PCR Detection of the Two Liberibacter Species Associated with Citrus Huanglongbing in São Paulo State, Brazil

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ABSTRACT. A new liberibacter species, *Candidatus* Liberibacter americanus, has been identified recently in sweet orange leaves collected in São Paulo State (SPS), Brazil and which showed blotchy mottle symptoms characteristic of huanglongbing (HLB). Primers GB1/GB3 were designed for PCR amplification of the 16S rDNA of *Ca*. L. americanus and effectively detected the new liberibacter in 214 of 218 symptomatic leaf samples. The leaves of two additional samples were infected with *Candidatus* Liberibacter asiaticus. Two other leaf samples, each from a single tree, contained both *Ca*. L. americanus and *Ca*. L. asiaticus. The samples came from 47 farms located in 35 municipalities. These data indicate that *Ca*. L. americanus is the major HLB agent in SPS. *Ca*. L. americanus was detected by PCR in several batches of *Diaphorina citri* (the Asian psyllid vector of *Ca*. L. asiaticus) individuals collected on symptomatic sweet orange trees infected with *Ca*. L. americanus in SPS, strongly suggesting that *D. citri* is the vector of *Ca*. L. americanus. *Ca*. L. americanus was also detected by PCR in symptomatic leaves of the ornamental rutaceous plant *Murraya paniculata*, which is widely distributed throughout SPS, and is the preferred host of *D. citri*. The detection of *Ca*. L. americanus in *M. paniculata* is relevant to HLB control in SPS.

There are two recognized causal agents of huanglongbing (HLB), *Candidatus (Ca.)* Liberibacter africanus in Africa and *Ca.* Liberibacter asiaticus in Asia (4, 6). Both are noncultured, sieve tube-restricted members of the α-subdivision of the *Proteobacteria*. A polymerase chain reaction (PCR) method (5) detects the two species in citrus leaves by amplification of their 16S rDNA with specific primers OA1+OI1/OI2c (Fig. 1). Both species yield an amplicon of 1160 bp, which can be differentiated by digestion of the amplicons with the restriction endouclease *Xba* I. The *Ca.* L. asiaticus amplicon contains one *Xba*1 restriction site, and yields two fragments (640 bp and 520 bp) upon restriction, while *Ca*. L. africanus has two such sites, and yields three fragments (520 bp, 506 bp and 130 bp). It is thus easy to identify the liberibacter species involved (5). The PCR method has been evaluated in many Asian and African countries for the detection of the two species, and whenever citrus leaves with symptoms of HLB were tested the correctly sized amplicon was regularly obtained.

In March 2004, leaf and fruit symptoms resembling those of HLB were observed in several sweet orange orchards in the Araraquara area of São Paulo State (SPS) (1). Leaf mottling or "blotchy mottle" (8), a characteristic feature of HLB, was the major foliar symptom (Fig. 2). Fruits were small and lopsided, showed strong color inversion, and contained many aborted seeds. In April and June 2004, the PCR-based detection technique was applied to 108 samples of leaves showing blotchy mottle symptoms in order to confirm the presence of HLB in SPS and identify the liberibacter species involved. Each sample came from a single tree, and surprisingly, only samples 34 and 51, respectively from a Lima sweet orange tree and a Murcott tangor tree, were positive for *Ca*. L. asiaticus (Fig. 3A). Test conditions

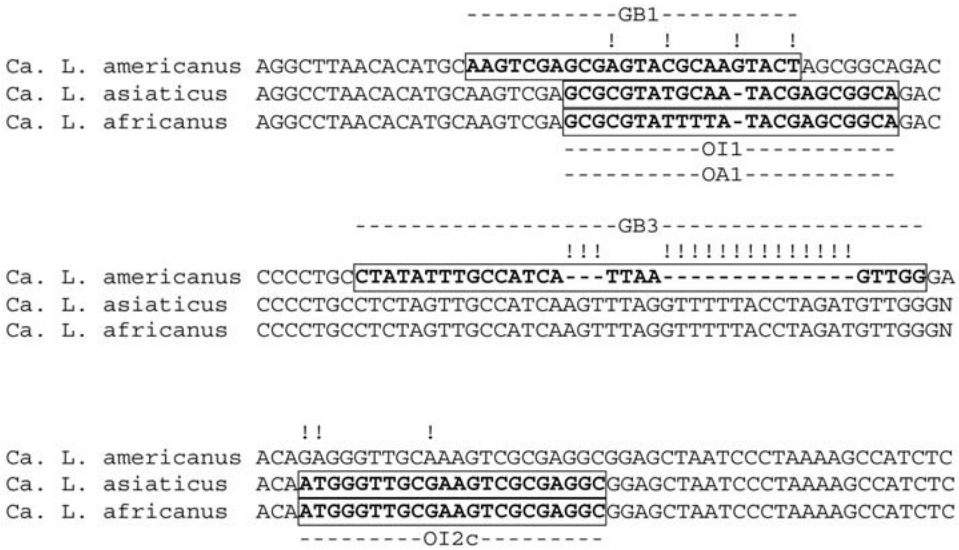

Fig. 1. Sequences of PCR primers for amplification of specific liberibacter 16S rDNA. GB1 and GB3 are forward and reverse primers respectively for *Ca*. L. americanus (amplicon size: 1027 bp). OA1 and OI2c are forward and reverse primers respectively for *Ca*. L. africanus (amplicon size: 1160 bp). OI1 and OI2c are forward and reverse primers for *Ca*. L. asiaticus (amplicon size: 1160 bp). Both forward primers OA1 and OI1 can be used in the same reaction mixture. The 1160 bp amplicons from *Ca*. L. asiaticus and *Ca*. L. africanus have respectively, 1 and 2 *Xba* I restriction sites, and can thus be identified by *Xba* I treatment. The symbol (!) indicates a mismatch between primers for *Ca*. L. americanus and primers for *Ca*. L. africanus and *Ca*. L. asiaticus.

were similar to those where symptomatic control leaves infected with *Ca*. L. asiaticus (Fig. 3A, AS) or *Ca*. L. africanus (Fig. 3A, AF) from the HLB collection in Bordeaux gave positive PCR reactions. Similar results were obtained previously, when Ca. L. asiaticus was detected by the same PCR technique in only two of ten leaf samples (2). These unexpected results suggested that HLB symptomatic leaves from SPS were infected with a novel HLB agent. This hypothesis was confirmed and led to the identification of the new liberibacter species *Candidatus* Liberibacter americanus (9, 11, 12).

Forward primer GB1 and reverse primer GB3 for amplification of 16S rDNA were designed from the 16S rDNA sequence (accession number AY742824) of *Ca*. L. americanus (Fig. 1). These primers, as well as the primers specific for *Ca*. L. africanus and *Ca*. L. asiaticus (OA1+OI1/OI2c) (4, 5), were used for the detection of the three liberibacters in each leaf sample as shown previously (10). A first aliquot of the DNA from a leaf sample was used for the detection of *Ca*. L. americanus with primers GB1/GB3 (lanes marked Am on Fig. 3B), leading to an amplicon of 1027 bp, and a second aliquot served for the detection of Ca. L. africanus and *Ca*. L. asiaticus with primers OA1+OI1/OI2c (Fig. 3B), giving an amplicon of 1160 bp.

PCR detection of *Ca*. L. americanus in citrus leaves. The first leaf DNA samples to be analyzed in this way were the 108 samples of April/June 2004. As before, 106 of the samples again tested negative for *Ca*. L. africanus and *Ca*. L. asiaticus, but all of them were now positive for *Ca*. L. americanus (Fig. 3B). Sample 51, was again positive for *Ca*. L. asiaticus, but negative for *Ca*. L. americanus. Interestingly, sample 34, previously positive for *Ca*. L. asiaticus, was now also positive for *Ca*.

Fig. 2. Sweet orange leaves from SPS showing a blotchy mottle which is characteristic of HLB, regardless of the causal liberibacter. Similar symptoms can also be obtained when phloem sap movement is impaired. Only leaves with liberibacter-induced blotchy mottle will give positive PCR reactions with the primers indicated in Fig. 1.

L. americanus (10). Thus, the Lima sweet orange tree which yielded leaf sample 34 was infected with both liberibacters. One hundred additional symptomatic leaf samples were analysed in the same way. Ninety-eight leaf samples tested positive only for *Ca*. L. americanus. Sample 121 from a 'Hamlin' sweet orange tree was infected with *Ca*. L. asiaticus (Fig. 4A, B, sample 121), and one 'Pera' sweet orange sample carried both *Ca*. L. asiaticus and *Ca*. L. americanus (data not shown).

As of January 2005, PCR identified *Ca*. L. americanus in more than 500 leaf samples from SPS. The tests have always been positive with symptomatic leaves. To test for *Ca*. L. americanus in symptomless leaves, leaves were collected from symptomless parts of symptomatic trees, from symptomless trees adjacent to symptomatic trees and from trees in a region not affected by HLB. As expected from previous studies with *Ca*. L. africanus and *Ca*. L. asiaticus all symptomless leaves tested negative for *Ca*. L. americanus. This result is not due to lack of sensitivity of the PCR technique, but reflects the uneven distribution of the liberibacters in recently infected trees. The results also emphasize the effectiveness of using symptoms to guide the selection of test samples. Therefore, indexing symptomless nursery trees or orchard trees for HLB by PCR may not provide an accurate infection status.

In summary, among the 218 symptomatic HLB leaf samples studied, 214 were positive for *Ca*. L. americanus, 2 for *Ca*. L. asiaticus, and 2 for *Ca*. L. asiaticus and *Ca*. L. americanus. As these samples came from 47 citrus farms within 35 municipalities and were well dis-

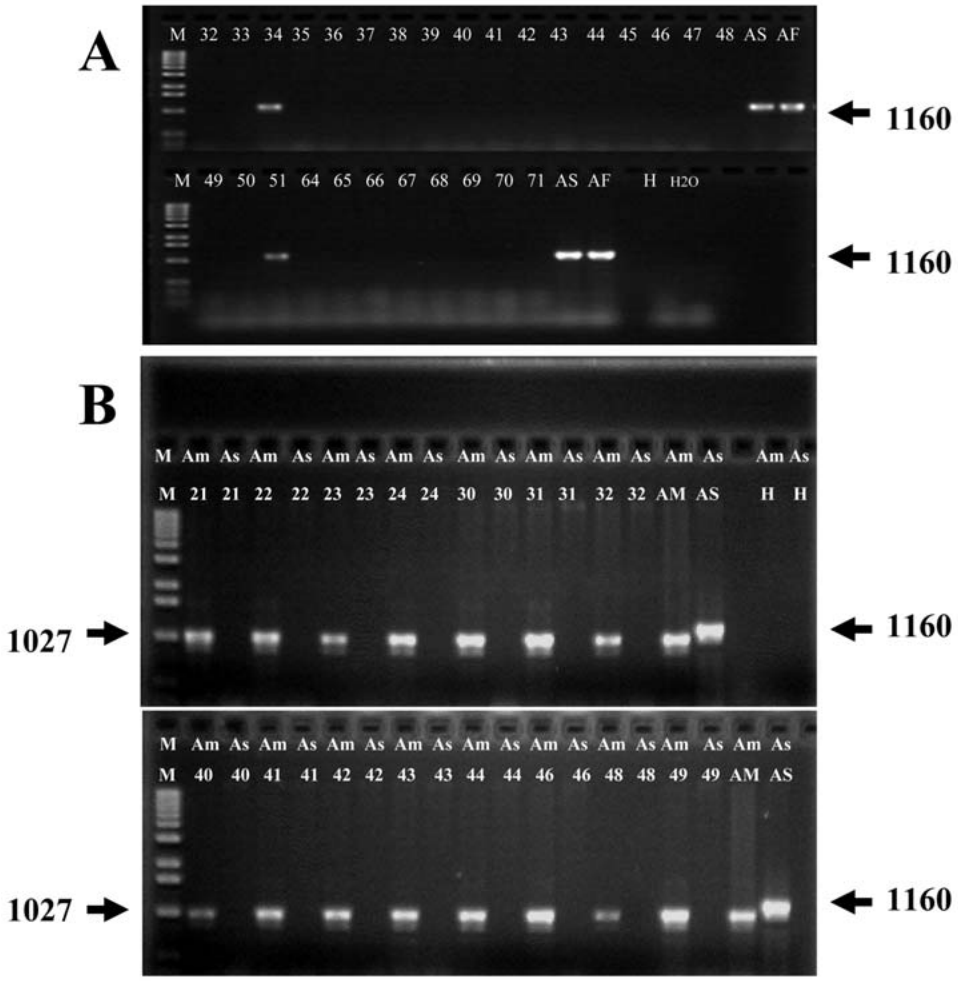

Fig. 3. Agarose gel electrophoresis of 16S rDNA amplified from DNA extracted from symptomatic citrus leaves from SPS. A. The DNA from each leaf sample was amplified with primers (OA1+OI1)/OI2c, specific for *Ca*. L. africanus and *Ca*. L. asiaticus. Lanes 1 to 51, and 64 to 71: DNA from the symptomatic leaf samples. AS and AF: DNA from symptomatic sweet orange leaves infected with respectively, *Ca*. L. asiaticus and *Ca*. L. africanus. Lane H: DNA from healthy sweet orange leaves. Leaves for AF, AS and H were from the Bordeaux greenhouse. Lane H_2O: amplification control in the absence of DNA. M: DNA size markers. Arrows on right point at 1160 bp amplicons, characteristic of *Ca*. L. asiaticus and *Ca*. L. africanus. The *Xba* I test identified the 1160 bp amplicons of samples 34 and 51 as *Ca*. L. asiaticus DNA. B. The DNA from each leaf sample was amplified with primers GB1/GB3 specific for *Ca*. L. americanus, as well as primers (OA1+OI1)/OI2c, specific for *Ca*. L. africanus and *Ca*. L. asiaticus. Am: primers GB1/GB3, As: primers (OA1+OI1)/OI2c. 21 to 24, 30 to 32, 40 to 44, 46, 48 and 49: DNA from the symptomatic leaf samples. AS, H and M: as in Fig. 3A. AM: DNA from symptomatic sweet orange leaves infected with *Ca*. L. americanus. Arrows on right: as in A. Arrows on left point at 1027 bp amplicon, characteristic of *Ca*. L. americanus.

tributed throughout the HLB-affected area of SPS, the results indicate that *Ca*. L. americanus is the major HLB agent in SPS. The four farms on which *Ca*. L. asiaticus was detected also had many trees that were infected with *Ca*. L. americanus. Among the 216 leaf samples infected with *Ca*. L. americanus, 208 were from sweet orange trees

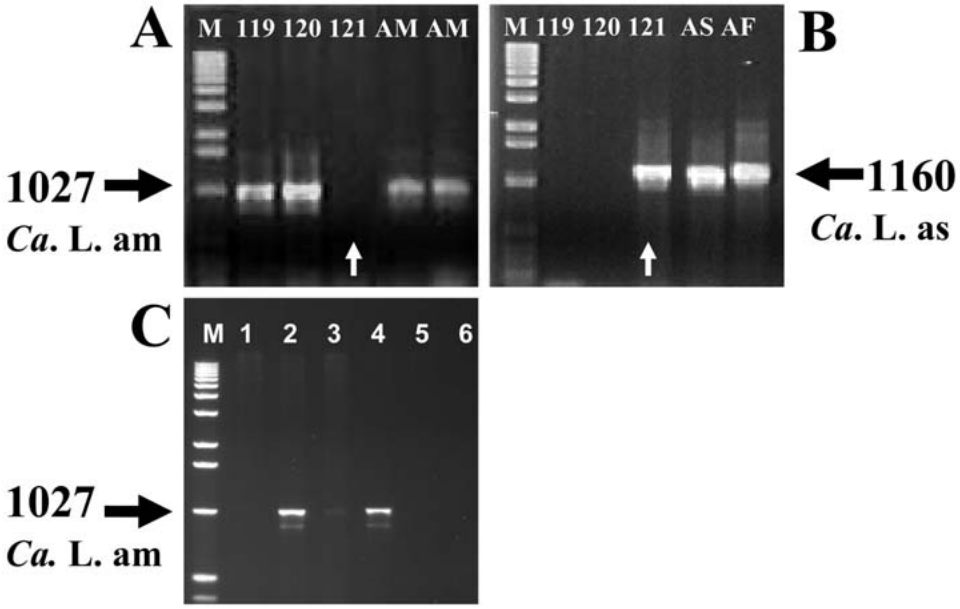

Fig. 4. Agarose gel electrophoresis of 16S rDNA amplified from citrus (A and B) and *Murraya paniculata* leaf DNA (C). 4A and 4B. DNA amplified from symptomatic sweet orange leaf DNA with primers GB1/GB3 specific for *Ca.* L. americanus (A) and primers (OA1+OI1)/OI2c, specific for *Ca.* L. africanus and *Ca.* L. asiaticus (B). Lanes 119, 120, and 121: DNA from symptomatic sweet orange leaf samples. AM, AS, AF, M, and right and left black arrows are as in Fig. 3B. White arrow in 4A: no amplification with DNA from sample 121, but in 4B, positive amplification. In 4C, DNA amplified from *Murraya paniculata* leaf DNA with primers GB1/GB3 specific for *Ca.* L. americanus. Lane 1: Fresh symptomless leaves. Lane 2: Fresh symptomatic leaves. Lane 3: Ten day old symptomatic leaves. All leaves are from the same plant. Lane 4: *Ca.* L. americanus-infected sweet orange leaves. Lane 5: Healthy sweet orange leaves. Lane 6: No DNA negative control amplification.

(Chamout, Hamlin, Lima, Natal, Pera, Valencia, and Westin), five from Ponkan mandarin trees, one from a Murcott tangor tree and two from Cravo mandarin trees. These proportions reflect the fact that sweet orange is by far the major cultivar in SPS.

PCR detection of *Ca.* L. americanus in *Diaphorina citri* psyllids. The Asian psyllid vector of *Ca.* L. asiaticus, *Diaphorina citri*, has been established in Brazil since the 1940s, and is present elsewhere in the Americas (3). In SPS, by the end of 2004, *Ca.* L. americanus was present in 46 municipalities, a rapid rate of spread suggesting that *D. citri* might be the vector. Psyllids were collected in August 2004 from three Pera sweet orange trees with severe symptoms of HLB and which were shown by PCR to be infected only with *Ca.* L. americanus. Insects were subdivided into batches of 10 psyllids for testing. *Ca.* L. africanus and *Ca.* L. asiaticus were not detected in any of these psyllids, whereas 27% (6/22) of batches yielded PCR products indicating the presence of *Ca.* L. americanus (10).

Psyllids also were collected from a severely infected orchard (~50% HLB trees) and analyzed in batches of ten adult insects. PCR detected *Ca.* L. americanus in 36% (27/76) of psyllid batches from symptomatic branches on symptomatic trees, from 36% (13/36) of batches from asymptomatic branches on symptomatic trees and from 14% (5/36) of batches from asymptomatic branches on

asymptomatic trees. Additionally, *Ca*. L. asiaticus was detected in only 4.5% (2/45) of psyllid batches, a proportion similar to the overall ratio of *Ca.* L. asiaticus-infected trees to *Ca*. L. americanus-infected trees in SPS. The data indicate that in a severely symptomatic orchard, infected psyllids are equally distributed among all branches in symptomatic trees and that infected psyllids, probably coming from symptomatic trees, can be found on asymptomatic trees. These results suggest that *D. citri* is a vector of *Ca*. L. americanus in SPS.

PCR detection of *Ca.* L. americanus in *Murraya paniculata* leaves. *M. paniculata* is an ornamental rutaceous shrub or tree which is widely planted throughout SPS and which is the preferred host of *D. citri*. *Ca*. L. americanus was detected by PCR only in symptomatic leaves from three of thirteen *M. paniculata* plants (7) as seen in Fig. 4C. Additionally, *Ca*. L. americanus was also detected in one batch of 10 psyllids collected from an *M. paniculata* plant (7).

Conclusion. The two sets of PCR primers used in this work, OA1+OI1/OI2c for *Ca*. L. asiaticus and GB1/GB3 for *Ca*. L. americanus, are very specific and detect only the homologous liberibacter. Regardless of the primers used, PCR methods involved have similar sensitivities. *Ca*. L. americanus was found to be infecting ~95% of the tested trees. The Asian citrus psyllid, present in SPS for more than 50 years, also was found to be infected with the American liberibacter, and is most certainly the insect vector of *Ca*. L. americanus in SPS. Psyllid batches infected with *Ca*. L. asiaticus were rare, reflecting the fact that most of the trees from which the psyllids were collected were infected with *Ca*. L. americanus. *M. paniculata*, the preferred host of *D. citri*, and which is present throughout SPS in and near citrus orchards and within non citrus areas, was shown to be a host of *Ca*. L. americanus, and thus represents an additional source of liberibacter inoculum for psyllids to acquire. On the basis of these results, it seems urgent to eradicate all *M. paniculata* plants present in citrus farms as part of a framework of efforts to control HLB. Removal of such plants from public parks, streets and avenues, as well as house gardens must also be seriously considered. Propagation of new *M. paniculata* plants should be prohibited.

It has been discussed elsewhere that *Ca*. L. asiaticus originated in Asia, *Ca*. L. africanus in Africa, and *Ca*. L. americanus in America (12). Based on current information, it is reasonable to think that *Ca*. L. americanus will be the causal agent if HLB appears in additional citrus growing countries on the American continent.

LITERATURE CITED

1. Anon.
2004. Greening chega aos pomares paulistas. Rev. Fundecitrus Ano XX, No. 123: 8-12.
2. Coletta-Filho, H. D., M. L. P. N. Targon, M. A. Takita, J. D. De Negri, Jr., J. Pompeu, and M. A. Machado
2004. First report of the Causal Agent of Huanglongbing ("*Candidatus* Liberibacter asiaticus") in Brazil. Plant Dis. 88: 1382.
3. Halbert, S. E., and C.A. Nuñez
2004. Distribution of the Asian citrus psyllid, *Diaphorina citri* Kuwayama (Rhynchota: Psyllidae) in the Caribbean basin. Fla. Entomol. 87: 401-402.
4. Jagoueix, S., J. M. Bové, and M. Garnier
1994. The phloem-limited bacterium of greening disease of citrus is a member of the α-subdivision of the *Proteobacteria.* Int. J. Syst. Bacteriol. 44: 397-86.
5. Jagoueix, S., J. M. Bové, and M. Garnier
1996. PCR detection of the two '*Candidatus*' Liberibacter species associated with greening disease of citrus. Mol. Cell. Probes 10: 43-50.

6. Jagoueix, S., J. M. Bové, and M. Garnier
1997. Comparison of the 16S/23S Ribosomal Intergenic Regions of "*Candidatus* Liberibacter asiaticum" and "*Candidatus* Liberibacter africanum", the two species associated with Citrus Huanglongbing (Greening) Disease. Int. J. Syst. Bacteriol. 47: 224-227.
7. Lopes, S. A., E. C. Martins, and G. F. Frare
2005. Detecção de *Candidatus* Liberibacter americanus em *Murraya paniculata*. Summa Phytopathol. 31: 48-9.
8. McClean, A. P. D., and R. E. Schwarz
1970. Greening or blotchy-mottle disease of citrus. Phytophylactica 2: 177-194.
9. Teixeira, D. C., A. J. Ayres, E. W. Kitajima, F. A. O. Tanaka, J. L. Danet, S. Jagoueix-Eveillard, C. Saillard, and J. M. Bové
2005. First report of a Huanglongbing-like disease of citrus in Sao Paulo State, Brazil, and association of a new liberibacter species, "*Candidatus* Liberibacter americanus", with the disease. Plant Dis. 89: 107.
10. Teixeira, D. do Carmo, J. L. Danet, S. Eveillard, E. C. Martins, W. C.de Jesus Junior, P. T. Yamamoto, S. A. Lopes, R. B. Bassanezi, A. J. Ayres, C. Saillard, J. M. Bové
2005. Citrus huanglongbing in São Paulo State, Brazil: PCR detection of the "*Candidatus*" Liberibacter species associated with the disease. Mol. Cell. Probes 19: 173-179.
11. Teixeira, D. do Carmo, C. Saillard, S. Eveillard, J. L. Danet, A. J. Ayres, and J. M. Bové
2005. *Candidatus* Liberibacter americanus sp. nov., associated with citrus huanglongbing (greening disease) in São Paulo State, Brazil. Int. J. Syst. Evol. Microbiol. 55: 1857-1862.
12. Teixeira, D. C., C. Saillard, S. Eveillard, J. L. Danet, P. T. Yamamoto, S. A. Lopes, A. J. Ayres, and J. M. Bové
2005. A new liberibacter species, *Candidatus* Liberibacter americanus sp. nov., is associated with citrus huanglongbing (greening disease) in São Paulo State, Brazil. In: *Proc. 16th Conf. IOCV*, 325-340. IOCV, Riverside, CA.