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Research Paper 研究报告

云南蔗区甘蔗线条花叶病毒分离物*NIa*基因形成新簇

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摘要：【目的】利用*NIa*基因，阐明甘蔗线条花叶病毒(*Sugarcane streak mosaic virus*, SCSMV)的种系发生关系，为预测SCSMV流行变异趋势及科学防控提供理论依据。【方法】从云南蔗区和国家甘蔗种质资源圃采集感病样品，RT-PCR扩增获得SCSMV *NIa*基因序列后，使用Splits Tree、RDP、PhyML、DnaSP等软件分析SCSMV中国分离物的系统发生、选择压力及基因流动等特征。【结果】共获得23条SCSMV *NIa*基因序列。这些序列间未发生重组，云南蔗区的部分序列形成1个新簇，且云南蔗区与国家甘蔗种质资源圃之间的基因交流不显著。此外，选择压力分析表明，*NIa*基因受很强的负选择压力作用。【结论】与*P1*、*HC-Pro*和*CP*等基因类似，SCSMV在*NIa*基因上也包含5个簇；SCSMV云南分离物具有较高的遗传多样性和清晰的地理相关性。

关键词：甘蔗线条花叶病毒，*NIa*基因，系统发生

甘蔗线条花叶病毒(*Sugarcane streak mosaic virus*, SCSMV)属于马铃薯Y病毒科(Potyviridae)禾本科病毒属(*Poacevirus*)。该病毒首先在甘蔗花叶病株上发现，自然条件下能够侵染甘蔗、高粱和一些禾本科杂草[1−4]。SCSMV侵染甘蔗引起长短不一的线条形花叶症状，发生普遍时能够引起甘蔗减产20%左右，目前在印度、印度尼西亚、泰国等南亚、东南亚国家广泛发生，已成为该地区甘蔗花叶病的主要病原[1,4]。2008–2011年，我们在云南省甘蔗产区首次发现SCSMV，后续调查发现，SCSMV在云南省甘蔗主产区发生越来越普遍，部分地区呈现流行爆发趋势[2,5−6]。因此，需要采取有效防控措施，控制SCSMV的进一步扩散和蔓延。

明确SCSMV种群的遗传多样性及分子进化可以为其防控提供指导。研究发现[7−8]SCSMV印度分离物的*CP*基因和*HC-Pro*基因具有较高的遗传多样性。我国分离的SCSMV云南分离物与印度分离

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物是2个独立遗传的种群，两者之间基因交换的频率很低[6]。我们的前期研究发现[6]，依据*P1*、*HC-Pro*和*CP*基因，SCSMV包含5个系统发育簇，而*NIa*基因只含有4簇，可能是由于*NIa*基因分离物的数目和多样性水平较低造成。本研究对采自云南省沅江、弥勒、红河、新平、常宁和国家甘蔗种质资源圃内的16个甘蔗花叶病样品的*NIa*基因进行克隆测序，基于*NIa*基因分析SCSMV的重组、系统发生、选择压力、种群结构以及基因流动，从而确定*NIa*基因的分子进化特征。研究结果能够为制定合理的SCSMV病毒病害防控策略提供依据。

1 材料和方法

1.1 病毒分离物

2008–2011年，从中国云南省沅江、弥勒、红河、新平、常宁等县市和国家甘蔗种质资源圃内(源自日本、印度尼西亚样品)采集具有典型花叶症状的甘蔗样品。新鲜叶片样品经RT-PCR法检测鉴定，将SCSMV阳性样品经冷冻干燥后，–80 °C保存备用。本研究选取其中不同地区具有代表性的16个样品，详细信息见表1。

1.2 克隆测序

利用RNA prep pure Plant Kit (天根)提取甘蔗叶片总RNA，方法参照试剂盒说明书。取2 μL总RNA用反向引物SCSMNIa-R (5′-CAAGTGCTCAACTCTTCGT-3′) 进行反转录获得cDNA，反应体系参照MLV反转录试剂盒(Promega)说明书。然后使用引物SCSMNIa-F (5′-ATTGGGATGATGGAAAACAG-3′)和SCSMNIa-R扩增*NIa*基因。PCR反应体系为：cDNA 2 μL，引物SCSMNIa-F

表1. 本研究中的SCSMV样品采集表

Table 1. The *Sugarcane streak mosaic virus* isolates collected in this study

Isolate	Origin	Collection time	Cultivar	Symptom
W23	Japan*	2010.8	Japan 1	Mosaic
W24	Japan*	2010.8	Japan 6	Mosaic
W32	Indonesia*	2010.8	Ti20-0	Mosaic
M55	Changning，China (常宁)	2008.6	Q170	Mosaic
M61	Yuanjiang，China (沅江)	2008.11	Unknown	Mosaic
M62	Xinping，China (新平)	2009.7	Yunyin10	Mosaic
M85	Honghe，China (红河)	2010.8	Yue79-177	Mosaic
M86	Mile，China (弥勒)	2010.8	Yun99-91	Mosaic
M112	Yuanjiang，China (沅江)	2011.6	ROC22	Mosaic
M116	Yuanjiang，China (沅江)	2011.6	Yun03-258	Mosaic
M117	Yuanjiang，China (沅江)	2011.6	Yun98-136	Mosaic
M118	Yuanjiang，China (沅江)	2011.6	De03-83	Mosaic
M119	Yuanjiang，China (沅江)	2011.6	Yunyin58	Mosaic
M121	Yuanjiang，China (沅江)	2011.6	Yunyin58	Mosaic
M124	Yuanjiang，China (沅江)	2011.6	SP81-3250	Mosaic
M126	Yuanjiang，China (沅江)	2011.6	Yun07-912	Mosaic

*: sugarcane samples stored in the Chinese national nursery of sugarcane germplasm resources (NNSGR).

(10 μmol/L)和SCSMNIa-R (10 μmol/L)各2 μL，10×PCR 缓冲液 5 μL，dNTPs (2.5 mmol/L) 4 μL，Long *Taq* DNA polymerase (5 U/μL) 0.5 μL，加ddH_2O至50 μL。PCR反应条件为：94 °C 5 min；94 °C 30 s，55 °C 30 s，72 °C 1 min，共30个循环；72 °C 10 min，4 °C保存。PCR产物经过凝胶纯化后克隆至pMD18-T载体(TaKaRa)上，并转化至大肠杆菌(*Escherichia coli*) DH5α感受态细胞中。经菌落PCR鉴定获得阳性重组质粒，筛选3–6个阳性克隆送至北京六合华大基因科技股份有限公司测序。测序结果通过峰图及序列比对分析排除由PCR扩增引起的突变。

1.3 重组分析

以TriMV (NC012779)作为比对分析的外组(outgroup)[9]。将GenBank中已登录的SCSMV *NIa*基因序列与本研究获得的序列一起进行分析。首先，利用CLUSTAL X2[10]和TRANSALIGN(由Georg Weiller教授惠赠)进行核苷酸序列和氨基酸序列的比对，得到能够正确编译出氨基酸的核酸序列。然后，使用Datamonkey (http://www.datamonkey.org/)中GARD和RDP 4.0软件包[11]中的RDP[12]、GENECONV[13]、BOOTSCAN[14]、MAXCHI[15]、CHIMAERA[16]、3SEQ[17]和SISCAN[18] 7个软件进行重组检测。各检测方法的参数采用默认值，Bonferroni校正的*P*-value为0.05和0.01，当某分离物有至少3个方法的检测结果$P<1.0\times10^{-6}$时，支持该分离物为重组体[19–20]。最后，删除分析中的外组序列，去除TriMV对SCSMV序列造成的gap影响，直接检测确认SCSMV在*NIa*基因区的重组位点。

1.4 系统发生与多样性分析

利用最大似然法(Maximum-likelihood, ML)、邻接法(Neighbor-joining, NJ)及邻接网法(Neighbor-net, NN)进行系统发生分析，所用软件分别为PhyML 3.0[21]、MEGA 6.0[22]和Splits Tree 4.11.3[23]。在ML法分析中，通过jModeltest 0.1.1[24]软件确定*NIa*基因序列的最适核苷酸替代模型为GTR+I+Γ4。在ML和NJ法分析中，支长皆用自举法(bootstrap)进行1000模拟复制计算检验。系统发育树由Tree View[25]展示。

使用DnaSP 5.0[26]计算不同种群分离物的核苷酸多样性(nucleotide diversity)和单体型多样性(haplotype diversity)。SCSMV *NIa*基因的核苷酸间的多样性分布通过SDT 1.0[27]中的Clustal W法计算获得。

1.5 选择压力分析

基于ML法计算*NIa*基因的d_N/d_S值(非同义突变和同义突变之间的比值)，预测其承受的选择压力。首先，利用Datamonkey中SLAC (single-likelihood ancestor counting)、FEL (fixed-effects likelihood)和REL (random-effects likelihood)检测*NIa*基因不同位置密码子的选择压力；其次，应用MEGA 6.0[22]中的Pamilo-Bianchi-Li method[28]计算不同系统发育分支(组)的选择压力。当$d_N/d_S<1$时，表示该组分离物处于纯化或负向选择压力下；当$d_N/d_S=1$时，表示该组分离物处于中性选择压力中；当$d_N/d_S>1$时，表示该组分离物受正向选择或多样化选择作用。

1.6 基因漂移分析

种群间的基因交流用F_{st}和N_m值来衡量。F_{st}的绝对值在0–1之间，当F_{st}的绝对值小于统计阈值0.33时，说明两个地区之间存在频繁的基因交流，反之，则交流的频率很低[6,29–31]。若$N_m<1$时，种群间很容易发生遗传漂变，故遗传漂变是促使群体发生遗传分化的主要原因；若$N_m>1$说明地缘关系较近或者种群间存在可以发生基因交流的渠道[31]。

种群之间的遗传差异用K_s*，*Z*和S_{nn}[32]衡量。K_s*由不同序列的数量决定，与序列的来源无关；*Z*值是秩统计量(rank statistic)，代表*Z*1和*Z*2的加权

和；S_{nn}是指近邻统计(nearest-neighbor statistic)，适用于样品采集来源于2个或者2个以上的地区，目的是计算在相同地理学空间上近邻序列出现的频率。如果Z和K_s*统计值很小，P<0.05，则拒绝无遗传差异的假设。上述统计由DnaSP 5.0[26]完成。

2 结果和分析

2.1 SCSMV *NIa*基因的序列特征与重组分析

在云南省不同蔗区和国家甘蔗种质资源圃内采集样品，经检测后，从中选取16个具有典型花叶症状的SCSMV阳性样品，对SCSMV的*NIa*基因进行克隆测序。共获得23条可用序列(登录号：KU314373–KU314395)。*NIa*基因的序列长度为714 nt。

将获得的23条序列与从GenBank中下载的43条SCSMV *NIa*基因序列进行重组分析。结果(图1)发现，除了SCSMV-TPT分离物，不同分离物间没有明显的网状交叉。表明不同分离物间未发生重组。此外，GARD和RDP 4.0重组分析也未发现重组位点。

2.2 系统发育树重建

在NN法构建的网状树中，SCSMV分成5簇(图1)。中国分离物集中在第1、2和5簇。值得指出的是，部分云南分离物形成1个新簇——第5簇(Cluster 5，图1)。这不同于之前的报道[6]。此外，ML法和NJ法生成的系统发育树具有相似的拓扑结构(ML树如图2所示)。可分为3组：第III、V和第I+IV组[11]，其中第V组又可以分为3个亚组(V-1、V-2和V-3)。由图2可知，中国SCSMV分离物分布于整个系统树，而印度分离物则集中于第V和 I+IV 组。

基于ML系统树分析发现，*NIa*基因处于强烈的负选择压力作用下(d_N/d_S值低至0.014)，Datamonkey检测结果显示，*NIa*基因中未发现正向选择作用位点。

2.3 多样性分析

图3中统计了SCSMV不同分离物在*NIa*基因上的核苷酸多样性分布。与系统发生分析结果相一致，SCSMV序列核酸相似性差异可以显著的分为3个组，不同组间*NIa*基因相似性高于80% (图3)。在系统发育所分的3个组中，第III和第V组分别具有较高的单体型多样性(0.978±0.054；0.980±0.007)和较低的核苷酸多样性(0.01147±0.00126；0.02661±0.00259)(表2)。相对于SCSMV印度分离

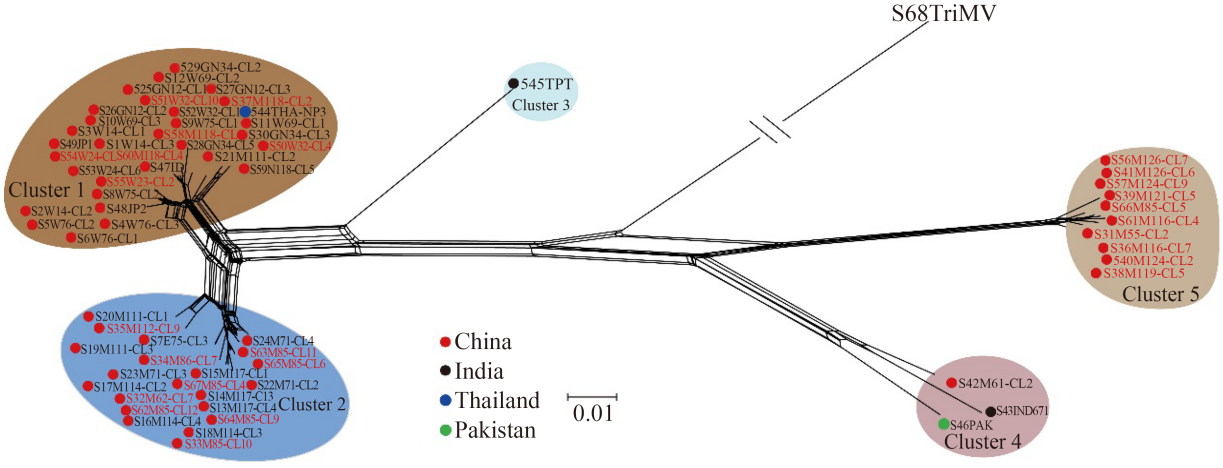

图 1. SCSMV *NIa*基因的网状树

Figure 1. Phylogenetic analysis of the *NIa* gene sequences of *Sugarcane streak mosaic virus* (SCSMV) using neighbor-net method. The SCSMV sequences with red colour were determined in this study.

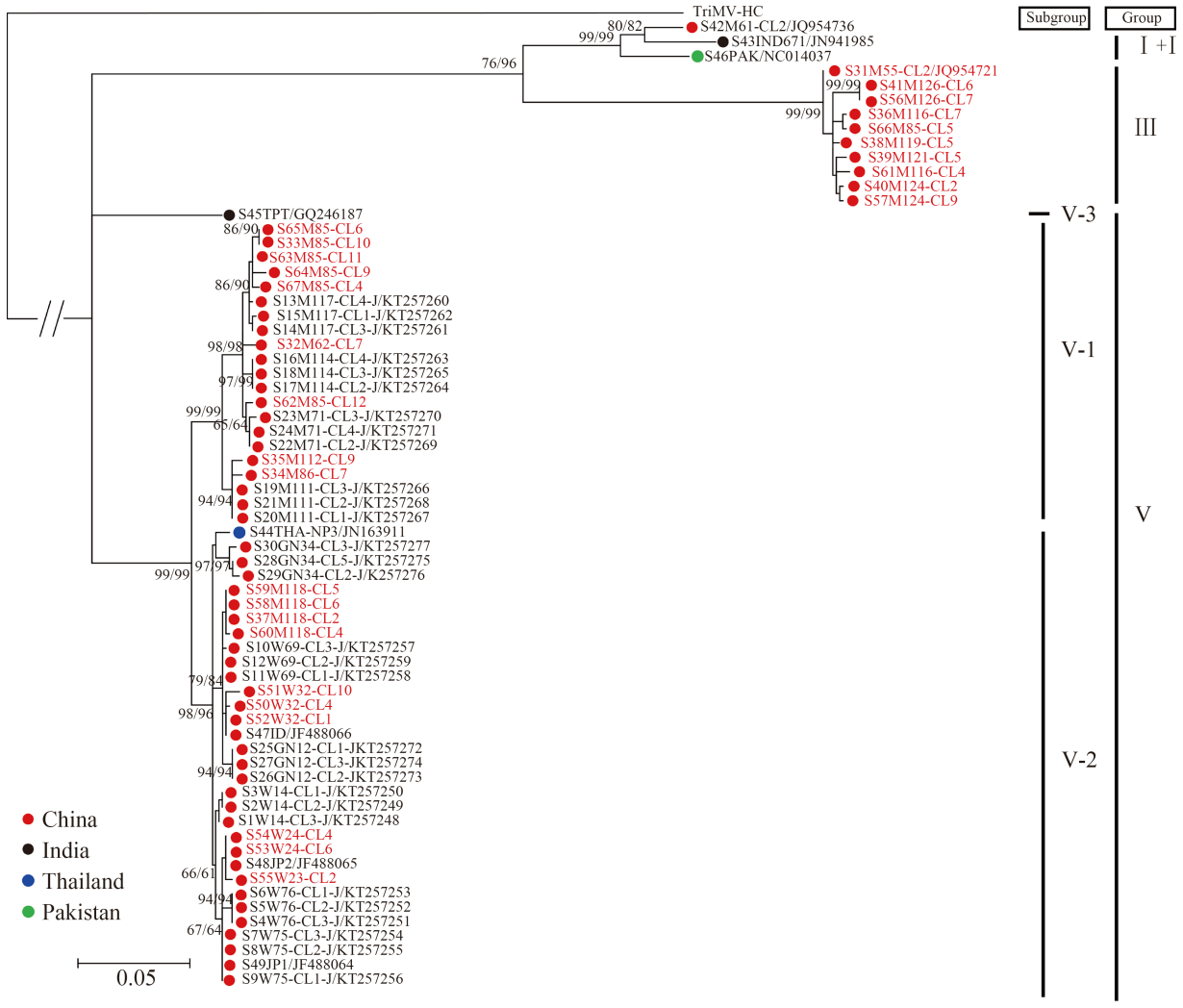

图 2. 利用ML法对SCSMV *NIa*基因的系统发生分析

Figure 2. Phylogenetic analysis of the *NIa* sequences of *Sugarcane streak mosaic virus* isolates using ML method. The SCSMV sequences with red colour were determined in this study.

表2. 甘蔗线条花叶病毒*NIa*基因的多样性分析

Table 2. Nucleotide diversity of *Sugarcane streak mosaic virus NIa* gene

Group	n	Haplotype diversity (H)	Nucleotide diversity (Π^a)
III	10	0.978±0.054	0.01147±0.00126
I+IV	3	1.000±0.272	0.05169±0.01451
V	54	0.980±0.007	0.02661±0.00259
China	63	0.985±0.006	0.07439±0.01084
India	2	1.000±0.500	0.18841±0.09420

[a]: nucleotide diversity was estimated by the average pairwise difference between sequences in a sample, based on all sites.

物，SCSMV中国分离物间遗传相似性程度更高(表3)。

2.4 基因漂移分析

通过DnaSP 5.0中K_s*、Z和S_{nn} 3个统计量分析SCSMV不同种群间的遗传差异。结果发现，SCSMV在不同组间的遗传差异显著，中国不同地区亚种群间的遗传差异也十分明显(表3)。基因流动分析中，SCSMV在云南蔗区和资源圃形成的种群间的F_{st}为0.352，大于0.33(表3)，表明SCSMV分离物在云南蔗区和资源圃间的基因流动

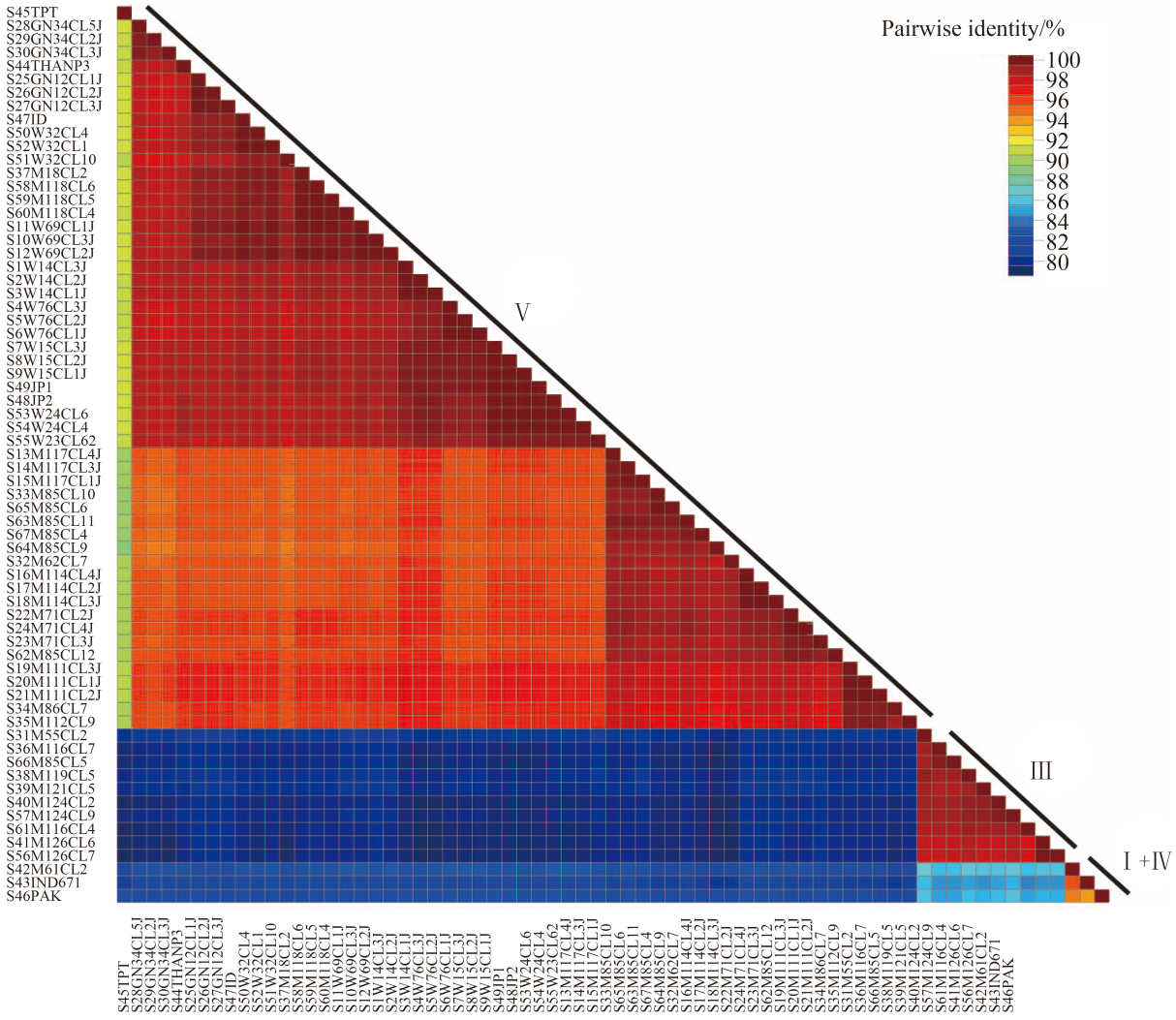

图 3. SCSMV *NIa*基因序列一致率分布图

Figure 3. The distribution of pairwise identity scores of the *NIa* sequences of *Sugarcane streak mosaic virus* isolates.

频率较低。SCSMV不同种群间N_m<1(表3)，说明SCSMV种群受到遗传漂变的影响。

3 讨论

SCSMV作为甘蔗花叶病病原之一，目前在印度、印度尼西亚和中国等蔗区的发生分布越来越广泛，并有在东南亚诸国广泛流行发生的趋势[4,6,8,33]。目前，针对该病毒的研究主要集中于遗传进化领域。相较于*P1*、*HC-Pro*和*CP*基因，*Potyviridae*基因组中*NIa*基因的保守性较高，重组发生的频率较低[6,19,29-30,34]。本研究测定了采自云南蔗区和国家甘蔗种质资源圃内的23个SCSMV *NIa*基因序列，经研究未发现其具有重组位点，这进一步支持先前的研究结果。

He等报道发现，SCSMV依据*P1*、*HC-Pro*和*CP*构建的网状树包含5簇，而*NIa*基因只含有4簇，原因可能是由于*NIa*基因系统发生分析中SCSMV分离物数量不足，无法完整显现其多样性分布[6]。本研究发现由云南分离物形成的1个新簇——第5簇，从而证明*NIa*基因与*P1*、*HC-Pro*和*CP*基因在进化上的一致性。

表3. 甘蔗线条花叶病毒*NIa*基因的基因漂移和遗传差异分析

Table 3. Gene flow and genetic differentiation of the *NIa* of *Sugarcane streak mosaic virus*

Region[the number of sequences]	Parameter[a]				
	K_s (*P*-value[b])	*Z* (*P*-value)	S_{nn} (*P*-value)	F_{st}	N_m
China[n=63] vs. India[n=2]	3.28388 (0.0190 *)	1009.58807 (0.0320 *)	0.95385 (0.2680)	0.07208	3.22
NN[n=27] vs. India[n=2]	1.89135 (0.0000 ***)	175.00000 (0.0040 **)	0.93103 (0.0550)	0.25235	0.74
YN[n=36] vs. India[n=2]	3.51550 (0.0010 **)	341.96667 (0.0780)	0.92105 (0.2720)	0.03656	6.59
YN[n=36] vs. NN[n=27]	2.82730 (0.0000 ***)	749.68054 (0.0000 ***)	1.00000 (0.0000 ***)	0.35185	0.46

[a], K_s* and *Z* are the sequence-based statistics considered by Hudson (2000). S_{nn} is the nearest-neighbor statistic. F_{st} is the interpopulation component of genetic variation of the standardized variance in allele frequencies across populations. An absolute value of F^{st}<0.33 suggests frequent gene flow. *N* is the population size of each subpopulation. m is the migration fraction per generation. [b], P<0.05 was considered as the criterion for rejecting the null hypothesis that there is no genetic differentiation between two subpopulations. *, 0.01<P<0.05; **, 0.001<P<0.01; ***, P<0.001.

系统发生显示，依据*NIa*基因SCSMV包含5个分组(亚组)，该结果与网状树形成的5簇相一致，具有清晰的地理特异性。SCSMV云南蔗区分离物分布在除V-3亚组外的所有分支，具有高度的遗传多样性。SCSMV资源圃分离物主要集中在V-2亚组，与蔗区分离物间有比较明显的遗传差异。结合SCSMV在云南蔗区与资源圃间的遗传差异和较低频率的基因交流，表明SCSMV在中国可能有2个不同的起源。

本研究确定了SCSMV云南分离物在*NIa*基因上的多样性、聚类分布和地理特异性特征，明确了SCSMV在云南蔗区和资源圃内具有不同的种群分布，为设计合理的甘蔗花叶病病害防治策略提供了理论依据。

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A novel phylogenetic lineage clustered by *NIa* gene of *Sugarcane streak mosaic virus* Yunnan isolates

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Abstract: [**Objective**] We assessed the phylogenetic relationship of *Sugarcane streak mosaic virus* (SCSMV) according to *NIa* sequences, to infer the prevalence and variation of SCSMV and to prevent and control this virus. [**Methods**] Leaf samples with mosaic symptom were collected from sugarcane-growing areas in Yunnan province and the Chinese national nursery of sugarcane germplasm resources (NNSGR). *NIa* sequences of SCSMV were determined by RT-PCR, and analyzed by Splits Tree, RDP, PhyML and DnaSP softwares, in aspect of phylogenetic, selection, and gene flow. [**Results**] We obtained 23 *NIa* sequences; clear recombination site was not found in *NIa*; a novel cluster formed by SCSMV Yunnan isolates determined here was found; strong purifying selection was found in *NIa* of SCSMV; and the gene flow of SCSMV subpopulations between sugarcane-growing areas in Yunnan province and the NNSGR was not frequent. [**Conclusion**] Similar with *P1*, *HC-Pro* and *CP* genes, SCSMV isolates could be divided into five clusters. *NIa* of SCSMV Yunnan isolates showed high genetic diversity and clear geographical distribution.

Keywords: *Sugarcane streak mosaic virus*, *NIa*, phylogenetic

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