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Molecular study of turnip mosaic virus population in the Czech Republic

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Abstract: Turnip mosaic virus (TuMV) is the most important virus of brassica crops. In our study, we compare the genetic structure of two Czech TuMV populations sampled in the country's 25-year interval of virus presence. The 21 isolates, mainly infecting rutabaga and horseradish, were collected from four farms under organic production, and nearly complete genome sequences, 9 596–9 787 nt in length, were obtained using Sanger sequencing for all of them. The analysis of variability and polymorphism showed differences in genetic structure but the relative stability of both populations and moderate negative selection as a factor affecting the current TuMV population. The newly collected isolates are characterised by a relatively high frequency of intralineage recombinants; interlineage recombinants were not detected compared to the 25-year-old population. The phylogenetic analysis allowed the classification of all Czech isolates into world-B phylogroup, with the prevalence of isolates of subgroup B2. The spread of isolates belonging to the other phylogenetic groups posing higher phytopathological risk, which were present in the old population and some surrounding countries, was not found.

Keywords: sanger sequencing; recombination; phylogeny; population changes; selection pressure

Brassica vegetables are important and very popular in the Czech Republic. In 2021, they occupied 14% of the vegetables cultivated area, with cabbage, cauliflower and broccoli, and kohlrabi being the most often grown (Němcová & Buchtová 2021).

Turnip mosaic virus (TuMV) is widespread in both temperate and subtropical regions (Ohshima et al. 2002), and it is considered to be the most important virus infecting brassica crops, causing production losses of up to 70% (Li et al. 2019). Their host range is not limited to Brassicaceae plants; the virus also causes problems for lettuce, spinach, and other bedding plants. TuMV is not seed-borne but is efficiently transmitted non-persistent by aphids, primarily by *Myzus persicae* and *Brevicoryne brassicae* (Špak 1992).

The *Turnip mosaic virus* is classified as a member of the genus *Potyvirus*. Its genome consists of approximately 9 835 nucleotides. It bears a large reading frame translated in one polyprotein (typically 3 164 amino acids in length) processed into ten proteins (P1, HC-Pro, P3, 6K1, CI, 6K2, NIa-VPg, NIa-Pro, NIb and CP); and small reading frame encoding protein PIPO (Nigam et al. 2019).

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TuMV is highly variable, with more than 500 genome sequences available from GenBank-NCBI. Until now, 12 pathotypes based on the symptoms and seven phylogenetic groups (basal-B, basal-BR, Asian-BR, world-B, Iranian 1 and 2, and OMs), which correlate well with their differences in pathogenicity and reflect their geographical origin, have been distinguished (Jenner & Walsh 1996; Yasaka et al. 2017).

The evolutionary and phylogeographic history is studied well, with the high mutation rates and frequent intralineage and interlineage recombinations identified as the important sources of variability (Tan et al. 2004; Ohshima et al. 2007).

In the Czech Republic, the occurrence of TuMV was first recorded on poppy plants (*Papaver somniferum*) in the mid-1980s (Špak & Kubelková 1990). In 1991, its occurrence on spinach plants (*Spinacia oleracea*) was published, in which the disease is responsible for reducing the quality and yield of fresh leaf mass (Chod & Jokeš 1991). Analyses of Czech TuMV isolates collected in the 90s of the last century from cabbage (*Brassica oleracea*), rape (*B. rapa*), horseradish (*Armoratia rusticana*), and garlic mustard (*Alliaria officinalis*) allowed their classification into host infecting type B, phylogenetic group world-B, pathotypes 3, 4, 5, 6, and 11 (Jenner & Walsch 1996; Kawakubo et al. 2021).

The aim of the present work was to study the current situation of TuMV population in the Czech Republic and the virus evolution after 25 years of its first collection here. The main goal was to answer the question of which TuMV phylogroups are currently present and if the previously detected interlineage recombinants infecting weeds (Tomimura et al. 2004) occur in cultivated crops; to obtain basic knowledge necessary for the application of the effective disease control strategies.

MATERIAL AND METHODS

Sampling, RT-PCR, and sequencing. Various vegetable crops were sampled in 2019 in Moravia, Czech Republic, at four organic vegetable farms in Jistebník, Pustějov, Sedlnice, and Lednice, in collaboration with the growers. The plants showing virus-like symptoms, mainly various chlorotic mosaics, were collected, and the TuMV presence was confirmed by the DAS-ELISA test using LOEWE® Biochemica GmbH kit (Navrátil & Šafářová 2022). Twenty-one turnip mosaic virus isolates representing a new set of Czech isolates were obtained from kohlrabi, rutabaga, and horseradish (Table S1 in electronic supplementary material); infection of savoy, cabbage, and cauliflower was not detected. The virus was not detected in the brassica crop fields under conventional farming systems in the region, including the Olomouc district (data not shown).

The RNAs were isolated from the infected leaves of the sampled plants using the NucleoSpin RNA plant kit (Macherey-Nagel, Germany). The 300–500 ng of isolated RNA was used to synthesise cDNA using Bioscript reverse transcriptase and random hexamers (both Bioline, UK).

The genomic sequences of studied TuMV isolates were amplified through the overlapping fragments using the specific primer pairs (Figure 1, Table S2) using My Taq polymerase (Bioline, UK). The reaction mix in a total volume of 25 µl consisted of MyTaq buffer (10×), 0.2 µM of each primer, 1 U of MyTaq polymerase and 2 µl of cDNA.

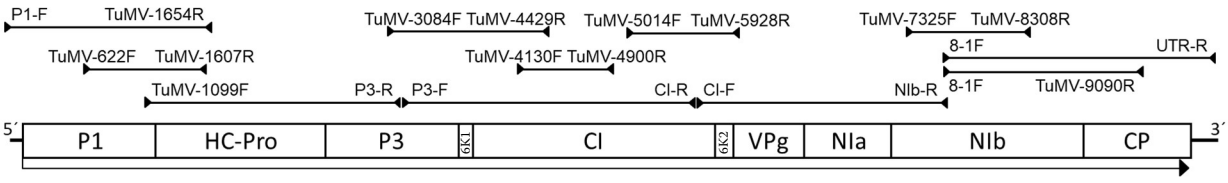

Figure 1. Schema of the TuMV genome with marked regions of ORF1 and the Sanger sequencing strategy used in this study

P1 – protein 1 protease; HC-Pro – helper component protease; P3 – protein 3; 6K1 – 6-kDa peptide 1; CI – cylindrical inclusion protein; 6K2 – 6-kDa peptide 2; VPg – viral protein genome-linked; NIa – nuclear inclusion a-protease; NIb – nuclear inclusion b; CP – coat protein

Arrow indicates ORF1 encoding polyprotein

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The conditions of the PCR reaction consisted of 94 °C for 2 min, followed by 40 cycles of 94 °C for 1 min, 52–62 °C for 1 min, and 72 °C for 1–3 min (both depending on the primer pair used); and a final extension 72 °C for 5 min. The PCR products were visualised in 1% agarose gel and isolated using a FastGene Gel/PCR Extraction kit (NIPPON Genetics, Europe).

The isolated amplicons were sequenced directly using BigDye™ Terminator (version 3.1) Cycle Sequencing Kit and an ABI PRISM3730 Genetic analyser in the Sequencing Centre, Institute of Experimental Botany, CAS, Olomouc. The obtained sequences were assembled into final contigs covering nearly complete genomes using Geneious Prime assembler and MEGA Alignment Explorer (version 7.0). The identity of isolates was confirmed by BLASTN analysis (Altschul et al. 1997).

Genetic variability and phylogenetic analyses. To study the TuMV population structure changes, the sequences of TuMV isolates obtained in this study were compared with nearly complete genome sequences of Czech isolates available in GenBank. These isolates were collected from different localities in the Czech Republic in 1993–1994 and were characterised by Kawakubo et al. (2021). The representative isolates of the TuMV phylogroups were also included in the phylogenetic analyses (Table S1). Multiple alignments using the ClustalW algorithm, the prediction of open reading frames, the model selections, the phylogenetic analyses using the Neighbor-joining algorithm and Tamura 3-parametric model for nucleotide and JTT model for amino acid sequences, and the tree visualisations were all performed using Mega (version 7) (Kumar et al. 2016).

In contrast to strictly bifurcating evolutionary dendrograms, SplitsTree provides a network-based approach. Screening and visualisation of the possible evolutionary relationships among the investigated accessions was performed in SplitsTree 4 (Huson & Bryant 2006). NeighborNet analysis was performed on uncorrected P-distances.

The estimation of genetic variability and basic evolutionary divergence–pairwise distance, intra- and inter-group mean distance analyses on the aligned sequences of ORF1 coding region and of the discrete genes on nt and deduced aa sequences were calculated using MEGA (version 7.0).

Analysis of population variability. The recombination events and potential breakpoints were identified in the RDP4 program (version 4.97) using RDP, GeneConv, Bootscan, MaxChi, Chimaera, SiScan and 3Seq methods (Martin et al. 2015). Default settings and the Bonferroni corrected *P*-value of 0.05 were applied throughout the analyses. Only recombinant events supported by at least six of seven methods with a *P*-value < 1.0×10^{-6} were considered significant.

The comparison of the new TuMV isolates (this study) and previously described (old) Czech isolates was done on the entire ORF1 region and the ten specific potyvirus protein-coding genes (P1, HC-Pro, P3, 6K1, CI, 6K2, NIa-VPg, NIa-Pro, NIb, CP). The genetic diversity of new and old isolate groups was estimated according to the four parameters: a number of haplotypes (H), haplotype diversity (Hd), average pairwise nt diversity (π), and a ratio of non-synonymous to synonymous nt diversity (ω = dN/dS). The genetic differentiation was examined using the three standard values: Ks*, Z*, and Snn, based on permutation statistical tests as well as three neutrality tests (Tajima's D, Fu and Li's D* and F*); all mentioned analyses were calculated using DnaSP (version 6.12.01) (Rozas et al. 2017).

RESULTS

The nearly complete genome sequences of 9 596–9 787 nt length for twenty of the TuMV isolates and two non-overlapping partial sequences of 4 895 and 4 736 nt lengths for the next TuMV isolate (T52) were obtained. All sequences were deposited in GenBank under Acc. No. OQ675593-OQ675613. To study the occurrence, population diversity and evolution of TuMV, these (new) isolates, collected from vegetable fields and the horseradish germplasm collection, were compared with the TuMV isolates collected in the Czech Republic 25 years earlier (old population), and for which genetic characterisation is available.

Genetic variability and phylogenetic analyses. The pairwise identity analysis showed that TuMV isolates from both groups (new/old) exhibited a similar trend towards intragroup identities in both their nt and aa sequences of complete ORF1 (92.6% and 93.4% in nt or 96.6% and 97.3% in aa, respectively) and of individual genomic regions (for details see Table S3). Intergroup *p*-distance at ORF1 was 9.1% in nt and 4.1% in aa. The parts that

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differed the most were P3 (13.4% in nt, 9.5% in aa), 6K2 (13.1% and 9.4%), and P1 (11.8% and 11.7%). The parts that differed the least were CP (5.0% and 1.8%) and NIb (5.1% and 1.8%) (Table S3).

The phylogenetic analyses of all complete or nearly complete sequences of Czech isolates and selected representatives of TuMV phylogenetic groups allowed discrimination of new Czech isolates within the common group world-B in Europe. The isolates of subgroup B2 were predominantly branching together with Czech isolates CZE5 and recombinant CZE4 and CZE8, showing close relation with isolates from Slovakia (HC9) and Poland (POL1, POL2). The isolates obtained from horseradish branched into the large subgroup B3, forming significantly different branches compared to the old, in GenBank available isolates, and isolate T41 belongs to the small subgroup B1 represented by other horseradish isolates, in relation to the old CZE18 isolate. The specific position within the B2 subgroup was also found for the isolate TGZ33. The analyses of phylogenetic relationships using SplitsTree showed a similar distribution of TuMV isolates, including the floating position of selected genes in the case of recombinants (Figure 2). Analysis of the ORF1 polyprotein generated a phylogenetic tree with the same branching (data not shown). Analyses of CP and NIb genes also allowed the construction of trees with similar topology, and only observed changes reflected recombination events (Figure S1 and Figure S2).

Analysis of population variability. The complete ORF1 sequences of the TuMV isolates aligned in the previous analysis (21 new Czech isolates and 47 other isolates available from the NCBI database, including 12 old Czech isolates) were screened for the possible evidence of recombination events using the RDP (version 4). A putative recombination event was identified in 18 of 21 new Czech isolates (Table 1). Recombination sites were detected in five genes of ORF1, for three of them, HC-Pro, and P3 genes, near the 5'end; and near the 3'end for NIb and CP genes. No recombination events were detected in the central regions of ORF1, specifically for 6K1, CI, 6K2, NIa-VPg and NIa-Pro genes. All recombination events occurred within the world-B phylogenetic group. The Polish isolates POL1 and CAR37A, and the Czech isolates T54, CZE8, and CZE12 were identified as major parents; the Polish CAR37A, Slovak HC9, and Czech T30, T41, TGZ53, CZE8 isolates as minor parents. For example, the CAR37A isolate (subgroup B1) was identified as the major parent, and the CZE8 isolate (subgroup B2) as the minor parent of the new Czech recombinant isolate T41 (member of the phylogenetic subgroup B1). Iso-

Table 1. List of recombination events detected in NEW Czech turnip mosaic virus group of isolates

Detected recombinant (Acc. No.)	Major parent (Acc. No.)	Minor parent (Acc. No.)	Start position	End position	RDP (version 4) P-value < 1.0×10^{-6}
TGZ33 (OQ675608)	POL1 (AB701728)	CAR37A (DQ648591)	$2\,001^{\text{HC-Pro}}$	$5\,912^{\text{VPg}}$	R, G, B, M, C, S, 3S
TGZ33 (OQ675608)	CZE4 (LC537538)	T41 (OQ675599)	$8\,731^{\text{CP}}$	$9\,454^{\text{CP}}$	R, G, B, M, C, S, 3S
T41 (OQ675599)	CAR37A (DQ648591)	CZE8 (LC537539)	$7\,262^{\text{NIb}}$	$8\,685^{\text{CP}}$	R, G, B, M, C, S, 3S
T53 (OQ675604)	T54 (OQ675605)	TGZ53 (OQ675613)	$7\,230^{\text{NIb}}$	$8\,636^{\text{CP}}$	G, B, M, C, S, 3S
T10 (OQ675593)	CZE8 (LC537539)	HC9 (MH469725)	$8\,811^{\text{CP}}$	$2\,999^{\text{P3}}$	R, G, B, M, C, S, 3S
T27 (OQ675594)			$9^{\text{5'UTR}}$	$2\,927^{\text{P3}}$	
T28 (OQ675595)			$8\,794^{\text{CP}}$	$2\,846^{\text{P3}}$	
T45 (OQ675600)			$8\,427^{\text{NIb}}$	$2\,961^{\text{P3}}$	
T47 (OQ675601)			$8\,208^{\text{NIb}}$	$2\,961^{\text{P3}}$	
T52 (OQ675603)			$8\,208^{\text{NIb}}$	$2\,961^{\text{P3}}$	
T53 (OQ675604)			$8\,811^{\text{CP}}$	$2\,999^{\text{P3}}$	
T54 (OQ675605)			$8\,892^{\text{CP}}$	$3\,000^{\text{P3}}$	
TGZ11 (OQ675606)	CZE12 (LC537542)	T30 (OQ675597)	587^{P1}	$1\,320^{\text{HC-Pro}}$	R, B, M, C, S, 3S
TGZ20 (OQ675607)			587^{P1}	$1\,320^{\text{HC-Pro}}$	
TGZ42 (OQ675609)			587^{P1}	$1\,320^{\text{HC-Pro}}$	
TGZ43 (OQ675610)			587^{P1}	$1\,320^{\text{HC-Pro}}$	
TGZ44 (OQ675611)			$9\,405^{\text{CP}}$	$1\,320^{\text{HC-Pro}}$	
TGZ49 (OQ675612)			587^{P1}	$1\,320^{\text{HC-Pro}}$	
TGZ53 (OQ675613)			$8\,972^{\text{CP}}$	$1\,348^{\text{HC-Pro}}$	

R – RDP; G – GENECOV; B – BootScan; M– MaxChi; C – Chimaera; S – SiScan; 3S – 3Seq

Genome regions in which the recombination event took place marked by an abbreviation in the index

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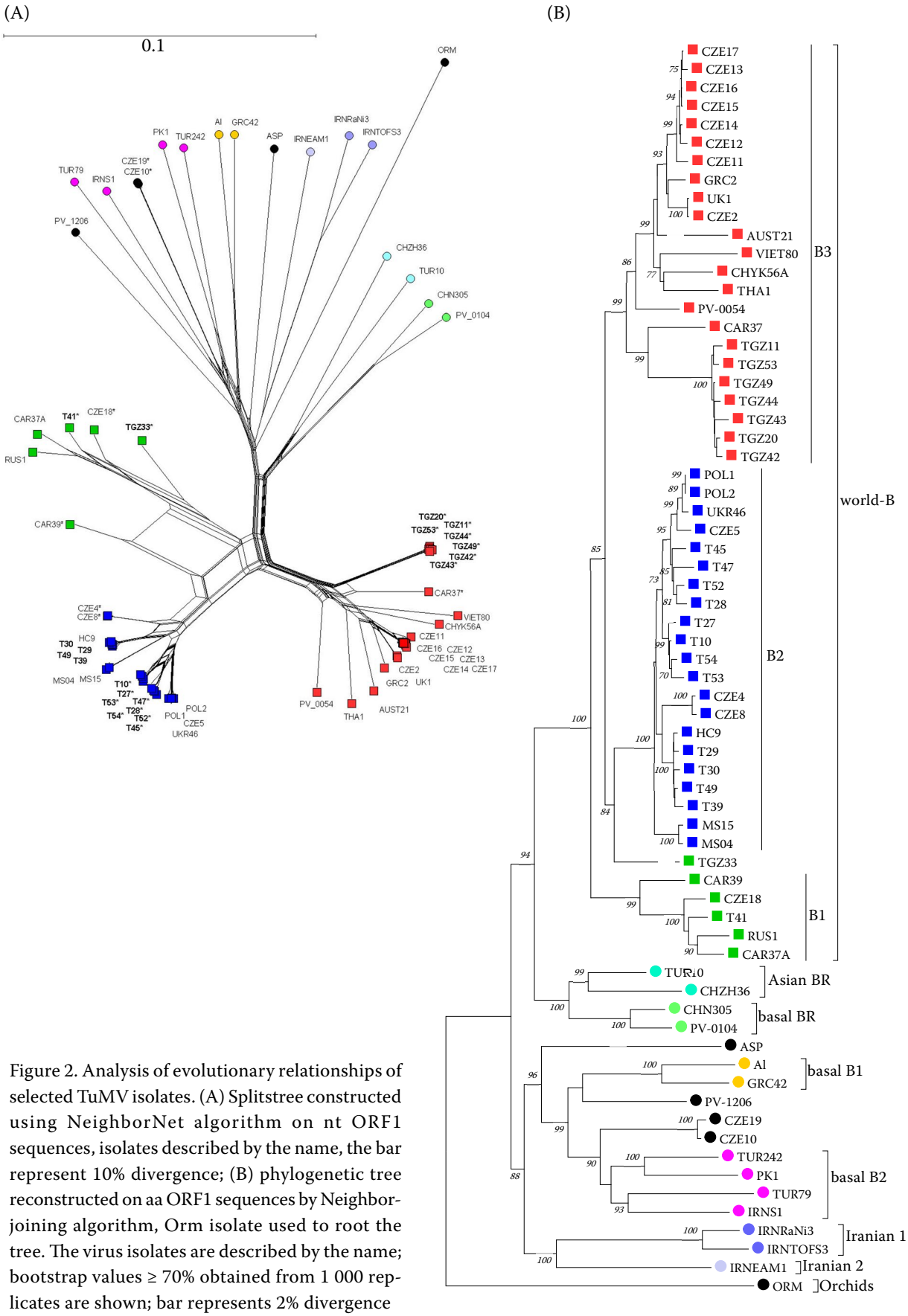

Figure 2. Analysis of evolutionary relationships of selected TuMV isolates. (A) Splitstree constructed using NeighborNet algorithm on nt ORF1 sequences, isolates described by the name, the bar represent 10% divergence; (B) phylogenetic tree reconstructed on aa ORF1 sequences by Neighbor-joining algorithm, Orm isolate used to root the tree. The virus isolates are described by the name; bootstrap values ≥ 70% obtained from 1 000 replicates are shown; bar represents 2% divergence

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lates T10, T27, T28, T45, T47, T52, T53, and T54 (all subgroup B2) evolved from isolate CZE8 (B2) as the major parent and isolate HC9 (B2) as the minor parent). Horseradish isolates TGZ11, TGZ20, TGZ42, TGZ43, TGZ44, TGZ49 and TGZ53 (all B3) originated from CZE12 (B3) as the major parent and T30 (B2) as the minor parent.

The results indicate more than one case of recombination events between CZE8 and HC9, CZE12 and T30, respectively. The multiple recombinations are associated with the origin of isolate TGZ33, which evolved from POL1/CAR37A and CZE4/T41, respectively. No recombinants were found between new Czech isolates and Asian-BR, basal-BR, basal-B, or Iranian phylogenetic groups.

The haplotype diversity of ORF1 of new and old Czech isolates were 1.000, and π value 0.07969 and 0.07172, respectively. The diversity of the ORF1 coding region is similar between both new and old TuMV isolates in all ten genes (Table S4). Haplotype diversity (Hd) among isolates of new Czech TuMV isolates was found to vary from 0.843 for 6K1 gene to 0.995 for P1, HC-Pro, and CI genes. π value varied from 0.03230 for the CP gene to 0.13466 for the P3 gene. An analogous situation was found within TuMV isolates within old Czech isolates; Hd values varied from 0.894 for 6K1 and 6K2 genes to 1.000 for P3, CI, and NIa-VPg genes. π value varied from 0.03502 for the CP gene to 0.11694 for the 6K2 gene. P3 and 6K2 genes are the most variable in both evaluated groups, and CP and NIb genes are the most conserved.

Any tested genes of both new and old TuMV isolates with a dN/dS ratio < 1 are considered under negative (purifying) selection. The relatively higher diversification pressure was not significantly noticed for the P1 gene of both groups. Strong negative selection was detected for NIa-Pro (ω = 0.015) and 6K1 (0.016) genes of new isolates and NIa-Pro (0.005) and HC-Pro (0.015) genes of old isolates (Table S4).

Tajima's D, Fu and Li's D*, and Fu and Li's F* neutrality tests were calculated for the ORF1 coding region and discrete genes of both new and old isolates (Table S4). The values were not significantly positive and/or negative, except for a significantly negative value for the P1 gene of new isolates ($p > 0.05$), indicating that new isolates were expanding in this gene. Positive values in most of the genes (P3, 6K1, CI, 6K2, VPg, NIb, CP) and complete ORF1 of the new TuMV population indicated its relatively stable state, contrary to the genes (P1, HC-Pro, P3, CI, NIb, CP) and complete ORF1 of the 25-years old population.

The Fst scores between new and old TuMV Czech isolates for the P1 and HC-Pro genes were higher than 0.25000, indicating their large genetic differences. Fst values for the other genes varied from 0.1496 for CP to 0.2514 for HC-Pro and 0.2270 for the whole ORF1 showing moderate genetic differentiation (Table S5).

Kst*, Z*, and Snn values obtained for each TuMV gene and the whole ORF1 between new and old Czech TuMV isolates were all statistically significant, supporting the hypothesis/estimation that new and old TuMV populations are diverse.

DISCUSSION

Turnip mosaic virus is like the other potyviruses, characterised by a relatively high frequency of mutations and recombination events resulting in the evolution of new viral variants, leading to changes in host range, pathogenicity and/or transmission by vectors. These and the recent changes in environmental conditions, i.e., global warming and climate instability, affect the relationships in the brassica pathosystem and pose risks to vegetable growers.

To characterise the genetic variability and micro-evolutionary trends of TuMV in the Czech Republic, the screening of current (new) isolates and their comparison with the 25 year old population was performed for the first time. Our results confirm the persisting prevalent occurrence of world-B TuMV isolates reported in Central Europe, namely in Poland, Slovakia, Austria and Hungary (Kozubek et al. 2007; Glasa et al. 2018), with the most brassica isolates belonging to the subgroup B2 and horseradish isolates belonging to the subgroups B3. The phylogenetic group classification of the current Czech population generally agrees with the already published characterisation of the old population, despite the fact that the TuMV was collected mainly from the different hosts, rutabaga, and none isolate was found in one of the previous primary sources, the Moravian Olomouc district.

The relatively high frequency of recombinant isolates in the new Czech TuMV population is surprising. Still, the total number of them could be affected by the frequent occurrence of recombinants in horseradish at the locality Lednice. Moreover, their high level of genetic identity, together with simi-

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lar recombinant events detected, implicates their similar origin. Most of the detected recombination events took place in the P1, HC-Pro, P3, NIb and CP genes, i.e. at the 3'- or 5'- end of the genome that are more prone to recombinations than the central part (Oshima et al. 2007; Kawakubo et al. 2021).

The recombination events are generally frequent in TuMV being reported from different countries and host plants, and both interlineage and intralineage changes have been described. In the case of the Czech Republic, the interlinkage recombinants between the world-B2 and basal-B2 groups were previously reported for the isolates CZE10 and CZE19 infecting *Alliaria oficinalis*. This situation is interesting as the current isolates belonging to the basal-B group were not found there. However, they were reported from a number of European countries, such as Germany, Italy, Greece, and Spain, isolated mainly from non-brassica hosts (Kawakubo et al. 2021). The interlineage recombinants have been repeatedly found in Asian countries, such as China or Vietnam, and there are connected/associated with the change of the virus epidemiology, and they are just known to be the cause of change of virus isolates virulence (Kawakubo et al. 2022).

The only recombinants found in the current Czech population were the intragroup recombinants within the world-B group, with the predicted parents coming from CZ and surrounding countries, i.e. Slovakia and Poland. This indicates a certain stabilisation of the TuMV population in the country and the Central European area. It represents the lower risk of TuMV evolution, its potential spread and widening of the host range, and the development of more severe damage to cultivated vegetable crops.

The analyses of the old and new Czech TuMV populations showed that the intragroup variability is quite similar, with the P3 and 6K2 as the most variable and NIb as the most conservative part of the genome. We can generalise that individual genes within the analysed groups of Czech isolates are under different negative selection, and the intergroup analyses show the genetic shift in the population structure based at least on the variability of P1, and HC-Pro regions, too. However, it could not be excluded that some effect of the different TuMV host origin of the sampled isolates, which mainly originate from the rutabaga, might be present. The P1 gene of both group populations is under less stringent negative selection ($\omega > 0.150$), in contrary to HC-Pro^{old}, 6K1^{new}, CI$^{new, old}$, NIa-Pro$^{new, old}$, NIb$^{new, old}$, and CP^{old} genes are under low stringent negative selection ($\omega < 0.03$). Similar results were earlier published by Tomimura et al. (2004), where the P1 gene had the largest dn/ds ratio while the NIa-Pro had the smallest, indicating that different protein genes are under different evolutionary constraints.

CONCLUSION

In the present study, the genetic structure of two populations of TuMV isolates, the old one represented isolates collected before 25 years and the new one represented by isolates collected at four sites in the Czech Republic, was studied in a microevolution and epidemiological context. The *p*-distance, $\omega = dN/dS$, Ks*, Z*, and Snn values as three neutrality tests (Tajima's D, Fu and Li's D* and F*) analyses showed that both populations are relatively stable, but they differ in their genetic structure and in the presence of isolates from different TuMV phylogenetic groups; the current population is generally under moderate negative selection which is also repeatedly reported from the other countries, too.

The frequency of recombinant isolates is relatively high, but only intragroup recombination event has been identified, and the presence of previously detected intergroup recombinants has not been confirmed. In the brassica vegetable crops, the only isolates found were of the group world-B, mainly the subgroup B2, which is common in Central Europe. Isolates of different phylogenetic groups, which could pose a potentially high epidemiological risk in the future, especially in connection with the spread of the ecological farming system, were not detected.

The potyviruses are a well-studied group of viruses, but concerning their ability to evolve under biotic and abiotic stress, further epidemiological studies of turnip mosaic virus are necessary to control this severe pathogen of brassica crops. The impact of wild and/or weed, mainly perennial, species and also horseradish should be evaluated, not only as the sources of infection (reservoirs of virus isolates) but also as mixing organisms for the TuMV recombination and the origin of new, more virulent isolates, to reduce the phytopathological risk.

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