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Research Article

Dynamics of emergence and spread of citrus huanglongbing disease in Iran

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Abstract

Introduction: Huanglongbing or Fruit Greening is one of the major citrus diseases in Iran that has spread to most of the country's citrus growing provinces. In this study, the prevalence trend of the disease in seven provinces was investigated and analyzed. **Materials and Methods:** Citrus orchards in Sistan-Balochestan, Hormozgan, Bushehr, Khuzestan, Fars, Kerman and Mazandaran provinces were visited and samples suspected of having this disease were collected, in 2007-2009 and 2017-2019 years. After DNA extraction from the samples, the PCR test was performed using the specific primers OI1/OI2c and A2/J5. **Results:** It was found that the percentage of infected specimens, infected areas and number of hosts of *Candidatus* Liberibacter asiaticus, the causative agent of this disease, has increased over this period. At the end of this period, some samples of oranges, tangerines, grapefruits, bitter oranges, Lisbon lemons and Mexican limes were obtained from the provinces: Kerman, Hormozgan, Sistan-Baluchestan, and Fars were infected with the disease. **Conclusion:** The study of the disease during these years showed that: the infected areas and the host range of the disease have expanded a lot during this period, and the spread of the disease in citrus cultivation areas in the south of the country has been with a gentle slope. The reduction of the disease-carrying psyllium population during these years seems to have been effective in slowing down the spread of the disease.

Key words: Orange, Tangerine, Grapefruit, Greening

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مقاله پژوهشی

پویایی‌شناسی وقوع و پراکندگی بیماری هوانگ‌لونگ‌بینگ مرکبات در ایران

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چکیده

مقدمه: هوانگ‌لونگ‌بینگ یا میوه سبز یکی‌از بیماری‌های مهم مرکبات در ایران است. روند گسترش بیماری در هفت استان در این تحقیق مورد بررسی قرار گرفت. **مواد و روش‌ها:** باغ‌های مرکبات استان‌های سیستان و بلوچستان، هرمزگان، بوشهر، خوزستان، فارس، کرمان و مازندران طی سال‌های ۱۳۸۶ تا ۱۳۸۸ و ۱۳۹۶ تا ۱۳۹۸ بازدید شدند و نمونه‌های مشکوک به بیماری جمع‌آوری و پس‌از استخراج DNA از نمونه‌ها، آزمون پی‌سی‌آر با استفاده از آغازگرهای اختصاصی OI1/OI2c و A2/J5 انجام شد. **یافته‌ها:** بررسی نمونه‌ها نشان داد که، مناطق و میزبان‌های باکتری *Candidatus* Liberibacter asiaticus، عامل بیماری، در این مدت افزایش یافته است، به طوری که در پایان این‌دوره در نمونه‌های درختان پرتقال، نارنگی، گریپ‌فروت، نارنج، لیمولیسبون و لیموترش از استان‌های کرمان، هرمزگان، سیستان و بلوچستان و فارس، بیماری ردیابی شد. **نتیجه‌گیری:** بررسی بیماری طی این سالها نشان داد که: مناطق آلوده و دامنه میزبانی بیماری گسترش زیادی یافته است و گسترش بیماری در مناطق کشت مرکبات در جنوب کشور با شیب ملایم بوده است. کاهش جمعیت پسیل ناقل بیماری طی این سال‌ها به نظر می‌رسد که در آهستگی روند گسترش بیماری مؤثر بوده است.

واژگان کلیدی: پرتقال، نارنگی، گریپ‌فروت، میوه سبز

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Introduction مقدمه

Huanglongbing (HLB), also known as ‘‘citrus greening,’’ is the most important bacterial diseases of citrus, which is distributed in citrus-growing areas worldwide, ranging from Asia to Africa, and America (Bove´, 2006). The disease was first reported from China at the end of the 19th century, then from other Asian and African countries, and in 2004 from Brazil one year later from Florida (Halbert, 2005; da Graca *et al.* 2015). Three *Candidatus* (*Ca.*) spp. of the pathogen are currently known. The most widespread Asian species, *Ca.* Liberibacter asiaticus Jagoueix, Bové, and Garnier, 1994, (CLas), is found in all HLB affected countries except Africa. The African species, *Ca.* L. africanus Jagoueix, Bové, and Garnier, 1994, (CLaf), and the American species, *Ca.* L. americanus Teixeira *et al.* 2005, (CLam), are so far restricted to Africa and Brazil, respectively (Teixeira *et al.* 2005a, Teixeira *et al.* 2005b). Most of the citrus species can be infected with HLB (Alizadeh 2004, Wang and Trivedi 2013, Safarpour *et al.* 2022). CLas species is the most important species causing this disease in the world.

CLas and Clam bacteria are transmitted through *Diaphorina citri* Kuwayama, the Asian citrus psyllid, and CLaf bacteria is transmitted through the African citrus psyllid, Trioza erytrea Del Guercio. (Bove 2006, Alizadeh 2009). The general symptom of this disease in newly infected trees is "yellow shoot" which is seen as a branch, with yellow leaves, inside the tree. The symptom on the leaf appears as light shades known as "blotchy mottle". Infected fruits are usually small, asymmetrical, with shriveled, pitted and brown seeds. they produce small, irregularly shaped fruit with a thick, pale peel that remains green at the bottom and tastes very bitter (da Graca *et al.* 2015).

The disease was reported in Sistan-Baluchistan and Hormozgan provinces in 2007 (Faghihi *et al.* 2009), then it was reported from several regions in Sistan-Baluchestan, Kerman and other citrus growing areas of southern Iran (Alizadeh *et al.* 2010, Salehi *et al.* 2012, Salehi and Rasoulpoor 2016). Integrated management of the disease is possible through insecticide applications to reduce psyllid populations, removal of infected trees to eliminate sources of bacterial inoculum, and the establishment of pathogen free nursery systems. (Parad and Rezaei 2016, Alizadeh 2017a, Alizadeh 2017b, Alizadeh 2017c, Alizadeh 2017d).

After the initial report on the distribution of HLB in Iran, the studies related to monitoring, survey and determining new areas of contamination continued as a research project. The present report is the results of this research project in the time periods of 2007-2009 and 2017-2019.

Materials and Methods مواد و روش‌ها

Sampling

Citrus orchards in different regions of Iran were visited from December to the end of July, every year, from 2007-2009 and 2017-2019. Samples suspected and showing symptom of HLB disease were collected from leaves, fruits and peduncles.

Detection of CLas in plant samples

After DNA extraction from plant samples (Ruangwong and Akrapisan 2006), PCR test was performed, using specific primer sets of this species (A2/J5 and OI1/OI2c). OI1/OI2c targeting the 16S rDNA locus and A2/J5 targeting the beta-operon locus of ribosomal proteins. (Safarpour *et al.* 2022) (Table 1).

جدول ۱. مشخصات آغازگرهای اختصاصی مورد استفاده در این مطالعه.

Table 1. Specific primers used in this study.

Primer name	Sequence	Annealing (°C)	Amplicon size (bp)	Target	Reference
OI1	5′-GCGCGTATGCAATACGAGCGGCA-3′	56	1160	16S rDNA	Jagoueix et al. 1996
OI2c	5′-GCCTCGCGACTTCGCAACCCAT-3′	56			Jagoueix et al. 1996
A2	5′- TATAAAGGTTGACCTTTCGAGTTT-3′	60	703	β-operon	Hocquellet et al. 1999
J5	5′-ACAAAAGCAGAAATAGCACGAACAA-3′	60			Hocquellet et al. 1999

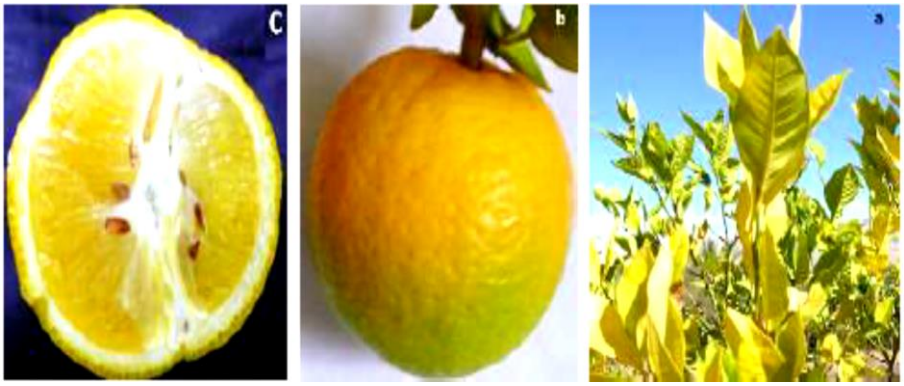
Sequencing and phylogenetic analysis

Four samples of the OI1/OI2c amplicons was purified, cloned and sequenced (by Codon Genetics, Iran) and edited using Bio-Edit and DNA STAR (SeqMan) software. The blast program was performed and the phylogenetic tree was drawn by neighbor-joining method and using MEGAX software (Kumar *et al.* 2018). *Rhizobium* sp. and *Phlomobacter fragariae* were used as outgroup species. The standard error was calculated using 1000 bootstrap replicates.

Results یافته‌ها

Disease symptoms and distribution

HLB disease symptoms were observed on orange, tangerine and grapefruit trees from Kerman, Hormozgan and Sistan-Baluchistan provinces during 2007-2009 and from Fars province in the following years (2017-2019) (Figures 1 and 2). Some symptoms similar to this disease were observed on citrus fruits of Mazandaran, Bushehr and Khuzestan provinces, but the presence of HLB disease agent was not confirmed in these provinces.



شکل ۱. a: برگ‌های شاخه‌های آلوده که به شکل "گوش خرگوش" درآمده‌اند، با رشد عمودی همراه با شاخه‌ها و برگ‌های راست، ایستاده و زرد. b: وارونگی رنگ و c: عدم تقارن میوه به همراه بذور چروکیده و قهوه‌ای در میوه پرتقال آلوده به بیماری HLB در منطقه سیرمند حاجی‌آباد در استان هرمزگان

Figure 1. a: Infected branches leaves form ‘‘rabbit's ears’’ symptoms, which consists of the vertical growth with upright shoots with erect chlorotic leaves. b: Color inversion symptoms on fruit. c: Lopsided fruit with wrinkled and brown seeds in orange fruit infected with HLB disease in Sirmand region of Hajiabad in Hormozgan province

شکل ۲. نشانه‌های بیماری HLB. نشانه بیماری در برگ و میوه پرتقال جمع‌آوری شده از استان سیستان‌وبلوچستان (A-D). A: نشانه پیسک (ماتلینگ) در برگ‌ها؛ B: وارونگی رنگ؛ C: عدم تقارن در میوه؛ D: بذرهای چروکیده و قهوه‌ای در میوه‌های آلوده. E: نشانه بیماری و خسارت پسیل آسیایی مرکبات روی لیموترش (در منطقه فاریاب جیرفت). نشانه بیماری روی میوه و برگ گریپ‌فروت (F و G) و نارنگی (H و I) به‌ترتیب درردوان و سیاهو در استان هرمزگان

Figure 2. Symptoms of HLB disease. Symptoms in orange leaves and fruits, collected from Sistan and Baluchestan province (A-D). A: Symptoms of mottling on leaves, B: Color inversion, C: Lopsided fruit, D: Aborted, wrinkled and brown seeds in infected fruits. E: Symptoms of HLB and Asian Citrus Psyllid damage on Mexican lime (in Faryab, Jiroft). Symptoms of HLB disease on fruits and leaves of grapefruit (F and G) and tangerine (H and I) in Rhodan and Siahoo in Hormozgan province.

HLB disease were observed in seven provinces during these periods (Table 2), and orange, tangerine, grapefruit, and lime were determined as the hosts of the disease in Iran.

جدول ۲. درصد نمونه‌های آلوده به *Candidatus* Liberibacter asiaticus از نمونه‌های دارای نشانه مشکوک به بیماری HLB جمع‌آوری‌شده از باغ‌های مرکبات جنوب ایران در بازه زمانی سال‌های ۱۳۸۶ تا ۱۳۸۸ و ۱۳۹۶ تا ۱۳۹۸.

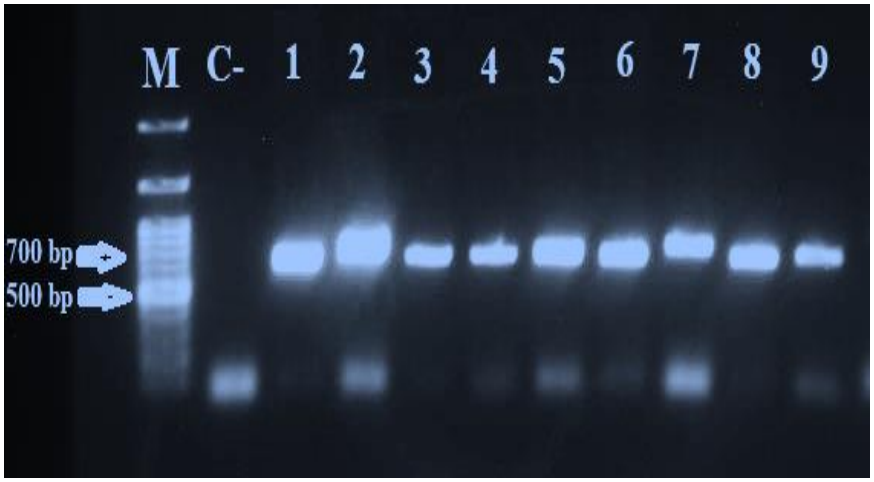
Table 2. Percentage of infected samples to *Candidatus* Liberibacter asiaticus among the specimens with suspected symptoms of HLB collected from citrus orchards in southern Iran in 2007-2009 and 2017-2019 periods

	CLas detection (2007-2009)			CLas detection (2017-2019)			CLas detection mean		
Province	samples Nos.	positive Nos.	positive%	samples Nos.	positive Nos.	Positive%	samples Nos.	positive Nos.	positive%
Sistan & Baluchestan	88	15	17.04	65	39	60	153	54	35.29
Hormozgan	94	35	37.23	91	66	72.52	185	101	54.59
Kerman	125	34	27.20	108	62	57.40	233	96	40.20
Fars	14	0	0	23	11	47.82	37	11	29.72
Khuzestan	8	0	0	21	0	0	29	0	0
Bushehr	2	0	0	20	0	0	22	0	0
Mazandaran	4	0	0	25	0	0	29	0	0

HLB disease was observed only in Sistan & Baluchestan, Hormozgan and Kerman provinces, in 2007 to 2009, and during 2017 to 2019 years, also detected in Fars province. In these years, the percentage of infected trees in Sistan & Baluchestan , Hormozgan, and Kerman provinces was 17.04, 37.23, and 27.20%, and in the next years, it was 60, 72.52, and 57.40%, respectively. CLas was detected in 29.72% of the samples of Fars province, for the first time, in this period. During two periods (2007-2009 and 2017-2019) the average incidence of HLB disease in symptomatic trees in Sistan & Baluchestan, Hormozgan, Kerman and Fars provinces was 35.29, 54.59, 40.20 and 29.72%, respectively (Table 2).

Molecular identification of the pathogen

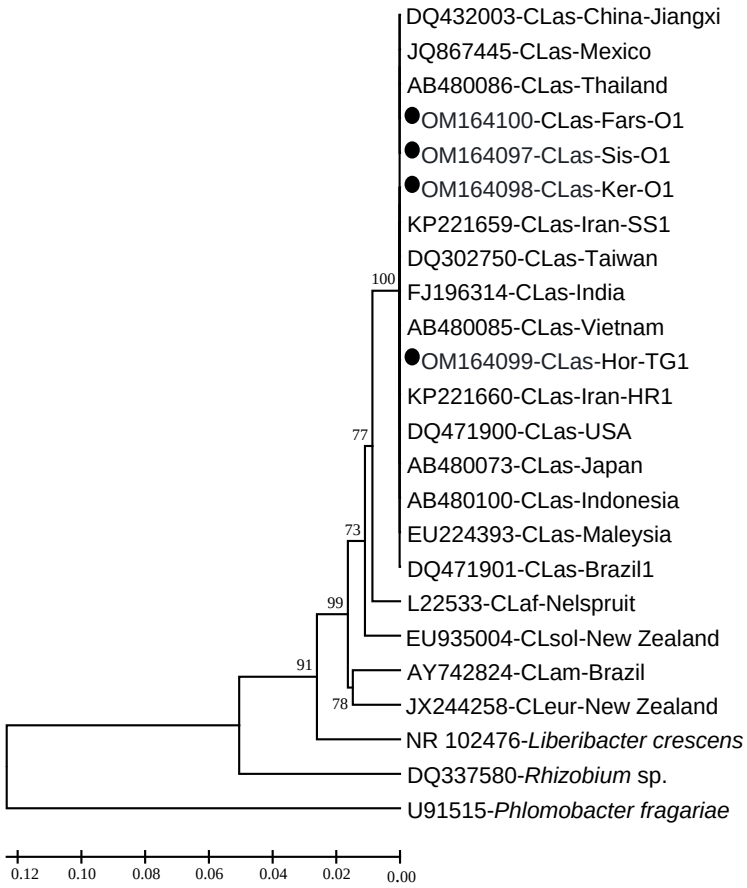
DNA samples from all symptomatic leaves were positive with both primer sets. The OI1/OI2c primers generated an amplicon of approximately 1,160 bp and A2/J5 primer set generated an ~703-bp amplicon (Figure 3).



شکل ۳. الکتروفورز محصول‌های PCR بااستفاده‌از جفت‌آغازگر A2/J5 در ژل آگارز یک‌درصد. راهک -C: شاهد منفی؛ راهک‌های 1 تا 9: باند ۷۰۰ جفت‌بازی، به ترتیب در نمونه پرتقال‌های سرباز، قصرقند، جیرفت، جیرفت، رودان، نارنگی‌های سیاهو و سیاهو، پرتقال‌های داراب و جهرم. M: نشانگر وزن مولکولی (Smobio, SM2300).

Figure 3. Electrophoresis of PCR products using A2 / J5 primers, in 1% agarose gel. C: Negative control; 1 to 9: Band profile (about 700 bp.) obtained from the samples of Sarbaz, Qasr-e-Ghand, Jiroft, Jiroft and Rudan oranges, Siahoo and Siahoo mandarin, Darab and Jahrom oranges, respectively. M: molecular weight marker (Smobio, SM2300).

Four derived sequences of 1167 bp for the OI1/OI2c amplicon have been deposited in the GenBank database under Accession Nos. OM164097, OM164098, OM164099 and OM164100. Sequence comparison revealed that these sequences shared >99% identity to the corresponding regions of '*Ca.* L. asiaticus' in the GenBank database. Based on the phylogenetic tree, four liberibacter isolates in this study are placed in the same group with CLas isolates from other parts of the world (Japan, America, Taiwan, Indonesia, Malaysia, Brazil and Mexico) and were separated from other liberibacter species such as CLaf, CLam, *Ca.* L. solanacearum, *Ca.* L. europaeus and *Liberibacter crescens* (Figure 4).



شکل ۴. درخت تبارزایی به‌دست‌آمده براساس ترادف‌های ژن *rRNA*۱۶ S از چهار جدایه باکتری عامل HLB. از گونه‌های Rhizobium sp. و *Phlomobacter fragariae* به‌عنوان گونه‌های خارج‌از گروه استفاده شد.) : جدایه‌های ایران در این مطالعه. CLas: *Candidatus* Liberibacter asiaticus؛ CLaf: *Ca*. L. africanus؛ CLam: *Ca*. L. americanus؛ CLsol: *Ca*. L. solanacearum؛ CLeur: *Ca*. L. europaeus

Figure 4. Phylogenetic tree obtained based on 16S rRNA gene sequences from four bacterial isolates associated with HLB. *Rhizobium* sp. and *Phlomobacter fragariae* were used as external species. •: Iranian samples in this study. CLas: *Candidatus* Liberibacter asiaticus; CLaf: Ca. L. africanus; CLam: Ca. L. americanus; CLsol: Ca. L. solanacearum; CLeu: Ca. L. europaeus

Discussion بحث

After reporting the occurrence of HLB disease in Iran, regular visits and sampling of citrus orchards in Sistan-Baluchistan provinces, Hormozgan, Kerman, Fars and other citrus growing regions of the country has been began. Preliminary investigations showed that the areas that were mainly infected with this disease are from the provinces of Sistan-Baluchistan, Hormozgan, and Kerman. The increasing geographic spread of the citrus psyllid vector and, as a result, the HLB disease, required the continuation of annual sampling, which peaked between 2016 and 2018. During this time period, visiting very sensitive and endangered areas, including the citrus growing areas of Fars province (where HLB disease was not seen despite the presence of Asian citrus psyllid in the region) was prioritized. The sampling of the second period from Fars province showed that about 47.82% of the trees with symptoms were infected with HLB disease.

These contaminations happened mainly in the areas that were faced with psyllid outbreaks in the previous years. The studies of the second time period showed that the spread of the disease has increased in areas where contamination was proven in the past (such as some areas south of Kerman, gardens in Nikshahr, Sarbaz and Qasrqand regions in Balochistan and Siahou region in Hormozgan province). Also, this disease has spread to Fars province, in areas such as Darab and Jahrom. Bushehr, Khuzestan and Mazandaran provinces were free of this disease in both periods. In these provinces, some symptoms similar to this disease were observed, but the existence of HLB disease was never confirmed. The possibility of the spread of this disease to citrus growing areas in the north of the country, where the Asian citrus psyllid has not been observed, is low, except through the transmission of infected seedlings or cuttings. Therefore, it is necessary to comply with quarantine rules (especially internal quarantine) and to carry out continuous surveillance and monitoring in the northern regions of the country as well as Bushehr and Khuzestan provinces. A number of trees with symptoms similar to HLB were not positive in the PCR test. This was expected due to the non-specificity of the symptoms of this disease as well as the similarity of its symptoms with other diseases, such as Stubborn and Tristeza.

Reports show that a few years after Asian citrus psyllid was detected in a region, HLB disease was also reported in that region. For example, HLB disease has been reported in Iran, America, and Cuba 11, 7, and 8 years after the first sighting of Asian citrus psyllid, respectively (Bové 2006, Gottwald 2010). If control measures and disease management are not carried out, the spread of the disease in young infected gardens (up to three years old) can reach more than 50% in three to five years and in older gardens, in five years or more. In general, the incubation period of the disease is longer in orchards older than ten years and the progress of the disease is slower than in young orchards (Gottwald 2010). The rate of disease progression is higher in the garden margins and inside the lines compared to the inner parts of the garden and between the lines (Shen *et al.* 2013).

Undoubtedly, if the invasion of Asian citrus psyllid was as severe as between 2011 and 2016, the south of Iran would have faced a widespread outbreak of this disease. Fortunately, in the last few years, due to the decrease in the population of Asian citrus psyllid (probably caused by repeated spraying or climate change), the rate of increase in the occurrence of HLB disease has decreased and the predictions related to the rapid spread of the disease have not been realized. Of course, recently, the role of reproductive organs, especially pollen, in the spread of this disease has been given serious attention (Wang *et al.* 2021). If the transmission of this disease agent is proved by pollen and contamination of seed embryos and other plant organs, which has been proposed by Wang *et al.* (2021), it is necessary to pay attention to the pollens originated from trees infected with this disease as a new way of spreading and transmitting the disease agent, in addition to vector control (Wang *et al.* 2021).

If the disease vector population continues to decrease sharply in citrus growing areas in the south of the country, the importance of this disease may gradually decrease. However, it is recommended that the continuous monitoring of this disease in all citrus growing areas in Iran, using the usual PCR methods, is still on the agenda of the Plant Protection Organization. In general, the spread of this dangerous and quarantine disease can be prevented by the following methods: control of Asian citrus psyllid in citrus orchards and prevent its further spread through quarantine and sanitary methods; Continuous monitoring of the disease agent and immediate destruction of infected trees in areas free of contamination.

Conclusion نتیجه‌گیری

The results of the monitoring of the mentioned two time periods showed that: 1. The spread of this disease has increased in the previously infected areas. 2. This disease has spread to new areas that did not exist before (such as Fars province). 3. Due to the reduction of the disease-carrying psyllium population, the overall spread of the disease has been slow and less than expected. 4. The Asian form of the disease (CLas) has been associated with this disease in all the places visited and sampled.

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