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Research Article

Identification of yeast and yeast-like symbionts associated with *Hishimonus phycitis* (Hemiptera: Cicadellidae), the insect vector of lime witches' broom phytoplasma

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Abstract: Witches' broom disease of lime (WBDL) is a lime disease that has destroyed several citrus orchards in Oman, United Arab Emirates and Iran. WBDL is caused by a bacterium *"Candidatus* Phytoplasma aurantifolia" which is spread through the citrus orchards by a leafhopper, *Hishimonus phycitis* (Distant) (Hemiptera: Cicadellidae). Leafhoppers are associated with symbiotic microorganisms which provide them with essential amino acids lacking in their diets. Yeast-like relationships with insects are known as common and obligate symbiotic relationship. A prerequisite for the development of future strategies for the symbiotic control of insect pests and insect-vector competence is the identification of insect-associated yeast-like symbionts. In the present study, yeast-like symbionts housed in *H. phycitis* were investigated in insects collected from 13 districts of citrus orchards distributed in southern Iran (Hormozgan, Kerman, Sistan-Balushestan and Fars provinces). Insects were collected from infected lime trees by a D-Vac and stored at -20 ºC up to the DNA extraction. Total DNA was extracted and PCR was conducted with specific primer sets targeting 18S rRNA and 26S rRNA genes of the symbionts. Results revealed that the vector harboured two yeast symbionts, namely Yeast like symbiont of *H. phycitis* (Hp-YLS) and *Candida pimensis*, with a similarity of (98-99%) to those reported from the other Cicadellids. These results substantiate the association of these two endosymbiotic microbiota with *H. phycitis* which may suggest their ecological interactions. To establish any endosymbiotic relationship and probable interfering in pathogen transmission, further studies are needed.

Keywords: *Hishimonus phycitis*, yeast-like symbionts, *Candida pimensis*

Introduction

Hishimonus phycitis (Distant) (Hemiptera: Cicadellidae) is the insect vector of witches' broom disease of lime (WBDL), a destructive disease of lime trees in southern Iran (Bove *et al*., 2000). WBDL is caused by "*Candidatus* Phytoplasma aurantifolia", a cell wall-less bacterium which is transmitted to the lime phloem during the sap meal of *H. phycitis* (Bagheri *et al*., 2009). Like other insect vectors, studies on the microbial community associated with *H. phycitis* is increasing, with

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the aim of detecting microorganisms which might be exploited for alternative control strategies. Insect endosymbiotic microflora have played a key role in their ecological and evolutionary success (Douglas, 2011). Most insect species harbour symbiotic microorganisms within specialized cells (mycetocyte or bacteriocyte), which can be scattered throughout different organs or tissues, or aggregated together, forming a mycetome. These mycetocyte endosymbionts are vertically transmitted to the offspring and this process generally occurs via transovarial transmission (Saachi *et al*., 2012). Several roles have been characterized for yeast and yeast-like fungi associated with insects including nutritional role and detoxification of toxic plant metabolites in the host's diet (Vega and Dowd, 2005).

Paratransgenesis, a strategy using transgenic microorganism associated with insects to express molecules interfering with pathogen transmission, is a new approach to reduce the burden of these diseases (Beard *et al*., 1998). Yeast and yeast-like symbionts (YLSs), which are commonly associated with insects, are offered as alternative microorganisms for transformation (Beard *et al*., 2002). A prerequisite for the development of future strategies for the paratransgenesis of this vector is to identify YLSs associated with *H. phycitis* whose characteristics appear promising.

Taking this fact into account, this study was carried out to identify yeast and yeast-like symbionts living in *H. phycitis*.

Materials and Methods

Insect collection

Adults of *H. phycitis* leafhopper were collected from Mexican lime (*Citrus aurantifolia* L.) trees at 13 sites in four major lime-growing provinces of southern Iran (Hormozgan, Kerman, Sistan-Baluchestan and Fars provinces) (Table 1) using a D-Vac aspirator. Specimens were preserved in absolute ethanol and stored at -20 ºC (Fukatsu, 1999).

DNA extraction

DNA was extracted from leafhoppers using a CTAB method in accordance with protocol of Reineke *et al*. (1998) with some modification. Each sample of DNA was dissolved in 50µL of double distilled water and stored at-20 ºC. The quality of the extracted DNA was verified on a 1% agarose gel and amount of total genomic DNA obtained was quantified using a Nanodrop (Allsheng, Nano-200, China).

Amplification of fungal rRNA genes and phylogenetic analysis

Specific forward primers (YLS-18S-F and YLS-26S-F) were selected which only amplify DNA of yeasts when used in conjunction with the universal primers (Table 2) (Nishino *et al*., 2016). In addition, ITS primers (ITS1, ITS2 and ITS4) were used to amplify yeasts. Yeasts DNA were amplified by PCR (PeqLab, USA) in 25 µL reaction mixture containing 12.5µL Master Mix, 1µL of each primer (10 pmol/µL), 1µL of extracted DNA and 9.5µL double-distilled water. Aliquots 5µL of each PCR product were visualized on a 1% agarose gel stained with Flouro dye. All PCR products were directly sequenced with both primers by Macrogen Sequencing Service (South Korea). In addition, a diagnostic PCR was conducted to calculate infection frequency of yeasts and YLSs housed in *H. phycitis*.

Phylogenetic analyses of 18S and 26S rRNA gene sequences were conducted by neighbor joining (NJ) methods using MEGA 6.0 software (Tamura *et al*., 2013). To assess statistical support for hypothesized NJ clades, bootstrap analysis was done with 1000 bootstrap replicates. The sequences of 18S and 26S rRNA genes were deposited in GeneBank (accession number AP589637-39) and other sequences used in phylogenetic analysis were downloaded from GeneBank.

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Table 1 Localities of sample collection sites for *Hishimonus phycitis* populations from *Citrus aurantifolia*, codes for collection sites and coordinates of collection sites in the tested localities.

Locality	Code	Number/Sex	Latitude	Longitude
Hormozgan/Roodan	Hr	2♂, 8♀	N27º 44'50"	E57º 13'75"
Hormozgan/Minab	Hm	6♂, 11♀	N27º 32'18"	E57º 10'05"
Hormozgan/Hashtbandi	Hh	8♂, 9♀	N27º 05'01"	E57º 23'75"
Hormozgan/Qale'e Qazi	Hq	4♂, 6♀	N24º 45'00"	E56º 54'58"
Hormozgan/Sirmand	Hs	2♂, 3♀	N27º 98'20"	E56º 12'36"
Hormozgan/Tashkooyeh	Ht	5♂, 3♀	N28º 14'66"	E55º 44'83"
Kerman/Kahnooj	Kk	7♂, 4♀	N27º 97'75"	E57º 71'15"
Kerman/Jiroft	Kj	4♂, 13♀	N28º 67'68"	E57º 68'52"
Kerman/Ali Abad	Kaa	5♂, 5♀	N28º 58'93"	E57º 84'31"
Kerman/Manoojan	Km	6♂, 4♀	N27º 51'96"	E57º 55'30"
Kerman/Jahad Abad	Kja	3♂, 4♀	N28º 54'71"	E57º 86'91"
Sistan-Baluchestan/Nikshahr	Sn	2♂, 2♀	N26º 14'13"	E60º 44'27"

Table 2 Primers and PCR conditions used in identification of yeast and yeast-like symbionts associated with *Hishimonus phycitis.*

Primer	Primer sequence (5'-3')	PCR condition
Y26S	F-GGTCCGTGTTTCAAGACGG R-GGATTGCCCCAGTAACG	94 °C: 2 min, followed by 35 cycles: 94 °C: 1 min, 55 °C: 30 s, and 72 °C: 1 min; and 5 min at 72 °C
Y18S	F-CACAAGTTATCGTTTATTTGATAGCACCTTAC R-GGCTGCTGGCACCAGACTTGC	94 °C: 2 min, followed by 35 cycles: 94 °C: 1 min, 64 °C: 30 s, and 72 °C: 1 min; and 5 min at 72 °C
ITS1	TCCGTAGGTGAACCTGCG	94 °C: 2 min, followed by 35 cycles: 94 °C: 1 min, 58 °C: 30 s, and 72 °C: 1 min; and 5 min at 72 °C
ITS2 ITS4	ATGTCGACCATAAGTCGAGCG TCCTCCGCTTATTGATATGC	

Results

The 18S *ca.* 700bp and 26S *ca.* 700bp ribosomal regions were amplified from *H. phycitis*, while the ITS sequence was not obtained. Phylogenetic analysis based on 18S rRNA gene revealed the presence of an YLS in *H. phycitis* [herein designated *H. phycitis*-YLS (Hp-YLS)] and had 99% similarity to YLSs reported from *Laodelphax striatellus* Fallén, *Nilaparvata lugens* Stål and *Sogatella furcifera* (Horváth). The 18S rRNA gene sequences resulting from *H. phycitis* populations was placed in the clade of YLS supported by 100% statistical support, with allied YLS sequences from *S. furcifera* (Fig. 1). The 26S rRNA gene of this endosymbiont was also amplified. Phylogentic analysis based on 26S rRNA gene showed that this sequence created a clade with YLS reported from *S. furcifera* with 95% statistical support (Fig. 1). Our diagnostic PCR survey of *H. phycitis* representing 13 populations and 126 individuals, detected Hp-YLS with 100% infection frequency in all populations examined.

DNA samples were subjected to PCR amplification of a 700bp region of the 26S rRNA gene using universal primers (see Table 2); then, the PCR products were sequenced, edited and compared with GenBank sequences using BLAST algorithms. After conducting BLASTN search, the sequences exhibited the most similarity (98%) with *C. pimensis* reported from *Chrysoperla comanche* gut. This sequence was placed in the *Candida* clade with 95% statistical support and allied with *C. pimensis* sequence isolated from *C. comanche* and *Chrysoperla carnea* gut. Infection frequency of *C. pimensis* was 100% as observed in Hp-YLS.

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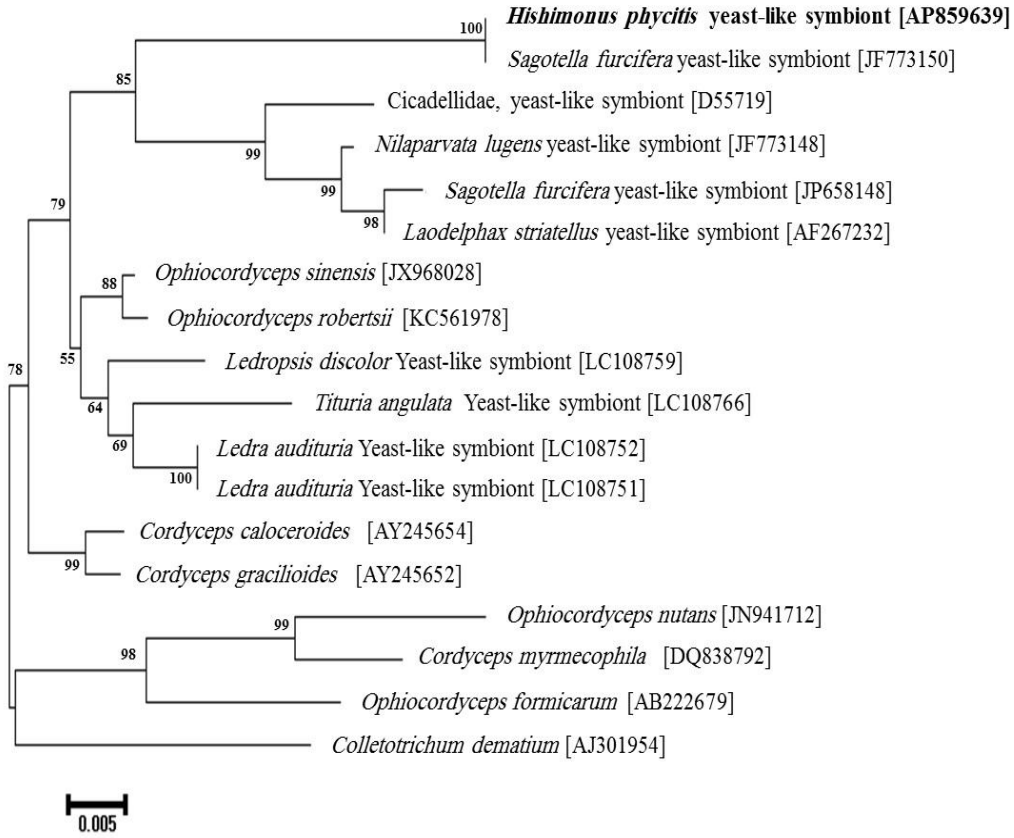

Figure 1 Phylogenetic relationship of YLS endosymbionts of *Hishimonus phycitis* to YLSs and some entomoparasitic fungi of other hemipteran insects on the basis of 18S rRNA gene sequences. A neighbour-joining (NJ) phylogeny inferred from 700 aligned nucleotide sites is shown. Bootstrap probabilities for the ML analysis at 50% or higher are shown at the nodes. The sequences obtained from the leafhoppers in this study is highlighted by boldface type, and the nucleotide sequence accession number is indicated in brackets. Scale bar shows branch length in terms of number of nucleotide substitutions per site.

Discussion

In this study, we examined fungal symbionts variation of 13 populations of the insect vector of lime witches' broom phytoplasma. Molecular phylogenetic analysis revealed that the fungal symbionts form a clade within the ascomycetous genus *Ophiocordyceps* consisting of entomoparasitic fungi. These results suggested the possibility that the fungal symbiont was acquired by the common ancestor of the subfamily Deltocephalinae and derived from an entomoparasite of the genus *Ophiocordyceps* (Nishino *et al*., 2016). The molecular phylogeny clearly showed that the fungal symbionts of the leafhopper vector were allied to the fungal symbionts of planthoppers. This phylogenetic pattern favors the idea of same evolutionary origins of the fungal symbionts from the genus *Ophiocordyceps* within the Hemiptera, and highlights the evolutionary connection between parasitism and mutualism in the insect-microbe symbiotic associations (Hongoh and Ishikawa, 2000). It should be noted that the fungal symbiont of aphids is phylogenetically very close to the fungal symbiont of leafhoppers, although their evolutionary origins must be independent suggesting the possibility that particular groups of *Ophiocordyceps* fungi specialized for hemipteran insects might recurrently serve as evolutionary sources of the fungal symbionts in the Hemiptera (Nishino *et al*., 2016).

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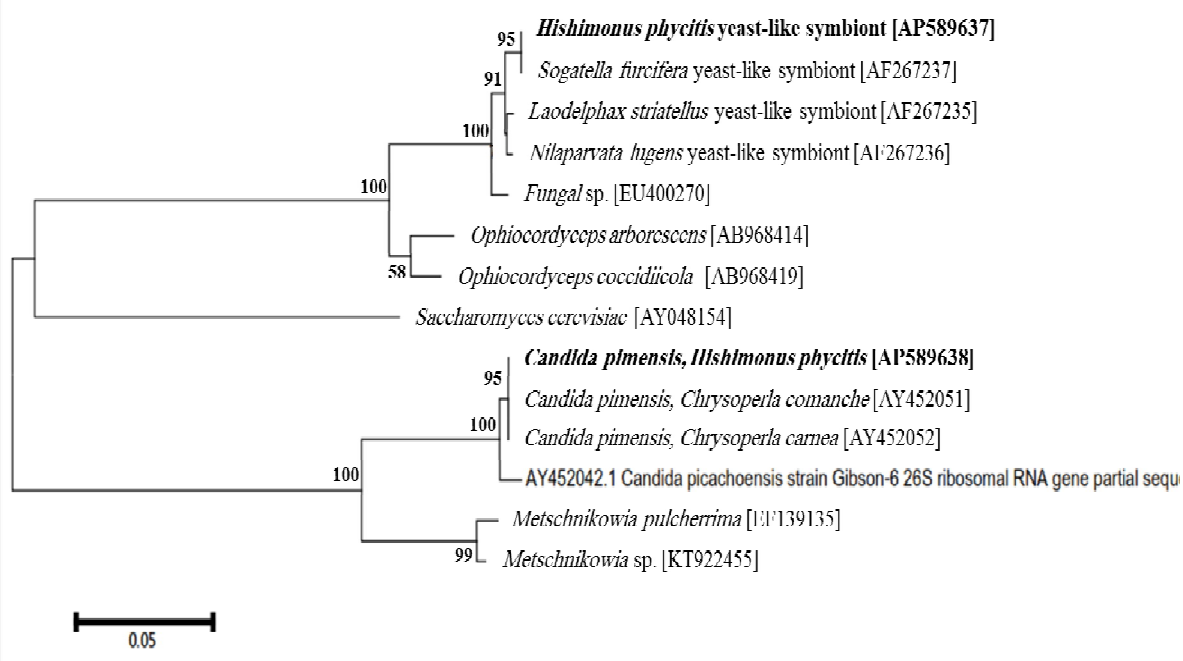

Figure 2 Phylogenetic relationship of YLS and *Candida pimensis* of *Hishimonus phycitis* to YLSs and fungi of other hemipteran insects on the basis of 26S rRNA gene sequences. A neighbour-joining (NJ) phylogeny inferred from 700 aligned nucleotide sites is shown. Bootstrap probabilities for the ML analysis at 50% or higher are shown at the nodes. The sequences obtained from the leafhoppers in this study are highlighted by boldface type and nucleotide sequence accession number in the figure. Scale bar shows branch length in terms of number of nucleotide substitutions per site.

Our results showed that there is another fungal symbiont namely *C. pimensis* in the vector body. Our results are in agreement with the previous studies which indicated that yeast like symbiont and another yeast present in multiple planthoppers from geographically distinct regions suggesting that these species are commonly associated with the insect. This was reported from *L. striatellus*, *N. lugens* and *Perkinsiella saccharicida* (Houghes *et al.,* 2011).

Yeast and yeast-like fungi associated with insects play several roles, the most important one is a nutritional role in which yeasts supply enzymes for digestion that result in improved nutritional quality, essential amino acids, sterols and vitamins. Yeasts also cooperate to detoxify toxic plant metabolites in the host's diet (Vega and Dowd, 2005). We previously found that *H. phycitis* harbored six bacterial endosymbionts which two of them (*Sulcia* and *Nasuia*) had obligatory relationship with their hosts (Unpublished data). Genomics of *Sulcia* and co-symbionts have suggested that these bacterial symbionts cooperatively provide essential amino acids and other nutrients for leafhoppers and other hemipteran insect hosts (Bennett and Moran, 2015). In view of the fact that vector feeds on lime trees carrying residues of many insecticidal compounds (Ibrahim *et al*., 2008), it is conceivable, although speculative, that this fungal endosymbionts may play a role in detoxification of toxic lime metabolites which deserves more investigation. Considering the much larger genome size and consequent broader metabolic capability of the fungal symbiont in comparison with the tiny-genome bacterial symbionts (McCutcheon, 2010; Moran and Bennett, 2014), it is conceivable, although speculative, that the fungal symbiont may play additional biological roles in the hemipteran hosts. Genomics of the fungal symbionts

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(Vogel and Moran, 2013; Xue *et al*., 2014) and physiological studies on normal and fungus-deprived insect hosts (Noda and Saito, 1979; Sasaki *et al*., 1996) are needed for deeper understanding of functional aspects of the insect-fungus symbiotic association.

Paratransgenesis utilizes microorganisms associated with insects to manipulate the vector competence of the host. To date, bacteria associated with insects are used in most paratransgenic approaches; however, recently, yeasts have been proposed as candidate microbes for this approach (Ricci *et al*., 2011). The symbiotic nature of both YLSs in planthoppers, and *Candida* yeast in other insects, has been elucidated (Sasaki *et al*., 1996; Vega and Dowd, 2005). Regardless of the nature of the symbiosis, the high prevalence of these microbes in *Hishimonus* populations suggests that these yeasts could be applied for paratransgenesis. The possible culturing and gene transformation for *C. pimensis* should be investigated.

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Statement of conflicting interests

The authors state that there is no conflict of interest.

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شناسایی درون همزیست‌های مخمری و شبه‌مخمری *Hishimonus phycitis*، ناقل فیتوپلاسمای عامل بیماری جاروک لیموترش

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چکیده: بیماری جاروک لیموترش که عامل آن یک فیتوپلاسما *Candidatus* Phytoplasma aurantifolia می‌باشد، یکی از مهم‌ترین تهدیدات باغ‌های لیموترش و سایر مرکبات حساس در ایران و برخی کشورهای دیگر محسوب می‌شود. ناقل این بیماری، زنجرک *Hishimonus phycitis* می‌باشد. زنجرک‌ها به‌دلیل تغذیه از شیره گیاهی و فقدان اسید آمینه‌های ضروری در رژیم غذایی خود، مواد غذایی لازم برای رشدو نمو را دریافت نمی‌کنند و بقای آن‌ها متکی به همزیست‌های درونی آن‌ها می‌باشد. همزیستی بین قارچها و حشرات بسیار شایع می‌باشد و تخصصی و اجباری است. این قارچها اصطلاحاً همزیست‌های شبه مخمری (YLS) نامیده می‌شوند و اعمال مختلفی مانند تامین آنزیم‌هایی برای هضم غذا و بهبود کیفیت غذا، تأمین اسید آمینه‌های ضروری، ویتامین‌ها و استرول‌ها را برای حشره انجام می‌دهند. این شبه‌مخمرها در سم‌زدایی ترکیبات سمی متابولیت‌های گیاهی موجود در غذای میزبان، مقاومت در برابر پارازیتوییدها، پاتوژن‌ها و برهم زدن تولیدمثل نیز نقش دارند. یکی از روش‌های نوین در کنترل بیولوژیک برای کنترل بیماری‌های قابل انتقال توسط حشرات، استفاده از روش کنترل همزیستی (Symbiotic control) است. برای انجام چنین روش مدیریتی، اولین گام شناخت میکروفلور درون همزیست‌های قارچی حشرات ناقل می‌باشد. پژوهش حاضر با هدف شناسایی درون همزیست‌های شبه‌مخمری زنجرک *H. phycitis* انجام شد. حشرات مورد مطالعه از استان‌های جنوبی کشور (هرمزگان، سیستان و بلوچستان، کرمان و فارس) با استفاده از دستگاه D-Vac جمع‌آوری شدند. تمامی نمونه‌ها تا زمان استخراج DNA در دمای ۲۰- درجه سلسیوس نگه‌داری شدند. ردیابی همزیست‌ها با استفاده از آزمون PCR و با استفاده از آغازگرهای اختصاصی ژن 26S و 18S rRNA همزیست‌های شبه‌مخمری گزارش شده در سایر زنجرک‌ها انجام شد. پس از حصول اطمینان از تکثیر قطعات موردنظر روی ژل آگارز یک درصد، قطعات تکثیر شده از ژل جدا و خالص‌سازی و سپس از هر دو انتها توالی‌یابی شدند. توالی‌ها با توالی‌های ثبت شده سایر گونه‌های بندپایان در مرکز ملی اطلاعات بیوتکنولوژی (NCBI) بلاست شدند. نتایج حاصل از بلاست قطعات توالی‌یابی شده، شباهت ۹۸ تا ۹۹ درصدی توالی‌های موجود را با درون همزیست‌های شبه‌مخمری Yeast like symbiont و *Candida pimensis* گزارش شده از زنجرک‌های خانواده Cicadellidae داشت. این نتایج نشان از پیچیدگی میکروفلور درون همزیست‌های حشره ناقل بیماری جاروک داشته و نشان می‌دهد که ممکن است یک رابطه تعاملی پیچیده بین همزیست‌های شبه‌مخمری و فیتوپلاسمای عامل بیماری جاروک در زنجرک *H. phycitis* وجود داشته باشد که می‌تواند قابلیت انتقال بیماری توسط این زنجرک را تحت تأثیر قرار دهد.

واژگان کلیدی: *Hishimonus phycitis*، درون همزیست‌های مخمری و شبه‌مخمری، *Candida pimensis*

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