

Incidence and Distribution of *Iris yellow spot virus* on Onion in Serbia

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ABSTRACT

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In a survey to determine the presence and distribution of *Iris yellow spot virus* (IYSV) in greenhouse ornamentals and onion field crops in 14 districts of Serbia as well as on imported ornamental plants, 1,574 samples were collected and analyzed by double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA). IYSV was not detected in nearly 1,200 plant samples collected from 39 genera of ornamentals grown in greenhouses in Serbia or imported from other countries during 2005 to 2007. The virus was detected in samples from an onion seed crop in the Sirig locality (South Bačka District) that showed symptoms resembling those caused by IYSV and in samples without IYSV-like symptoms from an onion bulb crop in the Obrenovac locality (City of Belgrade District). Mechanical transmission of IYSV isolates was difficult, and only the isolate 605-SRB could infect four plant species, but not in all replications. No virus transmission could be demonstrated in 5,000 tested seeds originating from IYSV-infected onion crops. For further confirmation of IYSV, the nucleotide sequence of its nucleocapsid (NC) gene was obtained by reverse transcription-polymerase chain reaction (RT-PCR) in symptomatic onion samples as well as in symptomless leaves of *Nicotiana benthamiana*. Four previously developed primers were tested to determine their suitability for routine detection of Serbian IYSV isolates. Phylogenetic analysis showed clustering of isolates 605-SRB and 622-SRB from the onion seed crop and isolate 283-SRB from the onion bulb crop into two distant clades. The analysis indicated that Serbian isolates of IYSV do not share a recent common ancestor and that they represent two distinct lineages of IYSV in Serbia. Considering that onion is one of the most important and traditionally grown vegetable crops in Serbia, IYSV represents a potentially devastating pathogen in this country.

In the last few decades, the genus *Tospovirus* within the family *Bunyaviridae* has become one of the most studied plant virus genera. *Iris yellow spot virus* (IYSV) is one of the several members of the *Tospovirus* genus (25). IYSV is considered an emerging virus whose world presence and distribution have recently dramatically increased (17,36,38).

IYSV is an economically important viral pathogen of onion (*Allium cepa*), but the infection of other cultivated and wild *Allium* species, several ornamental species, and a certain number of weeds has also been reported (17,36,37,46). IYSV is not reported to be seedborne and seed-transmitted in onion (27,31), but the question whether it can be transmitted through

seeds has often been raised because seed transmission could be affected by numerous factors.

Scape blight of onion with unusual symptomatology was observed for the first time in 1989 in Idaho and Oregon, USA, but a tospovirus associated with the disease was reported only in 1993 (20). Almost at the same time, an unknown tospovirus causing disease on onion was found in Brazil (42) and on iris and leek in the Netherlands (2,14). In 1998, the tospovirus isolated from iris in the Netherlands was identified and the name *Iris yellow spot virus* was proposed (12). Further characterization of onion and iris isolates of the tospoviruses identified in Brazil, the United States, and the Netherlands proved that it was the same virus, IYSV (32,41). The virus was reported soon afterward in Israel (18,19). Since then, IYSV has been reported in several parts of the world and in various states of the United States (17). Recently, first reports on finding of IYSV have come from Canada (21) and New Zealand (52). IYSV has caused considerable losses in the United States,

especially in the western regions of onion production, where since 2000 the virus has spread and its incidence has been increasing rapidly (36).

In the EPPO region, this newly characterized tospovirus gained significance in 1999, when it was included in the EPPO Alert List (3), because it could pose a risk for the cultivation of onion and other host plants. The present geographical distribution of IYSV is still under investigation, and in most cases the virus records were incidental findings. Until now, survey for the virus has been initiated only by several countries, and in almost all of them IYSV has been detected only sporadically and in restricted areas. In addition to the Netherlands and Israel, IYSV has been recorded in Slovenia (29), Italy (7,13), Poland (8), Spain (10,11), France (22,23), Germany (5,28), the UK (33), and Serbia (9).

Despite the fact that IYSV has increased its geographical distribution in the EPPO region and that further spread of IYSV would be expected, especially in field-grown onion, information on the present distribution and actual incidence level is uncertain and assessment of its economic impact is still missing. The current situation in the European countries concerning IYSV highlights the need for extensive research to gather more information to prevent its further spread in Europe.

The objectives of this study were to (i) gain an insight into the occurrence and distribution of IYSV in greenhouse-grown ornamentals and onion crops in Serbia, (ii) determine possible IYSV presence in suspected host plants of imported ornamentals, (iii) investigate possible seed transmission of IYSV in onion, and (iv) determine the genetic relationship of Serbian IYSV isolates with those from other parts of the world.

MATERIALS AND METHODS

Survey and sample collection. An intensive survey was conducted during 2005 to 2007 to determine the occurrence and distribution of IYSV on ornamentals grown in 14 districts of Serbia and onion grown in nine of them (Fig. 1). In 2005, during an initial survey, 453 samples of ornamentals were collected after visual

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inspection of approximately 1,170,000 plants produced in 59 greenhouses from 24 different localities. Collected ornamentals belonged to 35 genera: *Abutilon*, *Alstroemeria*, *Amaryllis*, *Begonia*, *Belis*, *Calla*, *Celosia*, *Chrysanthemum*, *Dahlia*, *Datura*, *Dianthus*, *Diascia*, *Dicentra*, *Gladiolus*, *Hibiscus*, *Impatiens*, *Iris*, *Lobelia*, *Lysimachia*, *Mimulus*, *Pelargonium*, *Peperonia*, *Petunia*, *Phlox*, *Primula*, *Ranunculus*, *Rosa*, *Saintpaulia*, *Salvia*, *Sinningia*, *Vanilla*, *Verbena*, *Viola*, *Zamioculcas*, and *Zinnia*. In addition, during 2005, approximately 18,700 imported ornamental plants were visually inspected, and 516 samples of *Iris*, *Alstroemeria*, *Eustoma*, and *Chrysanthemum* were collected and tested for IYSV presence. In 2006, 215 ornamental samples were collected from approximately 820,000 inspected plants grown in 39 greenhouses at 16 localities. Samples included plants from 14 genera: *Begonia*, *Chrysanthemum*, *Cyclamen*, *Dianthus*, *Impatiens*, *Iris*, *Lilium*, *Pelargonium*, *Petunia*, *Primula*, *Sinningia*, *Tagetes*, and *Vinca*.

During a 3-year period of visual inspection of 75 onion fields to record symptoms resembling those of IYSV, a total of 390 samples were collected. Onion production fields were diverse in size and production scale. Some fields were less than 5,000 m², while others were substantially larger, up to 20 ha. In 2005, a total of 52 samples of onion plants were collected from 20 bulb and 11 seed crops in the vicinity of 31 greenhouses for ornamental plant production. During 2006, a total of 149 samples of onion were collected from eight bulb and five seed crops, each from a different locality, and in 2007, 189 onion leaf samples were collected from 13 bulb and 18 seed onion crops from 16 localities.

Samples of ornamentals comprised of leaves from different parts of each plant exhibited various symptom types suggestive of tospovirus infection, such as yellow or necrotic spots and rings on leaves and tip necrosis. Symptomless onion leaf and scape samples were collected together with those showing symptoms of elongated, oval chlorotic or necrotic lesions. Both fresh and frozen (−80°C) samples were tested for IYSV presence by enzyme-linked immunosorbent assay (ELISA).

Serological testing. Samples were tested for the presence of IYSV utilizing a double-antibody sandwich (DAS)-ELISA kit (Loewe Biochemica, Sauerlach, Germany), and the ELISA procedure was performed according to manufacturer's instructions. Plant tissue samples were extracted in extraction buffer at a rate of 1:10 (wt/vol). After incubation with *p*-nitrophenyl phosphate (Sigma-Aldrich, St. Louis, MO) at 23°C for 1 to 2 h in dark, absorbance at 405 nm was measured with an ELISA microplate reader (DASsrl, Italy). Samples were considered positive if the absorbance value was equal to or higher than two times the absorbance of

the negative control. Commercial positive controls for IYSV (Loewe) and negative controls (extract from healthy onion leaf tissue, commercially available negative control from Loewe and extraction buffer) were included in each ELISA.

Bioassay. *Nicotiana benthamiana*, *N. tabacum* cvs. Samsun and Prilep, *N. rustica*, *N. glutinosa*, *Chenopodium quinoa*, *C. amaranticolor*, *Datura stramonium*, *Petunia × hybrida*, *Physalis floridana*, and *Gomphrena globosa* were mechanically inoculated with infected onion sap, using chilled 0.05 M phosphate buffer (pH 7) containing 1 mM Na-EDTA, 5 mM Na-DIECA, and 5 mM Na-thioglycolate (44). Mechanical transmission to test plants was performed using two ELISA-positive samples, 605-SRB from onion bulb and 283-SRB from onion seed crops, originating from the localities of Sirig and Obrenovac, respectively. A total of 10 plants of each

experimental species were inoculated, and the bioassay was repeated three times. Plants were kept in an insect-proof greenhouse at a temperature of 22 to 25°C and were inspected regularly for symptom development. Three weeks after inoculation, both symptomatic and asymptomatic plants were assayed by DAS-ELISA to confirm IYSV presence and to detect symptomless infections. Only the ELISA-positive symptomless leaves of *N. benthamiana* were further tested using reverse transcription-polymerase chain reaction (RT-PCR).

Seed transmission. Seed transmission was investigated using mature seeds from naturally infected onion plants showing severe and distinct symptoms. Infection of source seed plants with IYSV was confirmed by ELISA testing their symptomatic scapes. Seeds were sown in plastic trays with a sterilized mixture of soil and



Fig. 1. Map of Serbia showing districts surveyed for *Iris yellow spot virus* (IYSV) presence. Arrows indicate two localities where IYSV was found.

sand in an insect-proof greenhouse, and symptoms on seedlings were monitored on a weekly basis. The IYSV presence was determined in 10-week-old seedlings using ELISA. Leaf samples of a total of 5,000 seedlings were assayed in groups of five. Seedlings grown from seeds collected from healthy plants were used as a control.

RT-PCR detection and sequence analysis. Total RNA was extracted from freeze-dried symptomatic onion leaves and scapes and fresh symptomless leaves of *N. benthamiana* using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Tissue sample from healthy onion leaf was used as a negative control in RNA extraction and RT-PCR amplification. Specific nucleotide sequence of IYSV nucleocapsid (NC) gene was detected using RT-PCR with several previously developed primers (Table 1).

RT-PCR was carried out with the One-Step RT PCR kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The RT-PCR reaction mixture included 400 µM each of the four dNTPs, 0.6 µM each primer, and 1 µl extracted

RNA in a final volume of 25 µl. Cycling conditions were as follows: 35 cycles in a thermal cycler (Biometra, T-1 Thermocycler) applying 30 min at 50°C for reverse transcription, 30 s (15 min for the first cycle) at 94°C for denaturation, conditions for annealing depending on the used primers (Table 1), and 30 s (10 min for the last cycle) at 72°C for primer extension. Amplified products were analyzed by 1% agarose gel electrophoresis, stained with ethidium bromide, and visualized under a UV transilluminator.

The amplification products of predicted size, obtained using IYSV917L/56U primers, derived from the isolates 605-SRB and 622-SRB were sequenced directly after the purification with QIAquick PCR Purification Kit (Qiagen). Due to some nonspecific amplification, the fragment of expected size of the isolate 283-SRB was excised from the gel and subsequently purified using QIAquick Gel Extraction Kit (Qiagen) before sequencing. Sequencing was performed on an automated equipment (BMR Genomics, Padova, Italy) using both primers. The nucleotide sequences of the amplification products were deposited

in GenBank and compared with those of previously reported IYSV isolates (Table 2), which were retrieved online from the National Center of Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov/BLAST/>). Multiple sequence alignment was done using ClustalW (50). Selected sequences including sequences of all available European IYSV isolates were trimmed to 741 bp, the length of the shortest fragment. Calculation of the divergence in NC-gene nucleotide sequences of selected isolates and phylogenetic analysis were conducted using the MEGA4 software (49). Phylogenetic trees were constructed using the maximum parsimony method and bootstrap analysis with 1,000 replicates.

RESULTS

Virus detection and symptomatology. IYSV was not detected by ELISA in any of the 668 ornamental samples collected during 2005 and 2006, nor was it detected in 516 samples of ornamentals imported in 2005. In onion crops, symptoms resembling those of IYSV were observed in only one location. In July 2007, onion plants (cv. Stuttgarter) with chlorotic or necrotic, spindle and diamond-shaped lesions on leaves and especially on scapes (Fig. 2) were observed in an onion seed production field in the Sirig locality (South Bačka District). Affected plants were spread across the field with very high disease incidence, estimated at 80%. Disease occurrence was associated with a high population of *Thrips tabaci*. IYSV was detected by ELISA in 26 out of 34 symptomatic onion samples. Another outbreak was recorded in June 2007 in the Obrenovac locality (City of Belgrade District). Only two infected onion plants (cv. unknown) with chlorotic spots and streaks were found in an onion bulb crop with relatively low population of *Thrips tabaci*. IYSV was detected serologically in both samples.

Host range. Mechanical transmission of the virus from symptomatic onion samples was performed with variable success, depending on the virus isolate. The isolate from a symptomatic onion bulb sample (283-SRB) could not be mechanically transmitted to any inoculated plant. Isolate 605-SRB from an onion seed sample infected four experimental host species, but mechanical transmission occurred rarely and symptoms on test plants did not appear in all replications. *C. quinoa* (5 infected/30 inoculated) developed discrete chlorotic spots on inoculated leaves, and *N. tabacum* cvs. Samsun (4/30) and Prilep (2/30) developed mild mosaic within 5 and 14 days after inoculation, respectively. All inoculated test plants were assayed for further confirmation of IYSV by ELISA. Several symptomless *N. benthamiana* (7/30) plants were ELISA-positive, and IYSV infection was additionally confirmed by RT-PCR. No local or systemic symptoms occurred

Table 1. Primers used for *Iris yellow spot virus* detection

Primer	Sequence (5' to 3')	Annealing temp./time	Amplicon size (bp)	Ref.
IYSV917L	TAAAACTTAACAAACACAAA	40°C/1 min	896	43
IYSV56U	TCCTAAGTATTCACCAT			
IYSV-Pappu1 ^a	TAAACAAACATTCAAACAA	42°C/2 min	1,200	36
IYSV-Pappu2	CTCTTAAACACATTTAACAAGCAC			
IYSV-459	ACCAGAGGAAGCCCGCAG	54°C/30 s	459	51
TOS-R15	GGGAGAGCAATYGWGKYR			
IYSV-F	TCAGAAATCGAGAAACTT	50°C/1 min	700	16
IYSV-R	TAATTATATCTATCTTTCTTGG			

^a Reference sources included no primer name, and a suitable designation was allocated.

Table 2. Isolates with the nucleocapsid gene sequences of *Iris yellow spot virus* used

Isolate	Geographical origin	Host	Accession no.	Ref.
SgA	Saga, Japan	Lisianthus	AB180921	15
SgOniD1	Saga, Japan	Onion	AB180919	Unpub. data
T3	Shizuoka, Japan	Lisianthus	AB121026	15
Br10	Brazil	Onion	AF067070	41
Isra1 ^a	Israel	Lisianthus	AF271219	26
NL ^a	The Netherlands	Iris	AF001387	12
UK	Suffolk, UK	Lisianthus	AM900393	33
Slov1 ^a	Slovenia	Leek	AY377428	Unpub. data
IYSV-ALB	Spain	Onion	EF419888	11
IYSV-VAL	Spain	Leek	EF427447	12
Cip3	Northern Italy	Onion	FJ185142	Unpub. data
Chile ^a	Chile	Onion	DQ150107	45
WAPasco	Pasco, Wash., USA	Onion	DQ233469	36
Shallot	Grant Co., Wash., USA	Shallot	DQ233471	36
IDNampa	Nampa, Idaho, USA	Onion	DQ233472	36
Ica	Ica, Peru	Onion	DQ838584	35
Supe1	Supe, Peru	Onion	DQ838585	35
Guat2	Guatemala	Onion	DQ838590	35
Georgia2	Georgia	Onion	DQ838592	35
Georgia3	Georgia	Onion	DQ838593	35
New Zealand	New Zealand	Onion	EU477515	52
605-SRB	Sirig, Serbia	Onion	EU586203	9
622-SRB	Sirig, Serbia	Onion	EU750697	This study
283-SRB	Obrenovac, Serbia	Onion	EU727180	This study

^a Where no isolate code was supplied in the GenBank submission, a suitable isolate designation was allocated.

on *N. rustica*, *N. glutinosa*, *C. amaranticolor*, *D. stramonium*, *Petunia* × *hybrida*, *Physalis floridana*, and *G. globosa* after inoculation with the isolate 605-SRB, and the virus presence could not be detected by ELISA.

Seed transmission. No symptoms were observed on onion seedlings grown from seeds harvested from IYSV-infected plants, up to 10 weeks after seed emergence. All leaf samples (groups of five) from these seedlings were negative when tested by ELISA. These results indicate that the virus could not be transmitted through seeds from infected onion plants to seedlings.

Molecular detection and characterization. RT-PCR using different specific primer pairs detected IYSV presence in symptomatic onion samples, as well as in ELISA-positive symptomless inoculated leaves of *N. benthamiana*.

The suitability of four primer pairs for detection of Serbian IYSV isolates in one-step RT-PCR was also assessed. In no reactions was amplification recorded from the healthy onion control. Primers showed different specificity and ability to detect IYSV isolates from Serbia (Fig. 3). None of the Serbian isolates could be amplified with IYSV-F/R primers. Primers IYSV-Pappu1/2 amplified all isolates originating from the Sirig locality generating one clear band, but no isolate originating from the Obrenovac locality was amplified. Primers IYSV917L/56U and IYSV-459/TOS-R15, successfully detected IYSV in all tested samples and amplified cDNA fragments of predicted size. Although these two primer pairs were able to detect the virus in all tested samples regardless of their origin,

they differed slightly. Some nonspecific amplification was detected using IYSV917L/56U primers with two isolates from the Obrenovac locality, while IYSV-459/TOS-R15 primers amplified one clear band of 459 bp.

The sequences of isolates 605-SRB (GenBank Acc. No. EU586203), 622-SRB (EU750697), and 283-SRB (EU727180) proved to be identical at the nucleotide level of 86.5 to 97.8% with those from other parts of the world. Pairwise nucleotide sequence comparison revealed 99.2%

identity between isolates 605-SRB and 622-SRB. The sequences of these two isolates differed from each other only at six nucleotide positions, of which one was predicted to cause an amino acid substitution (99.6% aa identity). Isolate 283-SRB differed from isolates 605-SRB and 622-SRB at 29 (96.4% nt identity) and 27 (96.6% nt identity) nucleotide positions; the differences were predicted to cause nine (96.7% aa identity) and eight (97.1% aa identity) amino acid substitutions, respectively. Nucleotide sequences of iso-

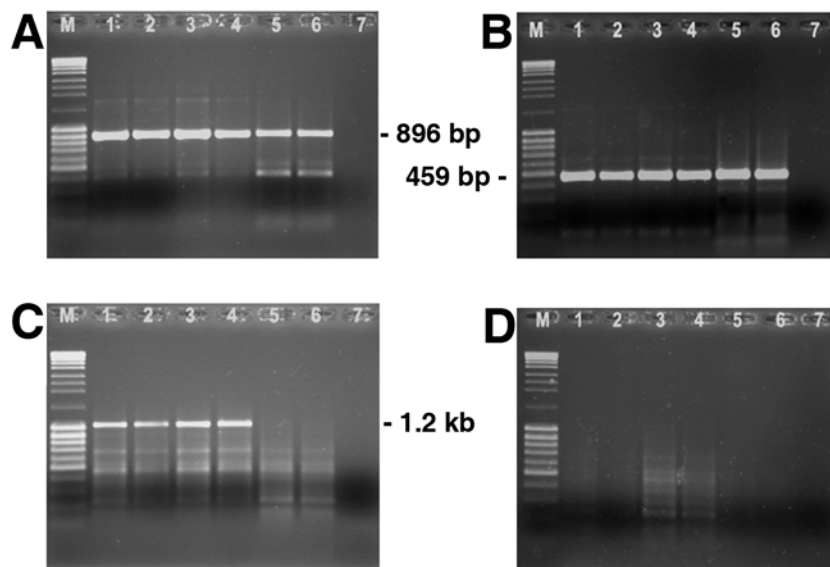


Fig. 3. Detection of nucleocapsid gene sequence of *Iris yellow spot virus* by reverse transcription-polymerase chain reaction (RT-PCR) with primers **A**, IYSV917/IYSV56U, **B**, IYSV-459/TOS-R15, **C**, IYSV-Pappu1/IYSV-Pappu2, and **D**, IYSV-F/IYSV-R primers. Lane M, MassRuler DNA ladder, Mix (Fermentas Life Sciences GmbH, Lithuania); lanes 1 to 4, field samples originating from the Sirig locality; lanes 5 and 6, field samples originating from the Obrenovac locality; lane 7, healthy onion plant.

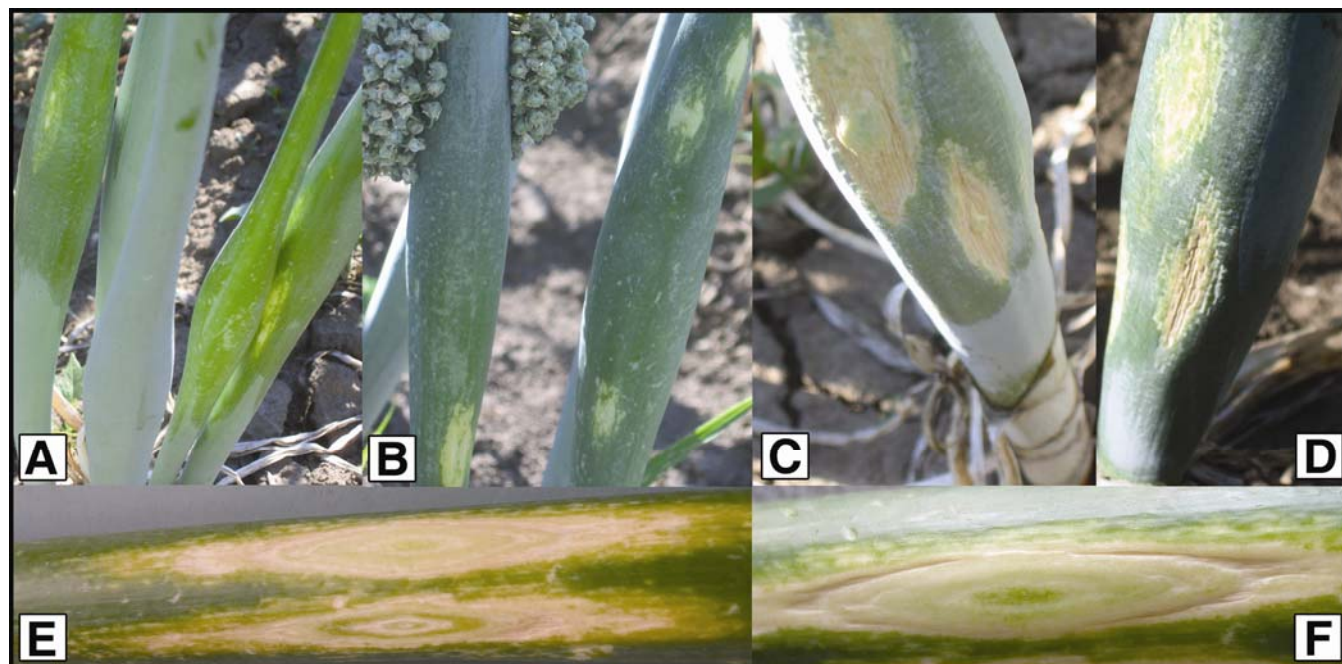


Fig. 2. Symptoms of *Iris yellow spot virus* infection in an onion seed crop. Characteristic symptoms on scapes include chlorotic spindle-shaped lesions with yellow centers (**A** and **B**), necrotic lesions with yellow or tan borders with (**C**) or without (**D**) distinct green centers, and typical diamond-shaped lesions with concentric alteration of green and straw-colored tissue (**E** and **F**).

lates 605-SRB and 622-SRB showed the highest homology with the Italian onion isolate of 97.6 and 97.8%, respectively. Sequence of isolate 283-SRB had the highest homology of 99.2% with Japanese lisanthus isolate. Sequences of the three Serbian isolates were most distant (86.5 to 87.3% nt identities) from the Slovenian leek isolate.

The consensus tree was inferred from 21 most parsimonious trees (Fig. 4). Maximum parsimony analysis resulted in six distinct clades with high nucleotide homology among sequences belonging to the same clade and with high bootstrap support. Two clades consisted of single isolates, clade 1 (Slovenian isolate) and clade 4 (Brazilian isolate). Clade 2 contained isolates from the Netherlands, the UK, and Israel (96.9 to 99.7% nt identities). Clade 3 had isolates from Italy, Spain, and 605-SRB and 622-SRB from Serbia (96.9 to 99.7% nt identities). Clade 5 consisted of Georgia and Peru isolates (98.4 to 99.6% nt identities), and in clade 6, isolates from the western United States, Japan, New Zealand, Guatemala, and Chile were clustered

(98.2 to 99.9% nt identities), and isolate 283-SRB from Serbia was within this clade. The phylogenetic tree also revealed a joint clustering of 283-SRB and Chile isolates in one branch, which could be considered as one subclade within clade 6.

DISCUSSION

There are still many uncertainties about IYSV epidemiology, although it is well known that the virus host range includes several edible and wild *Allium* species as well as some ornamental plants (17). In the EPPO region, among ornamentals, natural infection with IYSV was recorded on *Iris hollandica* (12,14), *Hippeastrum hybridum* (19), *Eustoma grandiflorum* and *E. russellianum* (4,26,33), and *Alstroemeria* (4) and *Chrysanthemum* sp. (8), while *Impatiens sultani* (15), *I. walleriana* (14), and *Petunia × hybrida* (15) were artificially infected. All these ornamental hosts are at risk along with *Allium* species. On the other hand, a systemically infected host, whether it is onion or ornamental, is a potential source of infection for other hosts (26). In view of the assumption that IYSV

is spread through international trade of ornamental propagation material, an intensive survey of greenhouse-grown ornamentals was conducted in Serbia. Testing for the presence of IYSV in numerous greenhouse-grown as well as in imported ornamentals did not reveal infection by the virus. With the exception of a previously reported outbreak of IYSV in an onion seed crop (9), the virus was found in one additional locality—in an onion bulb crop located more than 150 km from the previously reported locality. Besides ascertaining that the new disease of onion found in Serbia is caused by IYSV, this study extends the information provided in most recent reports about distribution of IYSV in European countries (5–7,10,22,28,33).

IYSV infection is often associated with characteristic symptoms on diseased onion plants such as straw-colored ringspots, diamond-shaped lesions, or necrotic eye-like spots (18,39,41). Some of these typical symptoms of diagnostic value were observed on onion plants in the Sirig locality. In the Obrenovac locality, onion plants did not express symptoms indicative of IYSV infection, and IYSV infection could only be confirmed by laboratory testing. A similar situation was reported in the Netherlands in 2006 (6), where, in contrast to many reports from other European countries, IYSV was mainly found in symptomless onion plants or in a few plants showing mild symptoms. These findings support consideration that IYSV could be more widely spread in Europe than is currently known and that its incidence might be underestimated at present. In this case, there is a possibility that IYSV is well-established in Europe but presently at low levels. However, further spread and increased impact could potentially take place if the conditions for virus outbreaks develop in the future. Although symptomatology is known to be associated with many factors, various symptoms found in onion fields led us to the assumption that there may be different strains of the virus present in Serbia.

Variation in biological properties and differences in host reactions among IYSV isolates have been reported on a number of hosts (12,26,41). This study confirmed that mechanical transmission of IYSV from infected onion samples is difficult. Furthermore, mechanical transmission of isolate 283-SRB was not possible. Although isolate 605-SRB infected four commonly used IYSV test plant species, transmission of the virus occurred rarely. Interestingly, this isolate caused symptomless infection of *N. benthamiana*, while most IYSV isolates caused systemic infection (12,14,26,41) or local lesions (48). The failure to mechanically transmit isolate 283-SRB to test plants did not let us compare it with isolate 605-SRB, and no correlation could be established between their phenotypes and molecular variability.

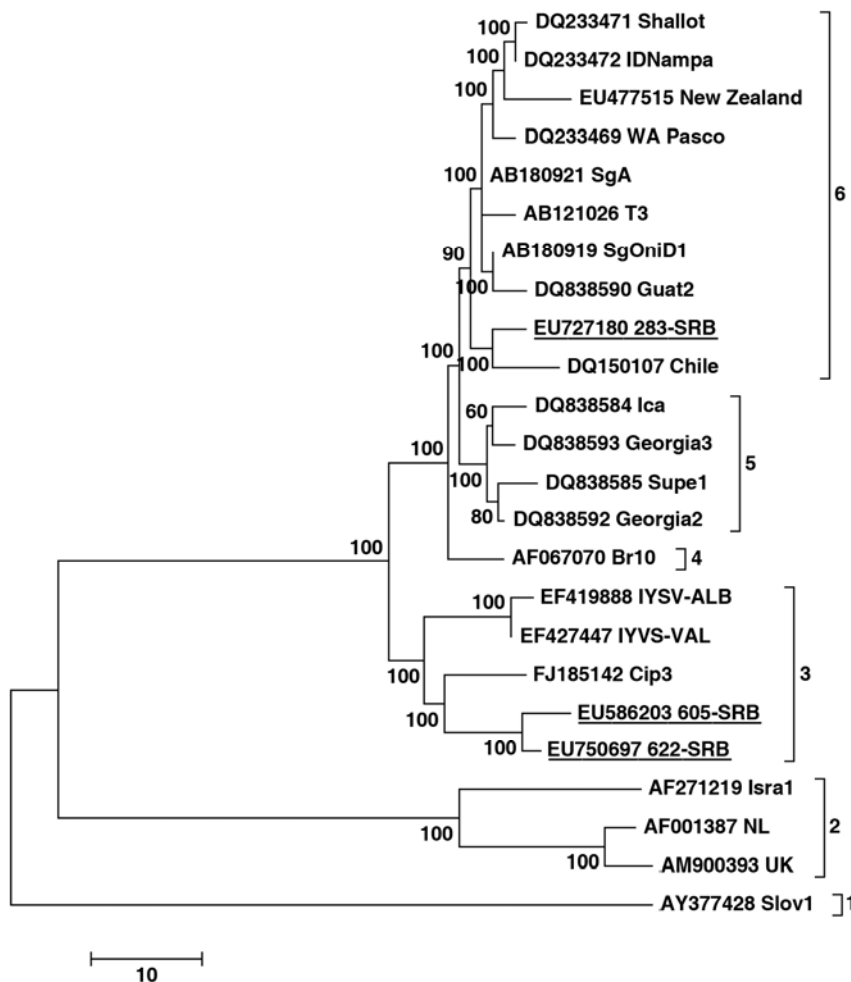


Fig. 4. Maximum parsimony tree based on nucleotide sequences of partial nucleocapsid gene of 24 isolates of *Iris yellow spot virus* (IYSV) using Slovenian IYSV isolate as the outgroup sequence. Phylogram was generated with MEGA 4 using default parameters. Percentages of parsimonious trees in which the associated taxa clustered together are shown next to the branches. Bar lengths are proportional to the number of base substitutions per site. The three Serbian isolates are underlined.

IYSV is not considered to be seedborne or seed-transmitted in onion (27,31). Robène-Soustrade et al. (43) were not able to detect IYSV in onion seed lots but found it in fairly high percentage in 45-day-old seedlings from a nursery. Also, it is well known that numerous factors affect seed transmission such as virus strain, host species and cultivar, environment during seed production, and time of infection. In view of these facts, a large number of seeds produced on IYSV-infected plants were tested. However, there is no evidence of seed transmission, which confirmed earlier findings that IYSV is not transmitted to onion seedlings from infected mother plants through the seed.

Both primer pairs IYSV-459/TOS-R15 and IYSV917L/56U were able to amplify both groups of Serbian isolates of different origin, each resulting in one distinctive band, and proved to be useful for routine molecular detection, as well as for field-based epidemiological studies. IYSV917L/56U primers amplified a larger product covering the entire NC-gene, and because of that they were used for further molecular characterization of the Serbian IYSV isolates. The other two primer pairs could not detect one or both IYSV isolate groups from Serbia. The absence of successful amplification with IYSV-F/R primers is due to observed differences in nucleotide sequences in both forward and reverse primer sites in both groups of Serbian isolates. IYSV-Pappu1/2 primers could not amplify isolates originating from the Obrenovac locality, also suggesting possible sequence differences at the primer site which, in this case, could not be confirmed due to the smaller size of sequences.

Sequence diversity among IYSV isolates has been reported in the United States (1,35,36), as well as the lack of diversity among Australian isolates (47). The phylogenetic analysis performed in this study showed high variation between IYSV isolates originating from two distant localities in Serbia. Two isolates originating from the Sirig locality (605-SRB and 622-SRB) clustered with European isolates from Italy and Spain, but isolate 283-SRB, originating from the Obrenovac locality, fell into a different and distant clade. Similarly, it was reported that an Oregon onion isolate had significant divergence from the others originating from the western United States, and it was grouped with IYSV isolates from the Netherlands and Israel (36). The analysis also indicated that Serbian IYSV isolates do not share a recent common ancestor and that there are two distinct lineages of IYSV in Serbia. The clustering of isolates from Serbia in distinct clades 3 and 6 suggests two introductions of IYSV at different times, as was similarly reported for isolates from California (36). The high sequence divergence among European IYSV isolates, their clustering into four different clades, and the occurrence of the

most divergent isolate so far from Slovenia indicate a longer association with the virus, such as was stated for Japan (47). Interestingly, clustering of one of the Serbian isolates into the clade with isolates from different parts of the world (the United States, Japan, New Zealand, and Chile) potentially indicated extensive and rapid spread of the virus throughout the world, probably due to increasing international trade. The nature and extent of the genetic diversity among IYSV isolates still remain unclear and speculative, apart from the knowledge that the genetic variation has not been associated with differences in vector specificity (12,34). Pozzer et al. (41) suggested that divergence between IYSV isolates from Brazil and the Netherlands reflected an adaptation of those isolates to different environmental conditions. Smith et al. (47) reported that genetic diversity among IYSV isolates from Brazil, Japan, Israel, and Australia were not associated with either the climatic conditions or original hosts. However, clustering of IYSV isolates based on geographic origin with some exceptions was also reported (17,36). Phylogenetic analyses based on IYSV NC-gene sequences may point to introduction of the virus into a particular region or country (26,36), the continent (47) or the occurrence of gene flow from one country to another (35). Sequencing and phylogenetic analyses of more isolates, especially from Europe, would provide additional information on the implications of high genetic diversity among IYSV isolates worldwide.

While IYSV has become a major constraint for the production of onion bulb and seed crops, especially in various states of the United States (40), Israel (19), and Brazil (41), the situation in Europe regarding its presence, distribution, and economic impact is still not completely understood. Despite the wide distribution of *Thrips tabaci* in natural vegetation and the fact that its most numerous populations in recent years were found in onion (30) and ornamental crops (24), until now outbreaks of the disease have been sporadic and limited. In European countries in which the virus is detected, its status can be described as: present, transient, incidental or isolated findings, or present without causing damage. There is no information on damage and economic impact, except that estimated incidence on onion was high in Slovenia (29) and that severely infected plants eventually died in Spain (11). However, most recent records of IYSV outbreak in several European countries such as Italy (7), Germany (5), the UK (33), and Serbia (9), and the virus occurrence in different areas of France on onion and shallot (22), as well as a new report on leek in Spain (10) could represent increasing incidence of IYSV in Europe.

Although IYSV was first recorded in Europe in 1992, and phylogenetic analysis

indicated its presence in Europe, it is not clear why the virus has not been such a serious problem so far as it has been in other parts of the world. This situation might be explained by differences in onion production systems. While in Europe onion is usually produced from plant sets, in the United States it is produced from transplants (10 to 20%) and seed (80 to 90%). Considering the most recent reports on records in new countries, increased incidence and rapid spread in Europe, its emerging status as well as a serious threat to onion production worldwide, further investigations are needed, especially on the efficiency of the vector with respect to the European ecotypes in transmitting the virus, the presence of susceptible host plants and alternative hosts. The results from this study, together with those of first reports on the presence of IYSV in European countries, indicate that the virus has been established in Europe and that it is necessary to prevent its further spread in onion as well as in other susceptible crops.

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