

以雄性不育胞质杂种‘华柚2号’ 为母本创制柚有性群体

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摘要:【目的】以具有胞质雄性不育特性的‘华柚2号’为母本与有核柚品种有性杂交, 以期转移其不育胞质进而实现二倍体水平的无核柚改良。【方法】以‘华柚2号’为母本, ‘沙田柚’和‘鸡尾’葡萄柚为父本分别配置杂交组合, 并对其子代遗传来源和单/多胚性早期鉴定。【结果】分别从‘沙田柚’和‘鸡尾’葡萄柚为父本的杂交组合获得实生苗1 018和687株; 用3对多态性SSR标记对实生苗的遗传鉴定表明均为父母本有性后代。以单/多胚性分子标记进行的早期胚性鉴定表明, ‘沙田柚’为父本的有性后代均为单胚性; 而‘鸡尾’葡萄柚为父本的后代, 单胚与多胚性比例为2.86:1。【结论】以‘华柚2号’为母本创制的有性后代为无核柚改良以及柑橘雄性不育恢复基因定位和克隆奠定了种质基础。

关键词: 柑橘; 细胞质雄性不育; 无核育种; 多胚性; 遗传鉴定

中图分类号: S666

文献标志码: A

文章编号: 1009-9980(2019)08-0961-07

Production of sexual hybrids with male sterile somatic cybrid pummelo ‘Huayou No.2’ as female parent

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Abstract:【Objective】Cytoplasmic male sterility (CMS) is common in higher plants and has significant advantages in production and basic research. Satsuma mandarin is a typical CMS type in citrus and has been contributed to the production of many excellent seedless varieties, such as ‘Shiranuhi’ ‘Harumi’ ‘Setoka’ and ‘Harehime’. Seedless breeding strategy by transferring the male sterile cytoplasm of Satsuma mandarin to seedy citrus cultivars via protoplast fusion was put forward and the produced somatic cybrid cultivar ‘Huayou No.2’ pummelo is a male sterile type with nuclear and chloroplast genomes from leaf parent ‘Hirado Buntan’ pummelo and mitochondrial genome from callus parent *Satsuma mandarin* ‘Guoqing No.1’. In order to breed seedless hybrid pummelo at the diploid level by transferring the sterile cytoplasm from ‘Huayou No.2’ pummelo, it was served as female parent to hybrid with seedy pummelo or grapefruit cultivars. In addition, most citrus genotypes have the capacity to produce clonal offspring via seed (apomixis), which is important for rootstock breeding. It was reported that CitRWP might be responsible for citrus polyembryony using genetic, genomic and transcriptomic approaches and a miniature inverted-repeat transposable element (MITE) insertion in the promoter region of CitRWP was co-segregated with polyembryony. The segregation ratios of both the phenotype and genotype in a segregating F₁ population were consistent with the single dominant gene model for the emergence of apomixes in mandarin. ‘Huayou No.2’ pummelo is monoembryonic, ‘Cocktail’ grape-

收稿日期: 2019-02-27 接受日期: 2019-04-16

基金项目: 国家自然科学基金重点项目(31530065); 国家重点研发计划(2018YFD1000106); 中央高校基本科研业务费(2662018PY007, 2662017PY019)

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fruit is polyembryonic and the embryony of their progenies may be segregated. The MITE marker can allow for inspection of the segregation ratio and early detection of polyembryonic individuals. 【Methods】Two sexual cross combinations were carried out with ‘Shatianyou’ pummelo and ‘Cocktail’ grapefruit as the male parents respectively. The mature fruits were collected; the seeds were extracted from the fruits and sown in the greenhouse. After the seedlings grew to ten-leaves old, the leaves were picked for extracting genomic DNA. The reported molecular markers were applied in identifying genetic origins and embryony. 【Results】1 018 and 687 seedlings were regenerated from the combinations of ‘Huayou No.2’ pummelo crossed with ‘Shatianyou’ pummelo and ‘Cocktail’ grapefruit respectively; the genetic origins of those seedlings were identified by three pairs of polymorphic SSR markers and the results showed that they were all of hybrid origin. The reported MITE marker was used to verify the embryony of the progenies, showing that the randomly detected 47 samples of ‘Huayou No.2’ × ‘Shatianyou’ pummelo were all monoembryonic while the ratio of monoembryonic and polyembryonic ones in the 687 ‘Huayou No.2’ × ‘Cocktail’ grapefruit’ sexual progenies was 2.86:1. 【Conclusion】The sexual progenies regenerated from the cross combinations with somatic cybrid ‘Huayou No.2’ pummelo as female parent are theoretically able to inherit the maternal sterile cytoplasm, and have the potential for producing seedless fruit, laying the material foundation for citrus seedless improvement at the diploid level. It also provides experimental material for the fundamental research of cytoplasmic male sterility, especially for the mapping and cloning of fertility restorer gene in citrus.

Key words: Citrus; Cytoplasmic male sterility; Seedless breeding; Polyembryony; Genetic identification

细胞质雄性不育 (Cytoplasmic male sterility, CMS) 是高等植物中普遍存在的现象, 表现为母性遗传、雌蕊正常和花粉败育, 可被显性核恢复基因恢复育性, 也称为核质互作不育^[1]。CMS 植物不能自交繁殖, 但可接受外源花粉受精结实, 有利于异质基因交流, 实现杂种优势利用。柑橘中, 温州蜜柑为典型的细胞质雄性不育类型, 日本以其为母本有性杂交已选育许多优良无核品种, 如‘清见’‘早香’‘不知火’等^[2-3]。CMS 是杂种优势利用和无核性状的遗传基础, 在柑橘生产中具有重要的利用价值。

‘华柚2号’是华中农业大学柑橘研究团队历时10余年, 基于提出的柑橘二倍体雄性不育胞质杂种创制的新思路^[4], 以‘国庆1号’温州蜜柑为愈伤亲本, 有核品种‘HB柚’ [*C. grandis* (L.) Osbeck.] 为叶肉亲本, 通过体细胞对称融合技术培育而成, 是国际上通过细胞融合技术直接培育的首例胞质杂种新品种, 2015年获农业部植物新品种权 (品种权号: CNA20101034.2)。遗传组成方面, 其核基因组来自‘HB柚’, 线粒体基因组来自温州蜜柑^[4-5]。果实品质方面, 与对照亲本HB柚相比, ‘华柚2号’表现为雄性不育、果实无核, 而果实糖酸品质未受影响, 有较好的推广应用前景。‘沙田柚’和‘鸡尾’葡萄柚均是品质优良的品种, 果肉爽脆, 风味浓甜, 但种子数较

多, 影响了消费者的食用体验和市场竞争力。以‘华柚2号’为母本与‘沙田柚’和‘鸡尾’葡萄柚有性杂交, 有望从中选育无核、酸甜适中等综合性状优良的杂种柚新品种。

种子多胚性是柑橘类植物的独特现象, 已被证明由单个基因控制的显性性状^[6]。Wang等^[7]利用单胚‘HB柚’和多胚‘Fairchild’橘的杂交分离群体和转录组测序等手段定位到在柑橘珠心胚上调表达的属于RWP-PK家族的候选基因CitRWP, 并发现多胚品种在该基因启动子区域比单胚品种多了一段约300 bp的微型可逆重复转座元件 (miniature inverted-repeat transposable element, MITE), 由此开发了可鉴定柑橘属单、多胚性状的MITE标记mite_p。Shimada等^[8]通过转基因验证了CitRWP的同源基因CitRKD1确实与柑橘多胚性相关。本研究创制的‘华柚2号’与‘鸡尾’葡萄柚的杂交群体可利用Wang等^[7]开发的MITE标记mite_p进行早期胚性筛选, 同时为柑橘珠心胚性状研究增添了宝贵的有性群体后代。

笔者以‘华柚2号’为母本与‘沙田柚’和‘鸡尾’葡萄柚授粉杂交, 对实生播种获得的后代进行遗传鉴定, 并利用可鉴定柑橘属单、多胚的分子标记对有性杂交后代进行早期胚性筛选, 创制的有性群体为

二倍体水平上柑橘无核改良奠定了种质基础,也为‘华柚2号’雄性不育及单多胚相关基础研究奠定了材料基础。

1 材料和方法

1.1 材料

杂交母本:‘华柚2号’(The cybrid between *C. unshiu* Marc. ‘Guoqing No. 1’ and ‘Hirado Buntan’ pummelo, 单胚), 定植于华中农业大学柑橘研究所种质资源圃。杂交父本:‘沙田柚’(*C. grandis* L. Osbeck., 单胚), 花粉采自广西特色作物研究院;‘鸡尾’葡萄柚(*C. paradisi* Osbeck., 多胚), 花粉采自华中农业大学柑橘研究所种质资源圃。

1.2 人工授粉

花粉制备和人工授粉参考解凯东等^[9]的方法, 制备好的花粉置于4℃避光保存。‘华柚2号’×‘沙田’柚及‘华柚2号’×‘鸡尾’葡萄柚组合的人工授粉分别于2013年和2015年的4月下旬进行。为避免外来花粉干扰, 授粉后进行套袋处理, 1周后摘除纸袋。

1.3 实生播种

实生播种参考梁武军等^[10]的方法。待11月份果实成熟后, 采摘果实并剥取种子, 1 mol·L⁻¹ NaOH

溶液浸泡10 min并用自来水洗净后, 剥去外种皮, 将其播于带营养土的小营养钵中, 覆1~2 cm细土, 覆膜后置于温室。待其长至有10片真叶时, 将其移入大营养钵, 加强苗期管理, 保证幼苗的正常生长。

1.4 基因组DNA提取

杂交亲本及其子代基因组DNA的提取参照Cheng等^[11]的方法。用NanoDrop1000紫外分光光度计(Thermo Scientific, USA)检测DNA浓度和质量, 并用TE缓冲液将DNA原液稀释到50~100 ng·μL⁻¹, 并置于-20℃保存备用。

1.5 PCR扩增及SSR分子鉴定

从已公开发表的SSR引物^[12-13]中筛选到3对亲本间多态性好的引物对2个组合的后代进行遗传鉴定(表1)。反应体系为10 μL, 包括1 μL DNA模板, 5 μL Master mix(Dream Tag Green PCR, 兴华基因), 0.15 μL正向引物, 0.15 μL反向引物, 3.7 μL灭菌双蒸水。PCR扩增程序为: 94℃预变性5 min; 94℃变性30 s; 55~58℃退火30 s; 72℃延伸30 s; 34个循环; 72℃延伸10 min; 最后4℃保存。扩增产物95℃加热变性5 min, 在恒定功率80 W条件下, 6%聚丙烯酰胺凝胶电泳1 h后银染显色。

表1 引物的序列信息
Table 1 Sequences of the primers

引物代号 Primer code	正向引物 5'—3' Primer 5'—3'	反向引物 3'—5' Primer 3'—5'	来源 Source
Ma2-1580	AAATGGACACTGCAGGCTTT	GCTGAGCAAAATGAAGCAAT	[12]
Ma3-183	ATCATTTCAACAAGGGACGG	TCGCCAATCAAACAACAAAA	[12]
Mest488	CACGCTCTTGACTTTCTCCC	CTTTCGCTGTGTTGTGCTGTT	[13]
mite_p	GTACCGAATTACCMCCCATAA	GTAGGATTGGGTATTGATG	[7]

1.6 有性后代单、多胚性的分子鉴定

2个杂交组合有性后代单、多胚性的分子鉴定参考张斯淇^[14]的方法。采用Phantamax Tag高保真聚合酶(Vazyme, 南京诺唯赞生物科技有限公司)的反应体系进行PCR扩增, 退火温度为55℃, 延伸时间为30 s; PCR扩增产物用1%琼脂糖凝胶(Regular agarose G-10, BIOWEST), 恒定电压110 V条件下电泳15 min后, 用Gel DocTM XR+成像仪(BIO-RAD)进行拍照。

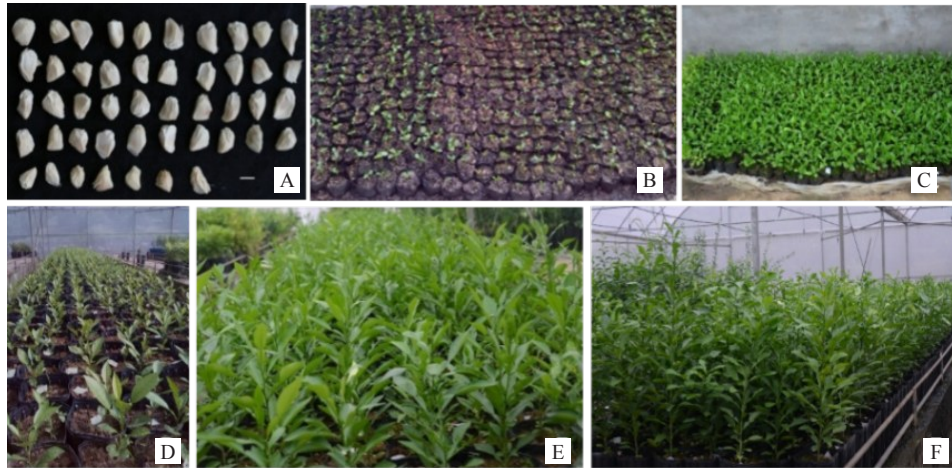
2 结果与分析

2.1 有性后代群体的获得

以‘华柚2号’为母本, ‘沙田柚’为父本, 授粉100朵花, 坐果21个, 坐果率为21.0%, 果实成熟后采摘果实剥取种子, 获得1 062粒种子, 平均种子数每果50.6粒(图1-A); 以‘鸡尾’葡萄柚为父本, 授粉84朵花, 坐果19个, 坐果率为22.6%, 获得900余粒种子, 平均种子数每果47.4粒。分别将所有种子实生播种到营养钵(图1-B), 最终萌发并再生植株1 018和687株(图1-C~E), 所有后代分别在2015年和2016年全部嫁接于枳砧(图1-F)并定植于田间。

2.2 有性后代群体的遗传鉴定

‘华柚2号’×‘沙田柚’组合筛选获得2对亲本间无条带重合的SSR引物, Ma2-1580和Ma3-183,



A. 单果种子; B. 播种后一个月; C. 播种后两个月; D. 移栽后一个月; E. 移栽后半年; F. 嫁接后一年。

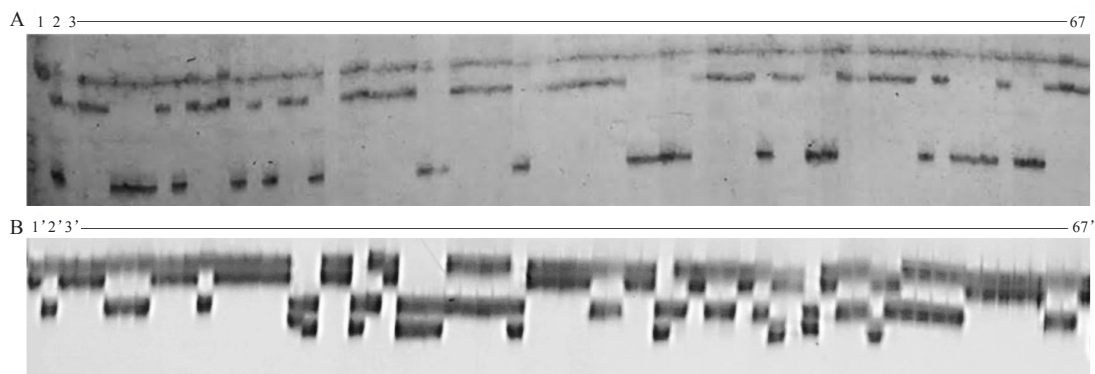
A. Seeds were extracted from one fruit; B. One month after sowing; C. Two months after sowing; D. One month after transplanting; E. Half a year after transplanting; F. One year after grafting.

图1 ‘华柚2号’×‘沙田柚’有性后代实生播种生长过程

Fig. 1 Growth of ‘Huayou No.2’ × ‘Shatianyou’ pummelo progenies by seed sowing

用这2对引物对所有杂交后代进行分子鉴定,表明在这2个位点,该组合所有杂交后代均含有父本特异条带,为父母本的有性后代(图2-A);‘华柚2号’×‘鸡尾’葡萄柚组合筛选获得1对父母本间无条带重

合的SSR引物Mest488,用该引物对所有杂交后代进行分子鉴定,结果表明该687株后代均含有鸡尾葡萄柚的特异性条带,为‘华柚2号’和‘鸡尾’葡萄柚的有性杂交后代(图2-B)。



A. 引物 Ma2-1580; 1. 华柚 2 号; 2. 沙田柚; 3~67. 随机选取的子代实生苗。 B. 引物 Mest488; 1'. 华柚 2 号; 2'. 鸡尾葡萄柚; 3'~67'. 随机选取的子代实生苗。

A. Marker Ma2-1580; 1. Huayou No.2; 2. Shatian pummelo; 3-67. Randomly detected seedlings. B. Marker Mest488; 1'. Huayou No.2; 2'. Cocktail grapefruit; 3'-67'. Randomly detected seedlings.

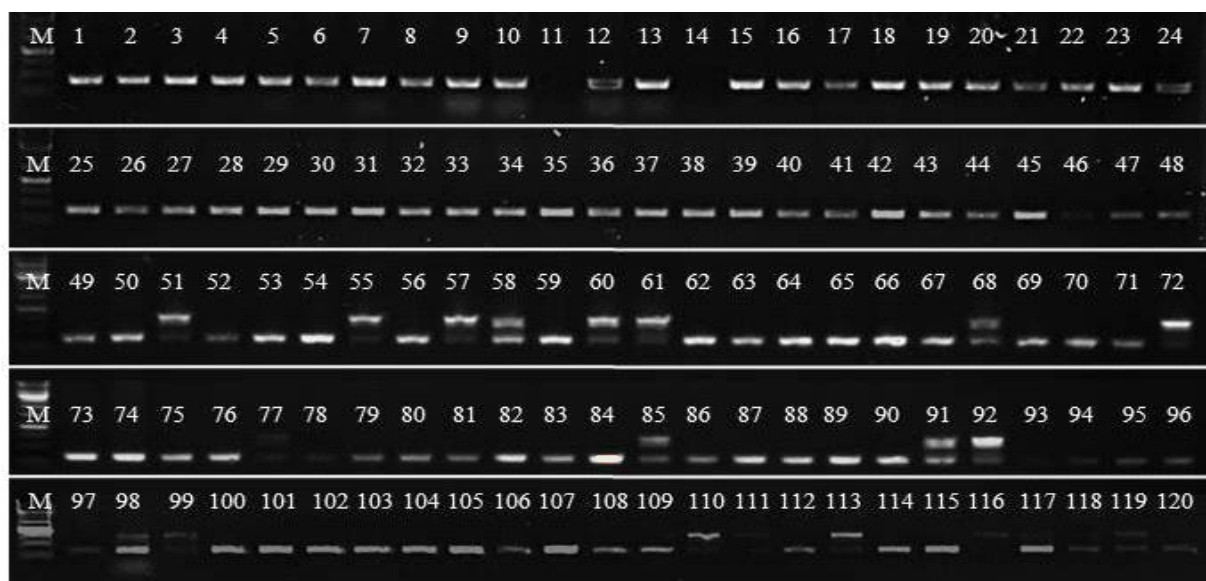
图2 2个有性杂交后代群体的分子标记扩增图谱

Fig. 2 SSR profiles of hybrids randomly selected from the two cross combinations

2.3 有性后代群体单、多胚性的分子鉴定

利用已发表的柑橘单、多胚性状的MITE分子标记对2个组合有性后代的胚性早期鉴定。从‘华柚2号’×‘沙田柚’组合随机挑选47株有性后代进行分析,表明所选的47株有性后代均未检测到MITE插入,推测其全部为单胚性有性后代(图3)。

同时用该标记对‘华柚2号’×‘鸡尾’葡萄柚组合的687株有性后代进行分析,表明所有有性后代中,178株后代检测到MITE插入,表明其为多胚性有性后代;而其余509株有性后代未检测到MITE插入,应该是单胚性有性后代,单、多胚性有性后代的分离比为2.86:1。



1. 华柚2号; 2. 沙田柚; 3~49. 华柚2号×沙田柚的部分子代; 50. 华柚2号; 51. 鸡尾葡萄柚; 52~120. 华柚2号×鸡尾葡萄柚的部分子代。M. 1 kb DNA ladder。

1. Huayou No.2; 2. Shatian pummelo; 3-49. Randomly detected seedlings from Huayou No.2 × Shatian pummelo; 50. Huayou No.2; 51. Cocktail grapefruit; 52-120. Randomly detected seedlings from Huayou No.2 × Cocktail grapefruit. M. 1 kb DNA ladder.

图3 MITE标记在2个有性杂交后代中的扩增图谱

Fig. 3 PCR profiles of hybrids randomly selected from the two cross combinations with the MITE marker

3 讨论

本研究以胞质杂种‘华柚2号’为母本,‘沙田柚’和‘鸡尾’葡萄柚为父本有性杂交,分别获得实生后代1 018株和687株。由于‘华柚2号’为单胚性品种,人工授粉后进行套袋处理,获得杂交后代理论上应均为双亲的有性后代。3对父母本间无条带重合的多态性SSR分子标记扩增结果表明,这两个杂交组合获得的所有植株均是父母本的有性杂交后代,与理论结果相符。

CMS为母系遗传,为杂交育种中杂种优势利用提供了简单高效的新思路。基于CMS性状的杂交育种已在多种作物,如大豆^[15]、水稻^[1]和油菜^[16]广泛应用。柑橘中,日本利用CMS雄性不育品种温州蜜柑与其他有核品种有性杂交,培育了许多少核或无核柑橘新品种^[3]。华中农业大学利用细胞融合技术培育的含有温州蜜柑雄性不育胞质的胞质杂种为柑橘无核育种提供了另一条高效途径^[4,17-19]。以‘HB柚’为对称融合亲本获得的具有CMS特性的新品种‘华柚2号’,遗传上类似于‘HB柚’的线粒体雄性不育突变体,以其为母本有性杂交创制的2个有性杂交群体不仅可用于选育果实品质优良且综合‘沙田

柚’或‘鸡尾’葡萄柚优良性状的无核杂种柚类新品种;而且可为柑橘CMS功能基因发掘和定位提供宝贵的研究材料。本研究团队已从细胞学、转录组学、蛋白质组学及小RNA测序等方法试图发掘和分离‘华柚2号’CMS相关的调控基因,揭示其核质互作雄性不育的分子机制^[20-21]。与此同时,若创制的2个有性群体后代出现不育和可育的分离,则可利用混合群体分离分析法(BSA)挖掘‘华柚2号’雄性不育恢复基因并揭示其分子机制。

本研究所选父本中,‘沙田柚’为单胚性品种,‘鸡尾’葡萄柚为多胚性品种,采用区分柑橘单、多胚性状的分子标记^[7]对这2个有性群体后代进行单、多胚性早期筛选具有可行性。柑橘珠心胚性状已被证明是一个质量性状,受一个显性位点的调控^[6-7],多胚基因型为显性杂合(Aa),单胚基因型为隐性纯合(aa)。本研究中,理论上‘华柚2号’×‘沙田柚’的有性后代应全是单胚,‘华柚2号’×‘鸡尾’葡萄柚的有性后代单、多胚分离比应接近1:1。MITE标记的扩增结果表明‘华柚2号’×‘沙田柚’组合随机分析的47株有性后代确实没有检测到MITE插入,全部为单胚性后代;然而‘华柚2号’×‘鸡尾’葡萄柚组合的687株有性后代,扩增检测到单、多胚的分离比为

2.86:1,原因可能为:(1)该MITE插入片段长度约300 bp,可形成茎环结构,不同于一般的MITE转座子,对扩增体系及扩增条件要求比较严苛,不易被扩增。本研究即使参照前人^[14]已优化的扩增体系及扩增条件进行扩增,仍有部分多胚子代不能重复扩增出MITE条带;换言之,被鉴定为单胚性的子代中可能混有部分多胚性子代,因此鉴定结果中单胚性子代较多;(2)前人^[14]利用该标记对124株已结果的‘HB柚’×‘Fairchild’橘有性后代群体的单、多胚性进行验证,发现在58株多胚后代中均能检测到MITE插入,66株单胚后代均未检测到MITE插入;该群体单胚性后代也略多于多胚性后代,推测单、多胚性状在不同群体可能易发生偏分离现象,且‘华柚2号’×‘鸡尾’葡萄柚组合后代群体较大,因此鉴定结果中单胚性子代远多于多胚性子代;(3)前人^[14]研究发现在‘红橘(多胚)×枳(多胚)’有性杂交群体后代中,多胚与单胚性后代的实际分离比不是理论值3:1,而是趋近2:1或1:1,且在控制柑橘多胚性状的候选区域,单、多胚表现型与位点基因型不连锁,推测枳中存在其他位点控制其多胚性状。Smith等^[22]用该MITE标记在21个栽培品种和4个以单胚性品种为母本与多胚性枳属杂种为父本的有性杂交组合共获得的79株子代群体进行验证,也认为柑橘属和枳属的多胚性状由不同位点控制。因此,从本研究结果看,‘华柚2号’×‘鸡尾’葡萄柚有性群体也可能存在不只一个位点控制其单、多胚性状。以上推测在该有性后代群体开花结果并观察种子胚性后将得以有效验证。

4 结 论

本研究以‘华柚2号’为母本与有核柚或葡萄柚有性杂交获得的实生后代均为父母本的有性后代,‘鸡尾’葡萄柚为父本的后代中单、多胚性分离比不符合1:1,推测该群体可能存在其他位点控制其多胚性状。这2个有性后代群体为柑橘无核改良以及雄性不育相关基础研究奠定了材料基础。

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