Population Structures of ‘Candidatus Liberibacter asiaticus’ in Southern China

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ABSTRACT


Huanglongbing (HLB) is a highly detrimental citrus disease associated with ‘Candidatus Liberibacter asiaticus’, a nonculturable alpha-proteobacterium. Characterization of the bacterial populations is important for development of disease management strategies. In this study, the ‘Ca. L. asiaticus’ populations in eight provinces in southern China where HLB is endemic were analyzed based on tandem repeat number (TRN) variations in a previously characterized genomic locus CLIBASIA_01645. Of the 224 HLB samples collected, 175 (78.3%) samples yielded single polymerase chain reaction (PCR) amplicons (the single amplicon group, SAG) and 49 (21.7%) samples produced multiple PCR amplicons (the multiple amplicon group, MAG). Variations in SAG are summarized by Nei’s diversity index (H) and ratio of TRN ≤ 10/TRN > 10 genotypes (R10).

Citrus huanglongbing (HLB, yellow shoot disease) is highly destructive to citrus production worldwide (1). According to Chinese literature, there was a severe HLB epidemic in the Chaoshan area of Guangdong Province in the 1930s (3), but the disease might have been there as early as the late 1880s (12). The disease is endemic in Guangdong and has been reported in 11 of the 19 citrus-growing provinces in southern China since the 1970s (8,19,21). The association of an alpha-proteobacterium, ‘Candidatus Liberibacter asiaticus’, with HLB was established in 1994 (10) and confirmed in China in 1996 (6,16). However, little is known about the biology of ‘Ca. L. asiaticus’ and the epidemiology of HLB, largely due to the inability to successfully culture the bacterium in vitro. The lack of efficient markers to discriminate the bacterial isolates poses a serious constraint for studying ‘Ca. L. asiaticus’ populations, hampering efforts for effective and efficient management of HLB.

Conserved genomic loci such as 16S rDNA sequences were first used to analyze the population of ‘Ca. L. asiaticus’ in China. Shan et al. (15) sequenced the 16S rDNA gene of ‘Ca. L. asiaticus’ to analyze eight citrus HLB samples collected from five provinces in southern China and noted very limited inter-provincial variations. Deng et al. (4) compared the loci of the 16S rRNA gene, rplAJ (β- operon of ribosomal protein genes), and an outer membrane protein gene (ompA) in ‘Ca. L. asiaticus’ samples from six different locations in Guangdong. The sequences of these three genes in the pummelo isolates from the inland Meizhou area were identical to those of sweet orange and mandarin isolates from the neighboring coastal Chaoshan area where HLB was first observed. In general, the extent of genetic variations at conserved loci was low, rendering it difficult to characterize bacterial populations of different origins with adequate resolution.

Recent research has focused on more variable loci as additional bacterial genomic sequences became available (7). Focusing on the ‘Ca. L. asiaticus’ population in China, Liu et al. (13) analyzed the variation of a prophage gene and found that the gene frequencies were significantly different between the ‘Ca. L. asiaticus’ population from Guangdong Province and that from Yunnan Province. A unique genomic region of ‘Ca. L. asiaticus’ showing locus mosaicism was recently characterized and the ‘Ca. L. asiaticus’ isolates in China could be divided into high and low altitude groups (18). Chen et al. (2) analyzed the tandem repeat number (TRN) variation at a genomic locus (CLIBASIA_01645) of ‘Ca. L. asiaticus’ and reported the bacterial population difference between Guangdong, China and Florida, U.S. While the Florida bacterial population was dominated by a TRN = 5 genotype (84.5%), the Guangdong bacterial population also dominated by a TRN = 7 genotype, but at only 47.6%. Katoh et al. (11) identified four tandem repeat loci showing high sensitivity in detection of ‘Ca. L. asiaticus’ diversity among isolates from Japan, Taiwan, and Indonesia. Locus CLIBASIA_01645 (designated as 005 by Katoh et al. [11]) was one of them. Indeed, Locus CLIBASIA_01645 had the highest overall Nei’s diversity value (11).

HLB research in China began in the 1940s (3,12). Before the recognition of ‘Ca. L. asiaticus’ (10), HLB was diagnosed by...
yellow shoot symptoms and confirmed by graft transmission (12). According to the HLB occurring timeline, it has in general been believed that HLB originated in Guangdong Province and spread to other provinces, presumably through infected propagating materials (12). In light of the success in differentiating the ‘Ca. L. asiaticus’ populations at locus CLIBASIA_01645 (2), this project used the same protocol to evaluate the population variations of ‘Ca. L. asiaticus’ in southern China (Guangdong and the nearby provinces). The unique structure of ‘Ca. L. asiaticus’ population in Guangdong was further confirmed. A different structure of non-Guangdong ‘Ca. L. asiaticus’ population was detected. Genotype aggregation and possible biological role of tandem repeat shuffling at locus CLIBASIA_01645 were discussed with genome sequence annotation information.

**MATERIALS AND METHODS**

**Bacterial sources.** Three to ten mature leaves from citrus trees with typical HLB symptoms and leaf mottling were collected and considered as an HLB sample, for DNA extraction. All samples were collected between April 2010 and November 2011 with locations illustrated in Figure 1, which included the following citrus species: Guangdong: *C. sinensis*, *C. grandis*, *C. paradise*, *C. reticulata*, *C. limon*, *C. aurantium*, *C. sunki*, Fujian: *C. reticulata*; Guangxi: *C. sinensis*, *C. reticulata*, *Fortunella margarita*; Guizhou: *C. reticulata*; Hainan: *C. aurantium*, *C. sunki*; Jiangxi: *C. sinensis*, *C. reticulata*, *C. grandis*. The presence of ‘Ca. L. asiaticus’ was confirmed by polymerase chain reaction (PCR) with primer set OI1/OI2c before the sample was selected for population analyses (10).

HLB samples from three orchards, one each in Guangdong, Hainan, and Yunnan provinces, were selected to test if differences among ‘Ca. L. asiaticus’ populations from different provinces exist. The orchard in Guangdong (Orchard GD, located in Huizhou, 0.67 ha of sweet orange [‘C. sinensis’], and 80% tree infection) was established with local nursery stock in 1996. The orchard in Hainan (Orchard HN, located in Chengmai, 2.4 ha of sweet orange [‘C. sinensis’], and 20% tree infection) was established with budwood from Guangdong in 2003. The orchard in Yunnan Province (Orchard YN, located in Ruili, 4.0 ha of lemon [‘C. limon’], and 8% tree infection) was established with budwood obtained from Chongqing City, where no HLB has been previously reported, in 2005. In Orchard GD, Asian citrus psyllids (Diaphorina citri) were also collected and stored in 90% ethanol to assay for ‘Ca. L. asiaticus’.

A larger region-wide comparison (Guangdong and non-Guangdong regions) of ‘Ca. L. asiaticus’ populations was then performed. In addition to Orchard GD, eight more locations in Guangdong Province were identified for collection of ‘Ca. L. asiaticus’ samples, collectively named as the Guangdong group (Fig. 1). In addition to samples from Orchard HN and Orchard YN, six more locations from the provinces of Fujian, Guangxi, Guizhou, Jiangxi, and Zhejiang (Fig. 1) were selected for sample collections. These samples were collectively designated as the non-Guangdong group.

**DNA extraction.** Sample DNA was extracted by E. Z. N. A. HP Plant DNA Kit (OMEGA Bio-Tek Co., Guangdong, China) using 0.2 g of leaf midribs. Psyllid DNA was extracted with the TIANamp Genomic DNA Kit (Tiangen Biotech Co., Beijing, China) under UV light. Three to ten mature leaves from citrus trees with typical HLB symptoms of yellow shoot and leaf mottling were collected and considered as an HLB sample, for DNA extraction. All samples were collected between April 2010 and November 2011 with locations illustrated in Figure 1, which included the following citrus species: Guangdong: *C. sinensis*, *C. grandis*, *C. paradise*, *C. reticulata*, *C. limon*, *C. aurantium*, *C. sunki*; Fujian: *C. reticulata*; Guangxi: *C. sinensis*, *C. reticulata*, *Fortunella margarita*; Guizhou: *C. reticulata*; Hainan: *C. aurantium*, *C. sunki*; Jiangxi: *C. sinensis*, *C. reticulata*, *C. grandis*. The presence of ‘Ca. L. asiaticus’ was confirmed by polymerase chain reaction (PCR) with primer set OI1/OI2c before the sample was selected for population analyses (10).

**PCR primers and procedures.** The previously published PCR primer set, LapGP-1f (5′-GACATTTCAACGGTATCGAC-3′) and LapGP-1r (5′-GCGACATACTCCTCCTT-3′) (2), was used in this study. PCs were carried out with a Bio-Rad Thermal Cycler in 25-µl mixtures containing 2.5 µl of 10x DNA polymerase buffer, 2.5 µl of 2.5 mM deoxynucleoside triphosphates (dNTPs), 1 µl of template DNA, 0.4 µl of Taq DNA polymerase at 2.5 U/µl, and 18.1 µl of H2O. The PCR procedure was programmed with an initial step of denaturing at 96°C for 1 min, followed by 35 cycles of each denaturing at 96°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s. PCR ended with a final extension at 72°C for 4 min. Amplicons were electrophoresed in 2.0% agarose gel and visualized by Goldview (Guangzhou Geneshun Biotech Ltd., China) under UV light.

**DNA sequencing.** Of the 25 µl of PCR amplicon, half was used for electrophoresis and the other half was used for sequencing. Single amplicon DNAs were sequenced directly with primers

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**Fig. 1.** Geographical locations where huanglongbing samples/‘Candidatus Liberibacter asiaticus’ isolates were collected in southern China.

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LapGP-1f/LapGP-1r. Multiple amplicon DNA samples were cloned with pEASY-T1 Cloning Kit (TransGen Biotech Co., Beijing, China). Ten transformed *Escherichia coli* colonies were selected and cloned plasmids were isolated using the TIANprep Mini Plasmid Kit (Tiangen Biotech Co.). Cloned fragments were characterized by electrophoresis after restriction enzyme digestion. DNA fragments of different sizes were sequenced in both directions with primers LapGP-1f/LapGP-1r through a commercial source. TRNs of ‘Ca. L. asiaticus’ isolates were identified manually from DNA sequences.

**Data analyses.** PCR results yielded two groups of electrophoresis profiles, the single amplicon group (SAG) and the multiple amplicon group (MAG). The population variation of ‘Ca. L. asiaticus’ was estimated and summarized by three parameters. For the SAG, Nei’s H value was calculated based on the formula, \( H = 1 - \sum M^2 \), where M is the frequency of allele i at the locus (14). Preliminary comparison among different geographical regions showed an obvious difference in TRN genotype distribution, particularly the TRN > 10 genotypes. The H value did not capture this orderly information. To better describe the bacterial population structure, all ‘Ca. L. asiaticus’ samples were arbitrarily divided into two groups, TRN ≤ 10 and TRN > 10, which were established empirically (2). The ratios of TRN ≤ 10 and TRN > 10 genotypes were calculated and designated as R10. For the MAG, parameters H and R10 could not be applied because not all amplicons from an isolate were sequenced. Therefore, the percentages of multiple amplicon samples for each geographical location were calculated and designated as P_{MAG}. Differences between bacterial populations were analyzed by the \( \chi^2 \) test.

A total of 194 citrus leaf samples (PCR positive for ‘Ca. L. asiaticus’ with primer set OI1/OI2c) were collected from 17 locations in eight provinces (Fig. 1). PCR results with primer set LapGP-1f/LapGP-1r showed that 162 samples (83.5%) were in the SAG and 32 samples (16.5%) in the MAG. Electrophoretic profile of SAG samples were identical to those presented previously (2) and, therefore, not presented here. An example of the electrophoresis profile of MAG samples are illustrated in Figure 2. In general, two to five DNA bands were visible with one band in higher intensity, probably due to its high copy number or better match at primer sites. The exact numbers of bands were difficult to determine, for example, sample JX1 (Fig. 2). Figure 3 lists the TRN genotypes derived from clones of nine selected MAG samples. Interestingly, most genotypes (18 out of 28) were TRN > 10. The P_{MAG} values of MAG samples are presented in Table 1.

Of all HLB SAG samples, 78 (48.1%) were from Guangdong and 84 (51.9%) were from non-Guangdong provinces (Table 1). No SAG samples were found in Jiangxi Province (0/12). From all SAG samples, 20 TRN genotypes ranging from 2 to 24, with the absence of 20, 21, and 22, were identified (Table 1). The mode (the highest frequency genotype) was TRN = 7 (37 samples), identical to the previous observation (2). However, the frequencies of TRNs = 6 and 8 genotypes also were high. The total number of samples with TRNs = 6, 7, and 8 genotypes was 98 (31 + 37 + 30, respectively), or 60.5% of all SAG samples. No TRN genotype preference was associated with any specific citrus host (data not shown), confirming previous reports (2).

Comparative characteristics of ‘Ca. L. asiaticus’ populations among three single orchards are presented in Table 2. Orchard GD had an H value of 0.50, contrasting to the higher H values of 0.83 and 0.88 in Orchard HN and Orchard YN, respectively. R_{10} in Orchard GD was 23 (23/1), significantly higher than that in Orchard HN (1.5, or 21/14) (\( P < 0.002 \)) and Orchard YN (2.3 or 16/7) (\( P < 0.017 \)). Similarly, the P_{MAG} in Orchard GD (0.0, or 0/78) was significantly different from that in Orchard HN (16.7 or 7/35) (\( P < 0.001 \)) and Orchard YN (11.5 or 3/23) (\( P < 0.002 \)) (Table 1).

Table 2 also shows region-wide ‘Ca. L. asiaticus’ population structures. The overall H value of all HLB samples from southern China was 0.86. The Guangdong ‘Ca. L. asiaticus’ population had an H value of 0.77 compared with 0.91 in the non-Guangdong population. The R_{10} of Guangdong samples was 25 or 75/3, significantly different from that of the non-Guangdong samples being 1.7 or 50/30 (\( P < 0.001 \)). Only one MAG sample was detected in the Guangdong ‘Ca. L. asiaticus’ population, which accounted for the P_{MAG} value of 1.3 (1/78), significantly different
(P < 0.001) from the $P_{\text{MAG}}$ value of the non-Guangdong samples (36.9 or 31/84). $P_{\text{MAG}}$ values were also calculated for each province except for Fujian, Guangxi, and Guizhou which had too few (<10) samples to be statistically significant (Table 1). $P_{\text{MAG}}$ for Jiangxi was 100 and $P_{\text{MAG}}$ for Zhejiang was 31.2. Sequence analyses showed that a small number of clones from MAG samples were of plant origin (data not shown). This did not affect the calculation of $P_{\text{MAG}}$ values since each sample (Fig. 2) always had a majority of ‘Ca. L. asiaticus’ DNA clones.

In Orchard GD, 30 psyllids that tested positive for ‘Ca. L. asiaticus’ were analyzed. Thirteen samples were in the SAG and 17 samples in the MAG (Table 1). The $H$ value of the SAG samples was 0.27 and all SAG samples were the genotypes of TRN ≤ 10. This is identical to the bacterial population from citrus trees in the same orchard. However, the psyllid samples had a high $P_{\text{MAG}}$ (56.7 or 17/30). Sequence analyses of cloned amplicons from two Asian citrus psyllids showed the presence of TRN = 7 genotype and also TRN > 10 genotypes (Fig. 3). Like citrus tree samples, some clones of MAG samples were of psyllid endosymbiont origins (data not shown).

**DISCUSSION**

Previously, the primer set LapGP-1f/LapGP-1r representing the locus of CLIBASIA_01645 was used to characterize ‘Ca. L. asiaticus’ populations in Guangdong and Florida (2). In this study, the same protocol was again used to analyze the ‘Ca. L. asiaticus’ population from Guangdong with new samples. The same distribution of TRN genotypes was observed, confirming that the protocol is repeatable and therefore reliable for differentiations of ‘Ca. L. asiaticus’ populations. Multiple tandem repeat loci were also used to evaluate the population diversity of ‘Ca. L. asiaticus’ (9,11). However, different genomic loci may have different evolution rates. Therefore, we were concerned that the sum or average of the variations from multiple loci may not have adequate discriminative power needed to resolve the highly similar ‘Ca. L. asiaticus’ populations. This study focused on characterizing the single CLIBASIA_01645 locus and further explored its utility in analyses of the bacterial populations.

An interesting finding from this study is the observation of multiple amplicons from CLIBASIA_01645. MAG was previously not found in the CLIBASIA_01645 locus in the bacterial populations from Guangdong and Florida (2). In fact, the same genomic locus was also used to study 84 Japanese, 4 Taiwanese, and 12 Indonesian isolates of ‘Ca. L. asiaticus’ and no MAG isolates were reported (11). However, doublet amplicons were observed from a Taiwanese ‘Ca. L. asiaticus’ isolate at another tandem repeat locus, named locus 001 (11). Further cloning and sequencing studies revealed that the doublet amplicons contained five DNA fragments with different TRNs. In this study, MAG isolates were mostly from non-Guangdong areas. It remains unclear what caused the multiple amplicon phenomenon. It could be due to co-infection/existence of multiple genotypes (5,11,18), but others, such as nonoptimized PCR amplifications, cannot be excluded. In fact, amplification of plant/insect symbiont DNA could be explained by occasional nonoptimized PCR events. Regardless, MAG data constituted part of the bacterial population variations and could be described by $P_{\text{MAG}}$.

Nei’s $H$ value is a widely used parameter for evaluation of population diversity in bacteria including the ‘Ca. L. asiaticus’ populations (11). However, as mentioned earlier, the $H$ values did not reflect the orderly distribution of TRN genotypes. For example, there could be no difference in $H$ values if the mode of genotype were arbitrarily swapped from TRN = 7 to TRN = 17 (Table 1), according to the formula for $H$ value calculation (14). Use of $R_{10}$ values overcomes the deficiency and significantly increases differentiation sensitivity. Indeed, $R_{10}$ and $P_{\text{MAG}}$ are sufficient to describe and compare ‘Ca. L. asiaticus’ population structures at the CLIBASIA_01645 locus (Table 2).

With data from both the previous study (2) and this study, it is apparent that there is an obvious TRN genotype aggregation in the ‘Ca. L. asiaticus’ population in Guangdong as reflected by the

**TABLE 1. Single amplicon group (SAG) and multiple amplicon group (MAG) of ‘Candidatus Liberibacter asiaticus’ samples from southern China and distribution of tandem repeat number genotypes in SAG**

| Province  | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 23 | 24 | Total | MAG | Total |
|-----------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|----|----|----|
| Guangdong | 1 | 5 | 3 | 19 | 23 | 2 | 1 | 2 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 84 | 31 | 115 |
| Orchard GD| 1 | 1 | 1 | 1 | 3 | 16 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 78 | 1 | 79 |
| Others    | 1 | 4 | 2 | 18 | 20 | 6 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 54 | 1 | 55 |
| Guangxi   | 1 | 2 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| Guizhou   | 1 | 2 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| Hainan (Orchard HN)| 1 | 2 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| Jiangxi   | 1 | 2 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| Yunnan (Orchard YN)| 1 | 2 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| Zhejiang | 1 | 2 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| Total HLB leaf | 1 | 1 | 10 | 9 | 31 | 37 | 30 | 5 | 5 | 9 | 4 | 1 | 3 | 5 | 1 | 6 | 1 | 1 | 1 | 1 | 162 | 32 | 194 |
| ACP<sup>a</sup> | 1 | 11 | 1 | 11 | 1 | 11 | 1 | 11 | 1 | 11 | 1 | 11 | 1 | 11 | 1 | 11 | 1 | 11 | 1 | 11 | 1 | 11 | 175 | 49 | 224 |

<sup>a</sup> Numbers in parentheses are samples selected for DNA sequencing analyses.

<sup>b</sup> Asian citrus psyllids (Diaphorina citri).

**TABLE 2. Population structures of ‘Candidatus Liberibacter asiaticus’ in southern China described by $R_{10}$, $P_{\text{MAG}}$, and $H$ values**

<table>
<thead>
<tr>
<th>Province</th>
<th>$R_{10}$&lt;sup&gt;a&lt;/sup&gt;</th>
<th>$P_{\text{MAG}}$&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Orchard-wide</td>
<td>0.50</td>
<td>23.0 (23/1)</td>
</tr>
<tr>
<td>Orchard GD</td>
<td>0.88</td>
<td>1.5 (21/14)</td>
</tr>
<tr>
<td>Orchard YN</td>
<td>0.83</td>
<td>2.3 (16/7)</td>
</tr>
<tr>
<td>Region-wide</td>
<td>0.77</td>
<td>25.0 (75/3)</td>
</tr>
<tr>
<td>Guangdong</td>
<td>0.91</td>
<td>1.7 (50/30)</td>
</tr>
<tr>
<td>Non-Guangdong</td>
<td>0.77</td>
<td>3.8 (125/33)</td>
</tr>
<tr>
<td>Southern China</td>
<td>0.86</td>
<td>3.8 (125/33)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Numbers within parentheses are defined as number of tandem repeat number (TRN) > 10 isolates/number of TRN > 10 isolates.

<sup>b</sup> Numbers within parentheses are defined as number of multiple amplicon group isolates/total number of isolates.
high $R_{DP}$ value. In fact, the low $P_{MAG}$ values could also be explained as the result of genotype aggregation, i.e., the trend towards a dominant genotype in each single tree. DNA sequences with tandem repeats are known to be highly dynamic in both prokaryote and eukaryote genomes. Research on *Saccharomyces cerevisiae* proved that intragenic tandem repeats generated genomic variations allowed the organism to be rapidly attuned to a particular environment (17). According to annotation (7), CLIBASIA_01645 encodes a bacteriophage repressor, a regulatory protein controlling phage/prophage activity. Stemming from this assumed regulatory role, CLIBASIA_01645 could generate different types of regulators (phage repressors) by varying TRN, and, therefore, change phage/prophage activities. Phages/prophages have been described in ‘Ca. L. asiaticus’ (20). There have been evidence that phage/prophage activities in ‘Ca. L. asiaticus’ are related to environmental differences (13,20).

Finally, this study considered the variation of ‘Ca. L. asiaticus’ population in southern China mainly in the context of two categories, Guangdong and non-Guangdong groups, and revealed only a possible trend of the bacterial evolution. The non-Guangdong group covered seven provinces, each of which could have its own unique bacterial population structure. It should be noted that sample sizes from Fujian and Guangxi provinces were too small to be representative. All samples from Jiangxi Province were in the MAG. Nevertheless, detailed population structures of ‘Ca. L. asiaticus’ in each province, as well as in other countries, need to be determined in the future. Of many importance issues that affect the ‘Ca. L. asiaticus’ population structure is the evolution rate of the CLIBASIA_01645 locus.

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**LITERATURE CITED**