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MOLECULAR CHARACTERIZATION OF COI GENE OF *IXODES RICINUS* (LINNAEUS, 1758) FROM SERBIA

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Abstract – The *Ixodes ricinus* tick is common in the central part of the Balkan Peninsula and is a vector of pathogenic agents causing diseases in humans and animals. Little is known about the genetic structure of *I. ricinus* in this region. We have investigated intraspecific variability of the COI gene among *I. ricinus* ticks collected from different regions of Serbia, and the correlation between the various types of habitat and genetic variability of ticks. The obtained COI gene sequences are the first barcoding sequences of *I. ricinus* ticks collected at localities in Serbia. Intraspecific variability of these COI gene sequences was very low, and there was no correlation between the various types of habitat and genetic variability of ticks. Samples from isolated localities (canyon/gorge) showed no genetic differentiations from the majority of samples from open areas.

Key words: Ixodes ricinus ticks; COI gene; genetic variability; barcoding; Serbia

INTRODUCTION

Ticks are obligate hematophagous ectoparasites of amphibians, reptiles, birds and mammals. Due to the ability to transmit a greater variety of infectious agents than any other blood-feeding arthropods they are consider to be second only to mosquitoes as vectors of medical and veterinary importance (Jongejan and Uilenberg, 2004). *Ixodes ricinus* (Linnaeus, 1758) is the most common tick species in Europe (Estrada-Peña et al., 2006). It belongs to the *Ixodes ricinus* complex containing 14 closely related species distributed in different regions of the world (Xu et al., 2003). As a vector it transmits a number of pathogens that cause various infectious diseases of humans and animals, such as Lyme borreliosis, tick-borne encephalitis, ehrlichiosis and babesiosis (Parola and Raoult, 2001; Gray et al., 2002).

In Serbia, *I. ricinus* is the most abundant and widely distributed tick species (Petrović, 1979; Milutinović, 1992; Milutinović and Radulović, 2002). Previous studies of *I. ricinus* ticks in Serbia were mainly focused on the morphology, distribution and ecology and the presence of pathogens and epidemiology of disease (Milutinović and Radulović, 2002; Milutinović et al., 2008; 2012; Tomanović, 2009, 2011, 2013). Considering the genetic structure of the species *I. ricinus* in Serbia, the only data are based on studies of analyses of polymorphisms of *Gpdh* gene and the gene for malate dehydrogenase (Radulović, 2005, 2010; Radulović et al., 2006,

Fig. 1. Map of Serbia with tick collecting sites (1-4 Belgrade area; 5 – Grivska; 6 –Virovo; 7 - Canyon of Veliki Rzav; 8 – Požega; 9 – Ivanjica; 10 - Đetinja Gorge; 11 – Dobra; 12 – Brnjica; 13 - Kajtasovo.

2012). Better understanding of the genetic variability of the vector is essential to understand the epidemiology of disease and evolutionary dynamics of disease and vector.

It has been shown that mitochondrial DNA (mtDNA) is a rich source of useful markers for the genetic characterization and studies of phylogenetic relationships of organisms at different taxonomic levels (Boore and Brown, 2000; Macey et al., 2000; Boore and Staton, 2002; Morisson et al., 2002; Lavrov et al., 2004). Mitochondrial genes are inherited only through maternal lineages and they evolve more rapidly than nuclear genes and have much higher proportion of coding sequence than nuclear genomes (Shao and Barker, 2007; Casati et al., 2008). As a source of genetic markers, mtDNA was used in several studies that analyzed phylogenetic relationships between ticks, and it was shown that these sequences were appropriate for distinguishing populations within a species (Caporale et al., 1995; Xu et al., 2003; Casati et al., 2008; Chitimia et al., 2010). The mitochondrial cytochrome C oxidase subunit I (COI) gene is used as the standard barcode for almost all animal groups (Hebert et al., 2003). Several studies have demonstrated that nucleotide sequences of the COI gene are suitable for phylogenetic studies and characterization of the genetic structure of *I. ricinus* (Caporale et al., 1995; Casati et al., 2008; Chitimia et al., 2010).

The aim of our study was to determinate the intraspecific variability of the COI gene among *I. ricinus* ticks collected from different regions of Serbia, and to investigate the correlation between the various types of habitat and genetic variability of ticks.

MATERIALS AND METHODS

Study area

Ticks were collected from 13 localities in Serbia, representing different habitat types of *I. ricinus* occurrence (Fig. 1).

In the Belgrade region, ticks were collected from four localities (Košutnjak, Avala, Titov Gaj, Makiš) that represent park-forests and recreation sites. Localities Avala, Košutnjak, Titov Gaj are under deciduous and coniferous forests and include areas of mostly deciduous submediterranean forests (Vukin, 2008). Makiš belongs to the coeno-ecological group of pedunculate oak and European alder forest type (*Alno-Quercion roboris* Horv. 1938) (Stojanović-Mitrović et al., 2013).

In western Serbia, samples were collected from six localities. The three localities belonging to the municipality of Arilje (Grivska, Virovo, Veliki Rzav Canyon). The countryside of Grivska is characterized by pure oak forests (*Quercus cerris, Q. petraea*) and mixed beech-hornbeam (*Fagus sylvatica*, *Carpinus betulus*) forests. Virovo represents agro-ecosystems with permanent anthropogenic influence. Veliki Rzav is a limestone canyon. The dominant forests in this region consist of beech trees *Fagus sylvatica* ssp. *moesiaca* and *Ostrya carpinifolia*. The Požega locality is situated in the valley of Požega, at the estuary of the rivers Đetinja, Skrapež and Moravica. We collected ticks along the Skrapež riverbanks. This area of willow and poplar forests is under permanent anthropogenic influence. Ivanjica is located in the southwest of Serbia. It lies on the banks of the Moravica River in a valley. The main vegetation types are oak forests dominated by *Quercus cerris*, *Q. petraea* and *Carpinus betulus*; beech forests with *Fagus sylvatica* ssp. *moesiaca*; mixed deciduous-coniferous stands dominated by *Abies alba, Picea abies* and *Fagus sylvatica* ssp. *moesiaca*, wet habitats dominated by *Carex* spp., mountain peat bogs characterized by *Sphagnum* spp., and mountain meadows with *Festuca pratensis, F. rubra, F. vallesiaca* and *Anthoxanthum odoratum* (Gajić, 1989). The Đetinja Gorge is located closed to the town of Užice. It is a small gorge, between huge layers of sedimentary rocks. Although it is constantly under human influence the fact that it is surrounded by vertical rocks enables the survival of numerous species, making them inaccessible.

In eastern Serbia, ticks were collected from two localities Brnjica and Dobra, belonging to Đerdap National Park. Brnjica and Dobra are located along the right bank of the Danube on narrow strips of forested hills. The predominant forest associations in this area are *Querco-colurnetum mixtum*, *Fago-colurnetum mixtum*, *Celto-Juglandetum* and *Syringo-colurnetum mixtum*. Locality Kajtasovo in Vojvodina belongs to the Special Nature Reserve Deliblatska peščara. This locality is characterized by proximity of the Danube. The countryside of Kajtasovo represents agro-ecosystems with permanent anthropogenic influence.

Samples

Samples were collected by dragging white flannel flags over vegetation in the period from April to May during the years 2011 and 2012. All ticks were morphologically indentified to the species level using the existing standard taxonomic key (Pomerancev, 1950).

DNA isolation

A total of 20 unfed adult female *I. ricinus* ticks were chosen for further molecular analysis. The collected ticks were stored live in a vivarium until DNA extraction. DNA was extracted from whole ticks using a GeneJet Genomic DNA Purification kit (Fermentas) according to manufacturer's instructions. DNA extracts were stored at -80°C until PCR amplification.

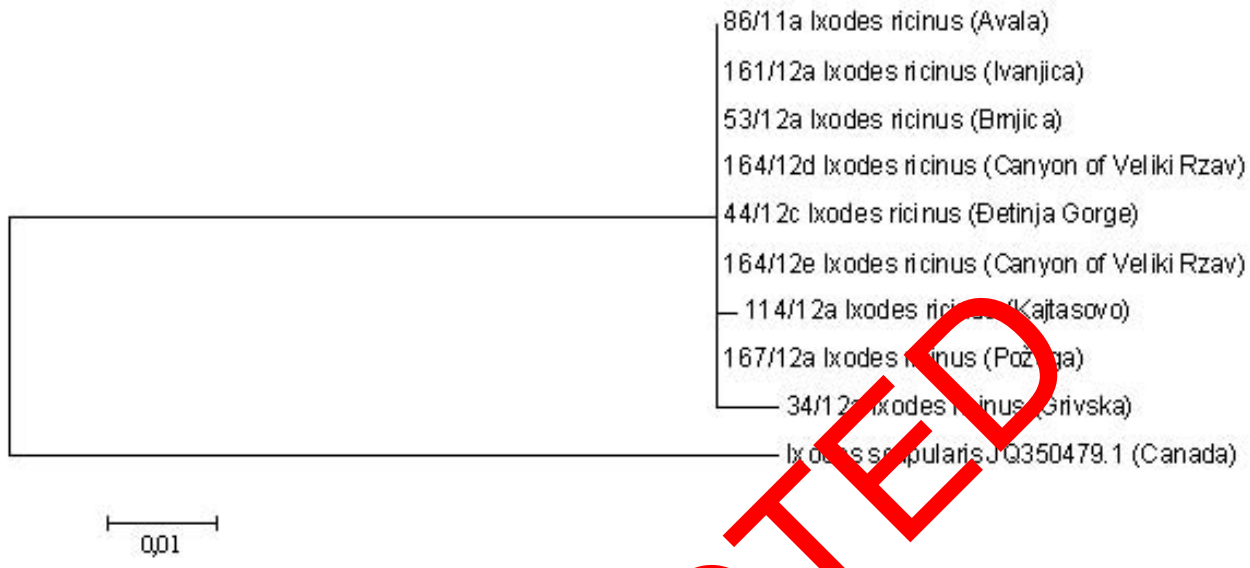

Fig. 2. A – Maximum likelihood tree based on *Ixodes ricinus* COI sequences from Serbia; B – Neighbor-joining tree based on *Ixodes ricinus* COI sequences from Serbia.

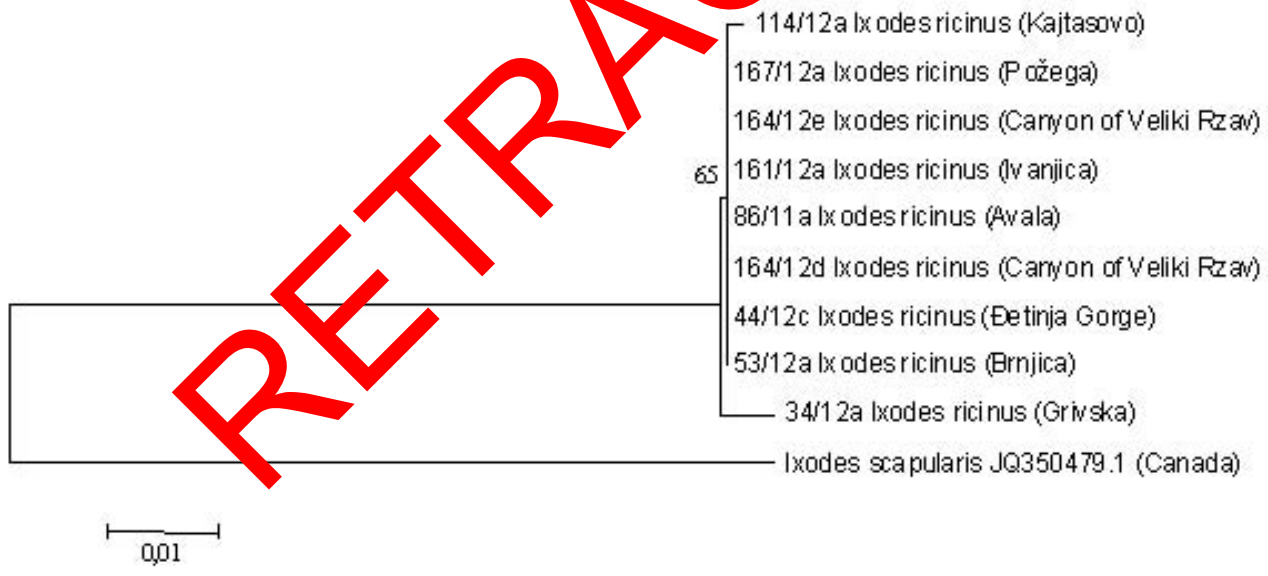

Fig. 3. Maximum likelihood phylogenetic tree based on COI gene sequences of ticks from Serbia and COI sequences taken from GenBank.

DNA amplification and sequencing

For amplification of cytochrome oxidase subunit I gene (COI) universal primers LCO1490 (forward: 5' GGTCAACAAATCATAAAGATA TTGG 3') and HCO2198 (reverse: 5' TAAACTTCAGGGTGACCAAAAAATCA 3') were used (Folmer et al., 1994). PCR reaction was performed with 3 µl of extracted DNA as template. Each 25 µl reaction consisted of 16.37 µl H_2O, 2.5 µl 10 mM $MgCl_2$, 0.5 µl dNTP, 0.13

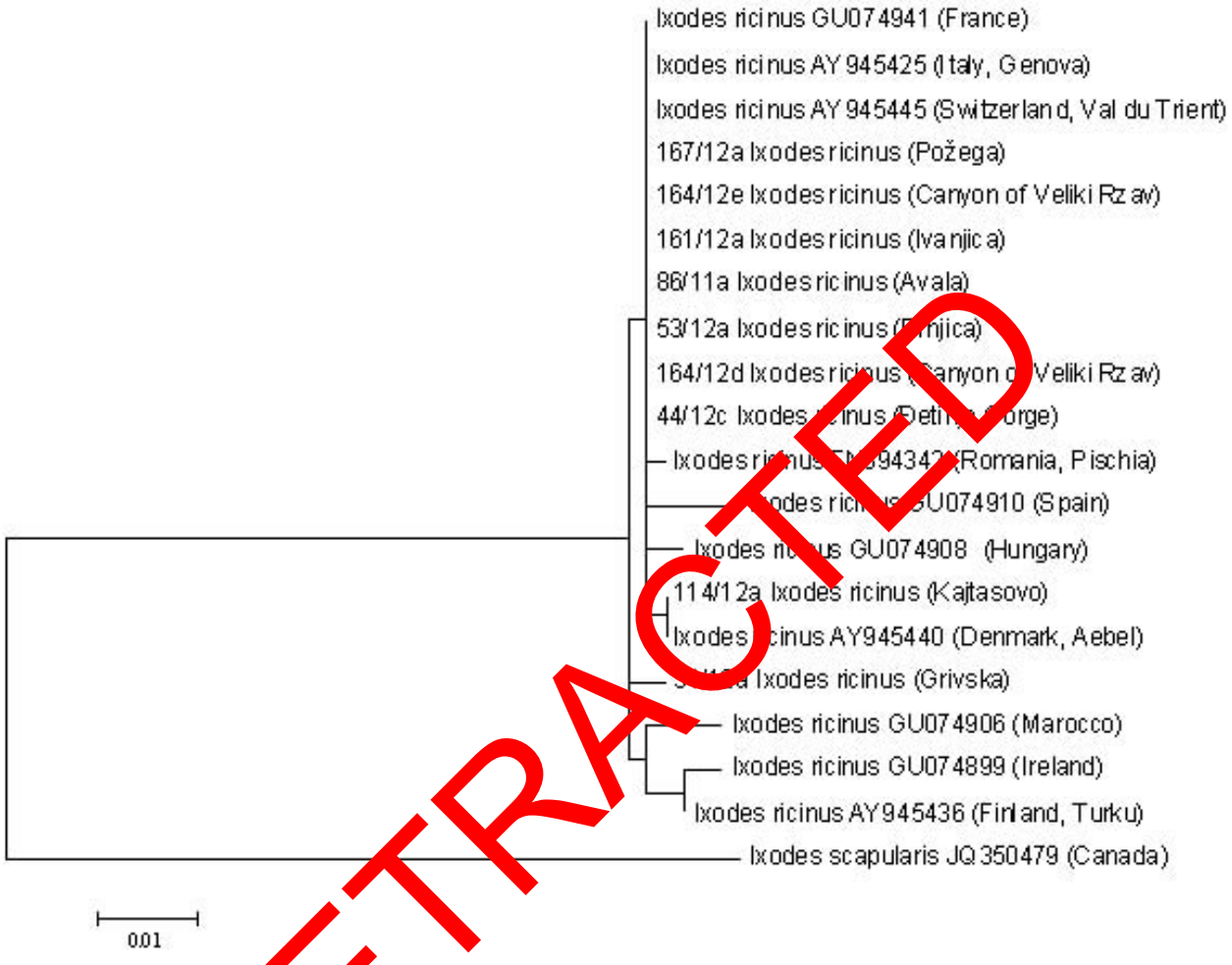

Fig. 3. Maximum likelihood phylogenetic tree based on COI gene sequences of ticks from Serbia and COI sequences taken from the GenBank

μl DreamTaq™ DNA Polymerase (5 u/μl) (Fermentas, Thermo Scientific Inc.) and 1.25 μl of each of the two primers. An initial denaturation at 94 °C for 3 min was followed by 35 cycles (denaturation at 95°C for 1 min, annealing at 49°C for 1 min and extended at 72°C for 1 min) and final extension at 72°C for 10 min. To confirm the efficiency of amplification, products were analyzed by 2 % agarose gel electrophoresis.

Products proven positive after agarose gel electrophoresis underwent sequencing. DNA sequencing, including primer walking, was performed by Macrogen Inc. Amsterdam Netherlands. The representative sequences were deposited in the GenBank database under accession numbers KC809972 to KC809977.

DNA sequences analysis

The standard nucleotide blast tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to align the obtained sequences with mtDNA sequences that were available in the GenBank. Additional alignment was performed using Clastal W Multiple Alignment (Thompson et al., 1994). Preliminary phylogenetic analyses were

Table 1. COI gene (*Ixodes ricinus*) GenBank accession numbers from different geographic origin used for phylogenetic analyses.

GenBank accession number COI	Geographic origin
AY945445	Switzerland (Val du Trient)
GU074941	France
FN394342	Romania (Pischia)
GU074899	Ireland
GU074910	Spain
GU074908	Hungary
GU074906	Marocco
AY945440	Denmark (Aebel)
AY945425	Italy (Genova)
AY945436	Finland (Turku)

performed with MEGA 5.1. (Tamura et al., 2011) using both neighbor-joining (NJ) and maximum likelihood (ML) tree reconstruction methods. For calculation of genetic distances between sequences, we used Kimura's two-parameter method (K2P) of base substitution. In phylogenetic analyses, *Ixodes scapularis* mtDNA COI sequence served as the out group (GenBank JQ350479.1).

RESULTS

A total of 20 unfed adult female *I. ricinus* ticks were chosen for analysis. COI gene sequences were amplified successfully in 13 out of 20 samples. PCR was not successful for the samples from the localities Košutnjak, Titov Gaj, Ivanjica and Virovo. Sequencing of the amplified fragments obtained 13 sequences of approximate length of 700 bp. Based on nine representative sequences of 533 bp length, phylogenetic trees (ML, NJ) were reconstructed (Figs. 2A and B). The average number of nucleotide substitution in the studied sequence of *I. ricinus* species obtained in this study was very low. Sequences 86/11a, 161/12a, 53/12a, 164/12d, 44/12c, 164/12e, 167/12a showed no variation; the 114/12a sequence differs in only one and the 34/12a sequence in three nucleotides. Calculated genetic distances (K2P) between specimens of *I. ricinus* from Serbia were very low (0.00%-0.06%).

One more maximum likelihood phylogenetic tree was reconstructed based on 9 COI gene sequences of ticks from Serbia and 10 COI sequences taken from the GenBank (Table I, Fig. 3). All ticks whose sequences were taken from the GenBank were collected from vegetation in open areas (meadows, pastures and along ecotone). Ticks collected from Požega, Arilje (Veliki Rzav Canyon), Ivanjica, Avala, Banjica and Đetinja Gorge did not show genetic differences in COI sequences compared to ticks from France (Gâvre forest in the Loire-Atlantique), Italy (Genova) and Switzerland (Val du Trient). The *I. ricinus* sequence from Kajtasovo (114/12a) was identical that from Aebel in Denmark, while the *I. ricinus* sequence from Arilje (Grivska) was different from all the other sequences analyzed in this study. Nevertheless, the total genetic variation in the sequence of COI gene was quite low (1.8%).

DISCUSSION

The universal DNA primers, LCO1490 and HCO2198 (Folmer et al., 1994), are frequently used in phylogenetic studies due to the ability to amplify successfully a 710 bp region of the mitochondrial cytochrome oxidase subunit I gene from a broad range of metazoan invertebrates (Folmer et al., 1994; Blanco et al., 2013). Obtained COI gene sequences are the first barcoding sequences of *I. ricinus* ticks collected at localities in Serbia.

The average number of nucleotide substitution among *I. ricinus* COI sequences obtained in this

study was very low. As one of the focal points of our attention, samples from two isolated localities, the Veliki Rzav Canyon and Đetinja Gorge, showed no genetic differentiations from the majority of samples from open areas. This result is in accordance with other studies of De Meeus et al. (2002) that found no genetic differentiation within *I. ricinus* s.s. samples originating from different regions of Switzerland separated by the Alps. Up to this study, single population genetic studies of *I. ricinus* in Serbia have been based on analysis of the polymorphism of genes encoding enzymes involved in the metabolism of carbohydrates and fats (*Gpdh* gene, encoding glicerol-3-phosphate dehydrogenase and *Mdh* gene, encoding malate dehydrogenase). According to our results, these analyses did not reveal significant differences between populations (Radulović, 2005, 2010; Radulović et al., 2006, 2012). In order to broaden the spectrum of analyzed localities and observe eventual relationships between different types of habitat and genetic variation, additional *I. ricinus* COI gene sequences from other European countries were included in phylogenetic analysis. Based on these results and the topology of the obtained phylogenetic tree, we conclude that there is no specific relationship between habitat type and genetic variability. Our results are in accordance with those of Casati et al. (2008). The study of Casati et al. (2008), based on a large set of mitochondrial genes (cytb, 12 S rDNA, COI, COII, and the highly polymorphic control region) provided no evidence of phylogeographic structure among 26 ticks collected from six European countries. This study showed that the number of nucleotide substitutions within tested *I. ricinus* s.s. ticks was low in all the five analyzed mtDNA markers, suggesting that there was no correlation between indentified haplotypes and their geographic origin. The studies based on allozyme data and microsatellite markers have provided similar results (Delaye et al., 1997; De Meeus et al., 2002). The results of Noureddine et al. (2011) clearly show that Eurasian *I. ricinus* populations have low levels of genetic variability (2.63% of the 6 963 sites were polymorphic) with low differences between the three analyzed spatial scales (local, regional and Eurasian scales), and the genetic variability is not spatially structured. Different ecological and species-specific factors can explain the observed low polymorphism level and weak phylogeographic structure of *I. ricinus* populations in Eurasia (Noureddine et al., 2011). The homogeneity among Eurasian *I. ricinus* s. s. complex can been explained by two factors. Ticks exhibit low mobility when they are not attached to the host. Host movements or migrations, especially that of birds, can promote homogeneity among *I. ricinus* s. s. populations. In addition, the absence of genetic structure among the Eurasian *I. ricinus* ticks may be a consequence of a recent rapid expansion of this species. Population expansion increases the retention of new mutations (Watterson, 1982) and creates an excess of haplotypes that only differ by one or a few mutations (Slatkin and Hudson, 1991; Rogers and Harpending, 1992). Xu et al. (2006) considered that the homogeneity among *I. ricinus* complex could be explained by the recent evolution of this group. Based on the low number of substitutions, they hypothesized that the *I. ricinus* complex is the most recently evolved group of ticks in the genus *Ixodes*.

Our study provides the first *I. ricinus* COI barcoding sequences from different localities in Serbia. This work is a first attempt to investigate intraspecific variability in the barcoding COI gene and to determine the phylogenetic relationships among the studied *I. ricinus* ticks. Knowledge about the genetic variability of disease vectors is important for understanding the epidemiology of disease and evolutionary dynamics of disease and vector. There is a need for further research involving different mtDNA markers, such as *cox1* and *nad5* sequences that were shown as useful genetic markers for the specific identification and genetic characterization of ticks in a study by Chitimia et al. (2010).

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