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Full Length Article

Characterization of Potato Golden Cyst Nematode Populations (*Globodera rostochiensis*) in Turkey

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Abstract

Golden potato cyst nematode, *Globodera rostochiensis* (Wollenweber) Behrens, is one of the most important soilborne pathogens causing economic losses in potato. The nematode is known to occur in several countries including Turkey and has a worldwide regulatory concern. In this study, identification and genetic diversity of *G. rostochiensis* specimens obtained from the main potato producing areas of Turkey were determined. Twenty-five of 35 soil samples collected from the provinces of Izmir, Nevşehir and Niğde were found to contain *G. rostochiensis*. The variation between *G. rostochiensis* populations was determined when examined according to ecological and pathogenic characteristics of nematode in Turkey. The cysts of *G. rostochiensis* were identified by measuring the morphological characters using perennial patterns, vulval cone, vulval basin and juveniles. Phylogenetic analysis of the Large Sub Unit (LSU) region of rDNA sequences was used to assess the inter or intra phylogenetic relationships between the nematode populations. The phylogenetic analyses demonstrated that the nematode specimens from Turkey cluster with *Globodera* spp. and signified the presence of single species of *G. rostochiensis*. As a result, morphological, morphometric and molecular methods were successfully combined for identification and characterization of *G. rostochiensis*. The frequency of *G. rostochiensis* in regulatory samples from potato-producing areas is becoming increasingly important. The morphological characterization has several complications in the detection of this quarantine nematode, using of this combination is beneficial for a reliable and quick diagnostic for these nematodes which is crucial for regulatory services and growers. The results might help to investigate different ecotypes of *G. rostochiensis* for comprehensive understanding about physiology, ecology, and biology of the genus *Globodera* for its effective management in Turkey. © 2020 Friends Science Publishers

Keywords: Cyst nematode; Genotypic variation; Molecular identification; Potato; Turkey

Introduction

The potato cyst nematodes (PCNs), *Globodera pallida* and *G. rostochiensis* (Golden potato cyst nematode), are two of the main threats to potato production around the globe. Although PCNs are important quarantine pests, they have been reported from all the continents where potato crop is grown (EPPO 2019). In the family Heteroderidae, the PCNs, *G. pallida* and *G. rostochiensis*, are some of the most important quarantine pests. These nematode species are successful plant pests because they can acclimatize to diverse environmental conditions (Turner and Evans 1998). The cysts are very resistant entities and juveniles and eggs present in the cysts stay viable for 30 years (Siddiqi 2000). The viability potato cyst nematodes juveniles decrease by 20–30% every year in the absence of host plants. In contrast, eggs present in the cysts remain viable for up to 30 years in diapause, if host plants are not present (Turner 1996). When host plant secretions are present and the soil temperature is above 10°C, J2 hatch from the eggs and move chemotactically toward the host plant roots (Franco 1979; Ali *et al*. 2017, 2018). Although it has been reported that PCNs normally only has one generation per year under favorable soil temperature in particular, they can have more than one generation per year (Jones 1950). Yield losses of potato cyst nematodes are reported to reach 70% but can vary according to the degree of tolerance of specific potato cultivars (Greco 1988).

The first report of *G. rostochiensis* from Turkey was published during 1996 (Enneli and Öztürk 1996). At that time the stringent quarantine procedures applied were considered effective in excluding the entry and spread of

this pest throughout the country. However, the pathogen was subsequently reported in the provinces of Afyon, Izmir, Kayseri and Konya after not being detected for almost 25 years (Ulutaş 2010). Moreover, it has been found in 77 countries in Europe (EPPO 2019). Spreading of *G. Rostochiensis* through seed potatoes has been also prohibited from Europe via Turkey Scheme (EPPO 2019)

Nowadays, it is important to determine the distribution and biodiversity of the indigenous cyst nematodes to develop effective management strategies. The aims of the study were to study the distribution, identification and genetic diversity of specimens of *G. rostochiensis* from the main potato producing areas in Turkey. Their systematics and characteristics were studied in detail by comparing and describing the morphology and taxonomic characteristics. The result will provide the comprehensive information about the distribution, ecology, physiology, and biology of sampled populations to determine effective management strategies and regulatory measures for *G. rostochiensis* in Turkey.

Materials and Methods

Nematode specimens

Specimens of PCN were obtained after the potato harvest from Izmir, Nevşehir and Niğde Provinces of Turkey. Soil samples were taken from potato fields from 0–30 cm using soil sampler. The potato cysts were isolated using Seinhorst cyst elutriator (Seinhorst 1964). The soil was washed through 840 μm sieve followed by 250 μm sieve and the content was collected onto filter paper with a funnel and drying purposes. The cysts were observed under Leica stereomicroscope (Seinhorst 1964). Cysts were first identified as *Cactodera* and *Heterodera* (lemon-shaped cysts) or *Globodera* and *Punctodera* (round or ovoid cysts) by Golden (1986) and Subbotin and Baldwin (2010).

Morphological identification

The morphological identification of the cysts nematodes is complicated; however, cysts and juveniles were used for morphological identification as they are the most widely used life stages to identify cyst nematodes (Golden 1986). Perennial patterns, vulval basin and vulval cone are specific and used for species identification, whereas body length, stylet length, knob shapes and labial patterns are important taxonomic characteristics of the second stage juveniles (Wouts and Baldwin 1998; Subbotin and Baldwin 2010).

Mounts of the vulval cones were prepared according to Bezooijen (2006). Second stage juveniles were fixed in formalin-glycerol fixative mounted on a glass slide and observed under a light microscope (Golden 1986). Morphological features of specimens were examined by light microscope (Leica, DM5500) and the LAS (Leica Application Suite) program was used for measurement. Species determination was made according to Wouts and Baldwin (1998); Subbotin and Baldwin (2010). Standard deviations and 95% confidence intervals were calculated as Fortuner (1984).

Molecular identification

Potato cysts were cut for extraction DNA of nematode under a stereomicroscope. One juvenile were picked, put in a 10 μL PCR reaction buffer (16 m*M* $[NH_4]2SO_4$, 67 m*M* Tris-HCl pH, 0.1% Tween-20) including 60 μg mL^{-1} proteinase K in a tube. Then the tube including second stage juvenile was incubated at 60°C for 65 min and then 5 min at 95°C. The extracted DNA was stored at -20°C until used.

The rDNA1 primer (5'-GTCGTGATTACCCGCTGAACTTA -3') and rDNA2 primer (5'- TCGGAAGGAACCAGCTACTA -3') were described by Holterman *et al*. (2008) for amplification of the LSU (28S) of ribosomal RNA region.

PCR amplifications were performed using nematode lysate (5 μL) and 0.5 μM of each primer, dNTPs at 200 μM, Taq buffer, 1 m*M* $MgCl_2$ and 1 U *Taq* polymerase in 50 μL of the final reaction volume in a tube. The cycling conditions followed were; denaturation at 94°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 60 s and were repeated for 42 cycles. A 7 min polymerization at 72°C followed the last cycle. Following amplification of DNA, 15 μL of each PCR amplicon was mixed with 5 μL 6x loading dye (Promega, Leiden, The Netherlands) and loaded on a 1% agarose gel in TAE buffer. After electrophoresis for 45 min at 120 V, the DNA in the gel were stained with 0.005% ethidium bromide (0.01 μg mL^{-1}) 15 min. The DNA in the gel was viewed on a UV-transilluminator and photographed.

After PCR process amplicon of the LSU region were transferred for sequencing (Macrogen, Amsterdam, the Netherlands) with an ABI 3500xL Genetic Analyzer. These sequences were then identified using the BLASTn algorithm on the NCBI website (https://www.ncbi.nlm.nih.gov). The sequences derived from this study were submitted into GenBank and received the number of accession shown in Table 1.

Phylogenetic analysis

Phylogenetic analysis was executed to determine genetic associations between the local specimens and selected specimens from database of the *Globodera* species in MEGA v.7.0 (Kumar *et al*. 2016), using method of neighbor-joining with 1000 replicates of bootstrap (Saitou and Nei 1986). The sum of branch length in the optimal tree is 0.0268. The confidence levels for the associated taxa grouped through bootstrap test are represented next to the branches (Felsenstein 1985). The distance of evolutionary among the taxa was calculated according to Tamura-Nei method (Tamura and Nei 1993). Our phylogenetic analysis

comprised of 30 DNA sequences. 1st, 2nd, 3rd plus noncoding codon positions were used to develop the tree. The positions with missing data and gaps were removed. In the final dataset of sequence base pair total of 673 positions were used.

Results

Nematode distribution

Thirty-five soil samples were collected from the main potato producing areas such as Izmir, Nevşehir and Niğde Provinces of Turkey to elucidate distribution, genotypic variation and molecular characterization of *G. rostochiensis* populations. Out of 35 soil samples, 25 samples contained *G. rostochiensis* (Table 1).

Morphological identification

All specimens examined from the samples collected in this study had morphology as described below for cysts and juveniles.

Cysts: Cysts were ovoid to spherical in shape, the color of cysts was light brown to slightly dark brown and they had a protruding neck (Fig. 1). The perineal pattern was circumfenestrate, with subterminal small anus at the surface of the V-shaped subsurface mark in the cuticle. There is no bullae, vulval bridge and under bridge. The ridges of cuticle were six to twelve between base of vulva and anus on the outer surface of the cyst, which were evidently visible under light microscope. The ridges were also changing to nearly not regular patterns in the area after anus and vulvabase and were modified to crescentic wavy ridges arise to the neck-area (Fig. 1). Punctuations were mostly present which were diverse in arrangement and intensity. Irregular subsurface dots were commonly found all over the body and were organized in parallel lines at a right angle to the along axis of some cysts (Fig. 1).

Fenestra diameter were 15–26 μm in Niğde and Nevşehir cysts, whereas Izmir cysts had larger fenestra (20–28 μm). Fenestra distance to anus was nearly the same for Niğde, Nevşehir and Izmir cysts (53–72, 59–68 and 55–70 μm, respectively), whereas the Granek's ratios were 2.03–4.2, 2.7–4.2, 2.2–4.6 for these cysts, respectively.

All morphological and morphometric characteristics of cyst had slight variation between Niğde, Nevşehir and Izmir cysts. The cysts from Niğde and Nevşehir were lighter with a less pointed cone tip when comparing to Izmir cysts. However, the fenestra diameter of Niğde and Nevşehir cysts was shorter than those of the Izmir cysts (Fig. 1).

Second stage juvenile (J2): The body of J2 was curved slightly on ventral side. The tail terminus was tapered to a fine point (Fig. 2). The head was with 3–4 annules along with medial lips with a labial disc a little be protruding from the rest of the body. The scanning electron microscopy demonstrated that the labial disc and medial lips were

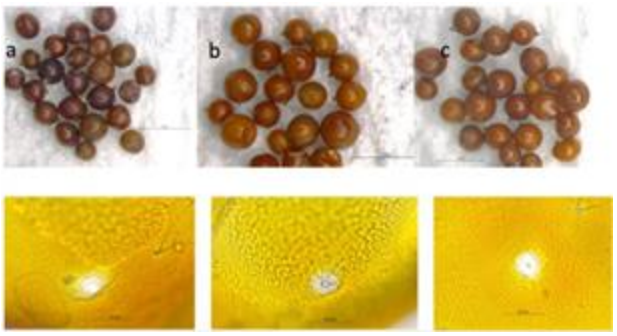

Fig. 1: Photomicrographs of cyst of *G. rostochiensis* and terminal areas of cyst representative specimen from **a**: Izmir; **b**: Niğde; and **c**: Nevşehir

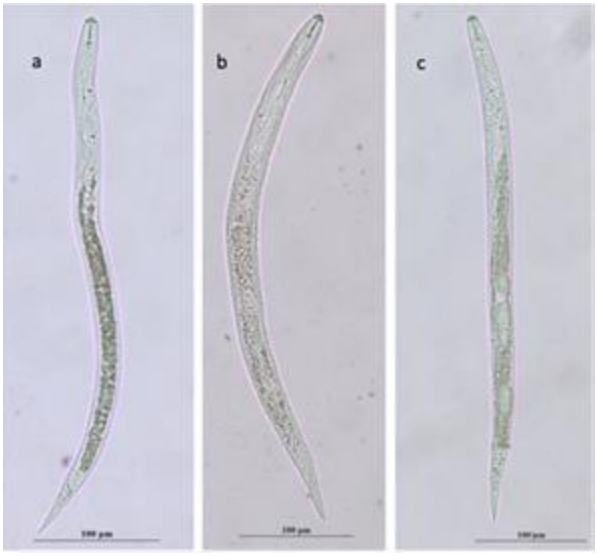

Fig. 2: Photomicrographs of *G. rostochiensis* second stage juveniles, the representative specimens from Turkey. **a**: Izmir; **b**: Niğde; and **c**: Nevşehir

rectangularly oval with the same height as those of lateral lips. The prestoma opening was rectangular and marginally elevated from the remaining part of medial lips and labial disc (Fig. 3). Similarly, the lips are rectangular, larger in size, occasionally with irregular shape, and bearing the amphid. The stylet was well stronged. The knobs of stylet were from rounded. Likewise, the distance between the stylet knobs and dorsal gland outlet ranged from 3.5 to 6.5 μm (Fig. 3). The lobe of the esophageal gland was about 35% of the body length. The genital primordium was somewhat posterior after the mid-body. However, the nerve ring was located shortly after median bulb with an excretory opening posterior to the nerve ring. The valve of median bulb was conspicuous. Cephalids, hemizonion and hemizonid were not present. The length of the annules was around 1.7 µm at the middle of body. The lateral field of body contained 4 crenated and areolated incisures which were extending outspreading from six annules

Table 1: Location and accession number of sequenced LSU (28S) domain region the rDNA of *G. rostochiensis* specimens from Turkey

No	Sample Code	Province	Locality	Geographic coordinates	Gen Bank accession number
1	3	Izmir	Tekke	38.3327, 28.0614	MK311329
2	13	Izmir	Karakova	38.2044, 27.9593	MK937714
3	114	Izmir	Yenicekoy	38.2289, 27.9382	MK937715
4	115	Izmir	Ocakli	38.2206, 27.9971	MK937716
5	51	Nevşehir	Bas	38.4037, 34.7391	MK937712
6	91	Nevşehir	Sivritas	38.6104, 34.9208	MK937713
7	8	Nevşehir	Gore	38.5575, 34.7039	MK311333
8	5	Niğde	Alay	38.2705, 34.6854	MK311330
9	100	Niğde	Orhanli	38.2964, 34.8904	MK937717
10	200	Niğde	Edikli	38.2230, 34.9634	MK937718
11	300	Niğde	Orhanli	38.2828, 34.8596	MK937719
12	400	Niğde	Alay	38.2600, 34.6835	MK937720
13	500	Niğde	Kiledere	38.3091, 34.6576	MK937721
14	600	Niğde	Karaatli	38.1398, 34.9546	MK937722
15	700	Niğde	Aslama	38.1359, 35.0564	MK937723
16	800	Niğde	Agcasar	38.3086, 34.7242	MK937724
17	900	Niğde	Agcasar	38.3253, 34.7123	MK937725
18	1000	Niğde	Golcuk	38.2063, 34.7878	MK937726
19	1100	Niğde	Hasakoy	38.2216, 34.6868	MK937727
20	1200	Niğde	Baglama	38.2343, 34.6706	MK937728
21	1300	Niğde	Tirhan	38.2400, 34.7016	MK937729
22	1400	Niğde	Ciftlik	38.2201, 34.4774	MK937730
23	1500	Niğde	Altunhisar	37.9958, 34.3672	MK937731
24	6	Niğde	Altunhisar	37.9958, 34.3621	MK311331
25	7	Niğde	Ciftlik	38.1892, 34.4862	MK311332

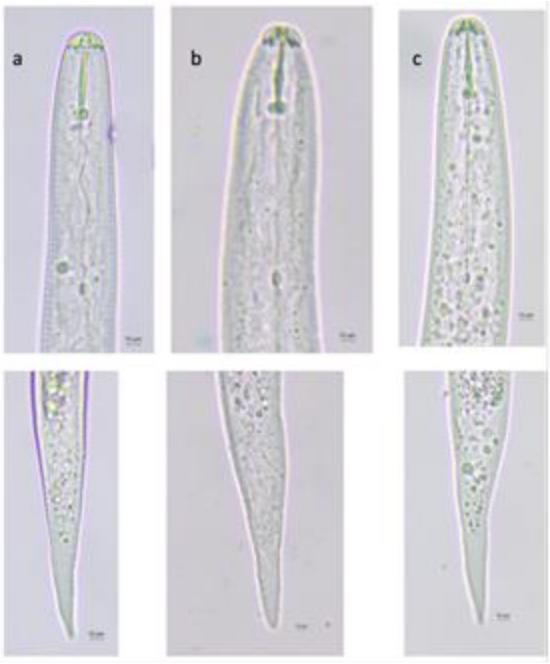

Fig. 3: Photomicrographs of the *G. rostochiensis* second stage juvenile heads and tails, representative specimens from Turkey. **a**: Izmir; **b**: Niğde; and **c**: Nevşehir

posterior to labial area, incisures. Most of the specimens had an indistinct phasmid. The tail end was apparently smooth and annulated (Fig. 3).

There was minimal variation in all morphometric characteristics of J2 from Izmir, Nevşehir and Niğde. Variance analyses revealed no significant distinguish in J2 body length and c' ratio, body size, stylet dimension, body size at the anus, tail size and hyaline tail distance. No differences were observed in the a and c ratios. J2 stylet length and knob shape were similar to those previously reported (Shahina and Maqbool 1995; Sirca and Urek 2004; Subbotin and Baldwin 2010).

Molecular identification

LSU of region of rDNA was amplified to characterize the specimens from Turkey. The sequence attributes of the studied isolates are given in Table 1 and Fig. 4 along with those of related *Globodera* isolates. Maximum sequence length of LSU region (547 bp) and minimum length of LSU region (286 bp) was demonstrated by the isolate Niğde 1100. The sequence from this isolate also contained the highest and lowest levels of thymine (36%) and ladenine (24%) respectively.

The phylogenetic comparison between already reported *Globodera* isolates showed that the specimens from Izmir, Nevşehir and Niğde and selected cyst nematode specimens from Italy, Poland, Slovakia and UK differed from other PCNs like *G. artemisiae* and *G. pallida* in the number of nucleotide per site, 0.042 and 0.054, respectively (Fig. 4). The sequences obtained from specimens from Izmir, Nevşehir and Niğde exhibited a substantial degree of sequence variation in comparison to *G. artemisiae* and *G. pallida* sequences. Moreover, when sequences from only the Turkish specimens (Izmir, Nevşehir and Niğde) and selected specimens (Italy, Poland, Slovakia and UK) are considered, the greatest similarities were found between

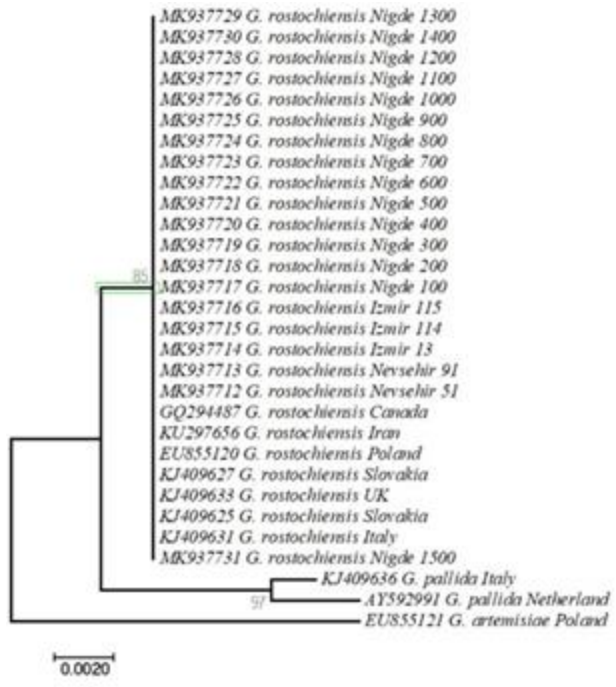

Fig. 4: Phylogenetic tree (neighbor-joining method) constructed through the LSU sequence alignment from 27 populations of *G. rostochiensis*. Bootstrap values (more than 60%) are given for the appropriate clades. Populations are designated with a number described in Table 1

Izmir, Nevşehir and Niğde.

Phylogenetic analysis

A number of sequences of *Globodera* species which are parasitic to solanaceous crops (Subbotin *et al*. 2011) were used for building an alignment along with the selected members from the other major clades of the circumfenestrate cyst nematodes. The sequences of *G. rostochiensis* (including specimens from Izmir, Nevşehir and Niğde obtained in this study and golden cyst nematode specimens from Italy, Poland, Slovakia and UK) and other *Globodera* species such as *G. artemisiae* and *G. pallida* were also included in the alignment (Fig. 4).

Phylogeny of selected *Globodera* species was developed on the basis of LSU sequences through neighbor-joining method are presented in Fig. 4. The resulting dendrogram was composed of the three major clades with adequate bootstrap support. The position of Nevşehir 51 and 91, and Niğde 1500 samples in the tree (Fig. 4) was supported by the previous investigations (Subbotin *et al*. 2011). These specimens occupied the position with a lineage of *Globodera* species from European countries, Italy, Poland, Slovakia and UK. The consensus phylogenetic tree, specimens from Turkey are included in the clade of *G. rostochiensis*, forming a monophyletic cluster with from the specimens from Europe.

Discussion

This is the first study for the detailed identification and authentic detection of local *G. rostochiensis* in potato fields in Turkey. Previous nematological research was limited to some sites in Izmir Province (Ulutaş 2010; Ulutaş *et al*. 2012), but in this research the authors did not target *G. rostochiensis* specifically. Similarly, *G. rostochiensis* was identified for the firstly in the provinces of Nevşehir and Niğde, which are the most important potato production areas of Turkey. Given that morphological and morphometric data alone are not sufficient to determine whether specimens are *G. rostochiensis*, molecular identification based on LSU sequence was performed to validate the morphological data. The molecular identification and phylogenetic analysis of the LSU sequences of the specimens from Izmir, Nevşehir and Niğde confirmed that they are the member of genus *Globodera*, and were similar to the previously described species in this genus. The explanation of morphometric attributes, plant-nematode interactions and phylogenetic association of these populations could be valuable for studying the evolution of this group of nematodes.

The coupling of morphological and molecular data led to the more reliable identification of the *G. rostochiensis* specimens from Turkey. The morphological and morphometric characteristics of the *G. rostochiensis* specimens examined in this study were quite similar. The cyst sizes determined from the isolates from the present study were smaller from already published isolates of this nematode species, which could be due to cysts of different age been used in those studies. For instance Subbotin *et al*. (2011) reported that cysts were more oval than round, which is consistent with the results of the current study. Similarly, *G. rostochiensis* cysts from Turkey were also smaller in size those from Pakistan (Subbotin and Baldwin 2010), which could be because these populations were from warmer climates which could influence the growth of females. The cysts from Izmir, Niğde and Nevşehir were similar to those previously reported by several scientists (Shahina and Maqbool 1995; Sirca and Urek 2004; Subbotin and Baldwin 2010).

Likewise, Knoetze *et al*. (2013) reported that the phylogenetic analysis of *G. rostochiensis* isolates could not display specific association with the different geographical origins of the isolates. This reveals that the phylogenetic relationships between the sequences from *G. rostochiensis* isolates were generally unclear.

The current study establishes the similar phylogenetic position of the specimens from Izmir, Nevşehir and Niğde with those from Europe, which indicates that introduction of *G. rostochiensis* to Turkey might have been from Europe. Quader *et al*. (2008) concluded that seven infested area of *G. rostochiensis* occurred in Australia. The analysis of the South African populations also does not indicate that they were the origin of Turkish *G. rostochiensis* populations. Our

results demonstrate that the LSU rDNA is a useful marker for identification of *G. rostochiensis* populations. However, relatively lower degree of evolution of ITS sequences from *G. rostochiensis* was found that demonstrated that these sequences are not a useful tool for studying the recent introductions of *G. rostochiensis* (Madani *et al*. 2010; Yu *et al*. 2010).

Conclusion

In conclusion, *G. rostochiensis* was only species in the most important potato growing areas of Turkey. It is recommended that annual survey of the potato growing provinces should be continued to closely observe the spread of this pest into new areas. Similarly, a comprehensive survey approach could help determine the origin of the pest, how it was introduced, and to where it might have spread in order to predict the where about of currently undetected infestations. This strategy would be applied immediately applied to all potato-producing areas in Turkey, and needs to use both morphometric and molecular identification.

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