgcaatgtctctaaatgggagc	
Me-14-3-3.seq	GCAATCTTCTCTCGGTTGCTTATAAGAAATGTTGTTGGCTCGCTGCTTCTTTGGCGTGTCTTTTCGCTATTGAGCAA	240
Mn-14-3-3-2475.seq	GCAATCTTCTCTCGGTTGCTTATAAGAAATGTTGTTGGCTCGCTGCTTCTTTGGCGTGTCTTTTCGCTATTGAGCAA	240
Mi-14-3-3.seq	GCAATCTTCTCTCGGTTGCTTATAAGAAATGTTGTTGGCTCGCTGCTTCTTTGGCGTGTCTTTTCGCTATTGAGCAA	240
Consensus	gcaatcttctctcggttgcttataagaaatgttgttggcctcgccttcttcttggcgtgtcttttcgctattgagcaa	
Me-14-3-3.seq	AAGACCGAAGCGGGAAGAAGACG.....ATGACCAAGGAATACCGTGAGAAGTTGAATCGAATTGCGTGACATTTG	314
Mn-14-3-3-2475.seq	AAGACCGAAGCGGGAAGAAGACG.....ATGACCAAGGAATACCGTGAGAAGTTGAATCGAATTGCGTGACATTTG	314
Mi-14-3-3.seq	AAGACCGAAGCGGGAAGAAGACG.....ATGACCAAGGAATACCGTGAGAAGTTGAATCGAATTGCGTGACATTTG	314
Consensus	aagaccgaagcg ggaagaagacg ..... atgac caaggaat accgtgagaag ttgaatc gaattg cgtgacat ttg	
Me-14-3-3.seq	CAATGGTGTATGAATCTTCGGACAATTCCTTATTTCGAAAGCTGGAACTCCTGATCTCAAAGTGTTCACCTTAAGA	394
Mn-14-3-3-2475.seq	CAATGGTGTATGAATCTTCGGACAATTCCTTATTTCGAAAGCTGGAACTCCTGATCTCAAAGTGTTCACCTTAAGA	394
Mi-14-3-3.seq	CAATGGTGTATGAATCTTCGGACAATTCCTTATTTCGAAAGCTGGAACTCCTGATCTCAAAGTGTTCACCTTAAGA	394
Consensus	caa gggttatgaaatcttcggacaat tcttatt tcgaaagctggaa ctctgattctcaaagtgtt taccttaaga	
Me-14-3-3.seq	TGAAGGGCGACTACTATCGTTATCTGCTGAGGTTTCCTGCTGATGACTGATGTTTTCGCAATTCGCAACAG	474
Mn-14-3-3-2475.seq	TGAAGGGCGACTACTATCGTTATCTGCTGAGGTTTCCTGCTGATGACTGATGTTTTCGCAATTCGCAACAG	474
Mi-14-3-3.seq	TGAAGGGCGACTACTATCGTTATCTGCTGAGGTTTCCTGCTGATGACTGATGTTTTCGCAATTCGCAACAG	474
Consensus	tgaagggcgactactatcgttatctgctgaggtttctctgctgatgactgatgttttcgcaattcgcaacag	
Me-14-3-3.seq	AGTTACCAAGAGGCTTCGATATTGCTAAGGACAAATATGCCGCCACTCATCCAATTCGCTCTTGGCTGCACAACTT	554
Mn-14-3-3-2475.seq	AGTTACCAAGAGGCTTCGATATTGCTAAGGACAAATATGCCGCCACTCATCCAATTCGCTCTTGGCTGCACAACTT	554
Mi-14-3-3.seq	AGTTACCAAGAGGCTTCGATATTGCTAAGGACAAATATGCCGCCACTCATCCAATTCGCTCTTGGCTGCACAACTT	554
Consensus	ag ttacca agagccttcgatattgctaagga aa atgcc cc actcatccaattcgtcttgg ttgctctcaactt	
Me-14-3-3.seq	TTCGGCTTCTACTTTGAGATTCGACAAATAAGCCCAAGGCTTGTGAGTTGGCTAAGCAGTCTTTTGAAGAGCCGTTG	634
Mn-14-3-3-2475.seq	TTCGGCTTCTACTTTGAGATTCGACAAATAAGCCCAAGGCTTGTGAGTTGGCTAAGCAGTCTTTTGAAGAGCCGTTG	634
Mi-14-3-3.seq	TTCGGCTTCTACTTTGAGATTCGACAAATAAGCCCAAGGCTTGTGAGTTGGCTAAGCAGTCTTTTGAAGAGCCGTTG	634
Consensus	ttcggcttctactttgagatctcgacaataagccaagccttgtcagttggctaaacagtcttttgaagagccggttg	
Me-14-3-3.seq	CGAGTTGGACACTCTTGGAGGAATTTGTACAAGGACTCGACTCTCATTATGCAACTTCTTCGCGACAATTTGACTCTG	714
Mn-14-3-3-2475.seq	CGAGTTGGACACTCTTGGAGGAATTTGTACAAGGACTCGACTCTCATTATGCAACTTCTTCGCGACAATTTGACTCTG	714
Mi-14-3-3.seq	CGAGTTGGACACTCTTGGAGGAATTTGTACAAGGACTCGACTCTCATTATGCAACTTCTTCGCGACAATTTGACTCTG	714
Consensus	c gaggttggacactcttgg aggaat ttgtacaaggactcgactctcattatgcaacttcttcgcgacaatttgactctg	
Me-14-3-3.seq	TGGACTTCTGATGGCGGCGTTGAGCAACCGGAGACGGCTCCATTCTTGCTGATGCCGAGGGCAAAATTA	785
Mn-14-3-3-2475.seq	TGGACTTCTGATGGCGGCGTTGAGCAACCGGAGACGGCTCCATTCTTGCTGATGCCGAGGGCAAAATTA	785
Mi-14-3-3.seq	TGGACTTCTGATGGCGGCGTTGAGCAACCGGAGACGGCTCCATTCTTGCTGATGCCGAGGGCAAAATTA	785
Consensus	tggacttctgatggcggcgttgagcaaccggagacggctccattcttgctgatgccgagggcaaaatta	

Mi-14-3-3 为 AAL40719.1 序列;Mh-14-3-3-2475 为北方根结线虫转录组数据库中的 2475 序列。

Mi-14-3-3 is AAL49719.1 from NCBI, Mh-14-3-3-2475 is the 2475 sequence from the northern root knot nematode transcriptome database.

图 8 象耳豆根结线虫 14-3-3 基因的比对

Fig. 8 The alignment of root knot nematode's 14-3-3 gene



广东、广西和福建的一些市县也先后报道有象耳豆根结线虫的发生,而随着全球气候变暖以及温室农业的发展,象耳豆根结线虫病害更有向温带地区蔓延的趋势<sup>[1]</sup>。因此,研究一种快速、高效的鉴定方法对象耳豆线虫的鉴定十分重要。传统的方法如会阴花纹的特征鉴定,会因为不同个体间的差异导致同一种线虫会阴花纹差别很大,利用这种方法直接鉴定会造成误差。本研究将线虫的形态学特征鉴定与分子生物学分析方法相结合就能更准确客观地鉴定出线虫种类。利用这种方法成功的在广州地区的番石榴果园鉴定出象耳豆根结线虫,这对以后对其他作物根结线虫的鉴定有重要的参考价值。

番石榴根结线虫的危害对于广州地区番石榴生产的影响越来越严重,对该病的系统研究刻不容缓。通过对广州地区番石榴根结线虫病的普查结果,明确了象耳豆根结线虫是寄生于广州地区番石榴上主要的优势种群之一,这为今后开展根结线虫病的综合防治,特别是对根结线虫抗性品种资源的筛选工作提供了重要依据。

笔者克隆了象耳豆根结线虫的14-3-3基因,并将该基因命名为*Me-14-3-3*。14-3-3蛋白基因可以通过调节信号转导来调节大量蛋白质的活性<sup>[12]</sup>。根据14-3-3基因的特性,对于象耳豆根结线虫而言,该基因片段在象耳豆根结线虫的生长发育繁殖中起到重要作用。14-3-3蛋白是真核生物体内的重要调节蛋白,蛋白的活性影响到象耳豆根结线虫的竞争力和环境适应力。成功克隆该蛋白基因片段,为进一步研究*Me-14-3-3*的具体作用和象耳豆根结线虫的致病机制奠定一定的基础。

## 4 结 论

从广州市及各区采集的线虫样本通过形态学鉴定,结合分子生物学技术分析,结果显示采样调查的侵染广州地区番石榴的线虫均为象耳豆根结线虫(*M. enterolobii*)。根据南方根结线虫及北方根结线虫14-3-3基因的序列设计简并引物,成功克隆象耳豆根结线虫14-3-3基因序列,将其命名为*Me-14-3-3*。对该基因编码的蛋白进行预测,推测该蛋白是不稳定存在的蛋白,聚类分析结果显示其与南方根结线虫14-3-3基因(AAL40719)聚为一支,与南方根结线虫的14-3-3b基因(AAR85527)为不同支系。该基因与象耳豆根结线虫的生长发育繁殖有关。

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