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Detection and genotyping of *Toxoplasma gondii* in wild canids in Serbia

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ABSTRACT

To gain insight into the population structure of the protozoan parasite *Toxoplasma gondii* in wildlife in Serbia, multiplex nested PCR- RFLP was performed on *T. gondii* DNA positive samples of heart tissue of three wild canid species, including red foxes (*Vulpes vulpes*), golden jackals (*Canis aureus*) and grey wolves (*Canis lupus*). Out of 110 samples (28 red foxes, 72 golden jackals, 10 wolves), 30 (27.3%) were positive for *T. gondii* DNA, of which 24 were genotyped (nine red foxes, 13 golden jackals and two wolves). The results showed a large dominance of lineage II (n = 20, 83.4%) over lineage III (n = 4, 16.7%) strains. Lineage II comprised four archetypes, 14 variants and one which remained undetermined, while within lineage III there were three archetypes and one variant strain. However, a notable difference in diversity was observed among the examined canids, with eight (88.9%) of the nine strains detected in red foxes distinct, vs. six (46.1%) distinct strains of the 13 detected in golden jackals. Since anthropogenic food sources are a major reservoir of archetypal lineage II and III *T. gondii* strains, the results suggest that the golden jackal may be outcompeting the red fox, forcing it to hunt wild mammals and birds for sustenance, thereby increasing its chances of infection with variant strains. In addition to presenting the first data on the *T. gondii* population structure in wild canids in Serbia, this is the first report ever on *T. gondii* genotypes in golden jackals, a species with an increasing presence in Europe and another important reservoir of domestically circulating *T. gondii* strains.

1. Introduction

Toxoplasma gondii is a ubiquitous food and waterborne protozoan parasite whose definitive hosts are only Felidae, but is capable of infecting a remarkably wide range of species which serve as intermediate hosts. Sexual replication and subsequent genetic recombination occur only in the intestine of the cat, resulting in the production of highly infectious oocysts, which are shed in the faeces into the environment. In intermediate hosts asexual replication yields clonal progeny which form tissue cysts that can persist for the lifetime of the host mostly in the muscles and the brain [1,2]. Transmission of *T. gondii* occurs primarily through ingestion of tissue cysts in contaminated meat or of oocysts via contaminated water, soil and fresh produce [3,4]. Any animal whose diet includes meat is at risk of infection with tissue cysts, while grazers and ground feeding birds are particularly exposed to infection with oocysts.

The genus *Toxoplasma* features only one species, of which there are a number of different strains. *T. gondii* strains can be identified by biallelic polymorphism at particular loci using multilocus restriction fragment length polymorphism (RFLP). Strains that have identical alleles at all loci are considered archetypes, while those displaying combinations of alleles are considered variants. The majority of strains can be grouped by haplotype, while related haplogroups make up the six major clades of *T. gondii* [5]. Most European and North American *T. gondii* strains belong to one of the three major lineages (types I - III) which occur globally, and one minor lineage (haplotype 12) which occurs in North American wildlife. South and Central America are regions with the greatest strain diversity whereas in contrast, Europe and North America are regions dominated by lineage II strains [5,6]. In Europe, lineage I strains are extremely rare [5,7], while lineage III seems to be more frequent in animals in southern and south-eastern regions [8–11]. Isolates from humans and domestic animals tend to be archetypal strains, whereas isolates from wildlife tend to be variants and recombinants of the three major lineages, or haplotype 12 [12,13]. Consequently, there is a dichotomy between the *T. gondii* strain populations from domestic animals and humans, and wild animals [14,15]. Moreover, Jiang et al. (2018) reported that strain diversity in wildlife decreases as the proximity to human settlements increases, adding more

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evidence to the proposed impact of mankind on the geographical distribution and perhaps even the evolution of genetic diversity and virulence of this parasite [16].

Our group has previously genotyped a number of strains from humans and domestic animals in Serbia [9,11,17–19]. However, our knowledge of genotypes from non-domestic animals is limited to only three *T. gondii* isolates from feral pigeons from the city of Belgrade, two of which were lineage II archetypes and one a lineage III variant [9]. To gain insight into the population structure of *T. gondii* in wildlife in Serbia, we genotyped *T. gondii* DNA detected in three canid species, including the mesopredators golden jackal (*Canis aureus*) and red fox (*Vulpes vulpes*) and the apex predator grey wolf (*Canis lupus*). The distribution ranges of golden jackals and red foxes in Serbia overlap to a great extent across a variety of different habitats, including those close to human settlements. In contrast, grey wolves inhabit remote, densely forested mountainous areas of the country. Therefore, these wild canids exploit different resources of the food-web which allows them to become reservoirs of *T. gondii* strains occurring both in the domestic and in the sylvatic cycles.

2. Materials and methods

2.1. Study area and samples

In collaboration with local hunting organizations, hearts of 72 golden jackals, 28 red foxes and 10 grey wolves ($n = 110$) were obtained. All animals were legally hunted in Serbia between 2013 and 2016. Heart sections were kept frozen at −20 °C until DNA extraction. The locations of the hunting areas from which the animals originated where grouped into five geographical regions (R1-R5, Fig. 1). Thirty-seven samples originated from R1, 43 from R2, 18 from R3, 10 from R4 and five from R5. In terms of human population density, R1 is the most highly populated, since it includes the capital city of Belgrade, followed by R2 and R3, which are similarly populated, while R4 and R5 have the lowest population density.

2.2. Sample preparation and DNA extraction

Total nucleic acids were extracted from a roughly 2 cm by 1 cm strip of heart tissue. The tissues were cut using sterile surgical scissors and placed into 2 ml microfuge tubes pre-filled with 1.4 mm diameter ceramic beads (Omni International, Kennesaw, GA, USA) and 1 ml of Trizol reagent (Thermo Fisher Scientific, Waltham, MA, USA). The tissues were homogenized using a bead beater instrument (Omni international, Kennesaw, GA, USA), set to the maximum (number 5) for 3 min. The level of homogenization was monitored visually. Samples which were insufficiently broken down were subjected to an additional homogenization cycle using identical settings. All samples were successfully homogenized after two cycles. Total DNA was extracted according to the Trizol reagent manufacturer's protocol for DNA extraction.

2.3. Detection of T. gondii DNA by real time qPCR

The *T. gondii* 529 bp repeat element (AF146527) was detected as previously described [19,20]. Briefly, each PCR reaction contained 10 µl of Taqman Universal Master Mix (Applied Biosystems, Foster City, CA, USA), 0.25 mM of each forward and reverse primers (5′-AGA GAC ACC GGA ATG CGA TCT-3′; 3'-CCC TCT TCT CCA CTC TTC AAT TCT-5′), 0.10 mM of the specific TaqMan probe FAM-ACG CTT TCC TCG TGG TGA TGG CG-TAMRA (Invitrogen, Life Technologies, Carlsbad, CA, USA) and 3 µl of extracted gDNA as template in a final reaction volume of 20 µl. The thermal cycling program consisted of the following steps: 5 min at 95 °C for initial denaturation, followed by 45 cycles of 15 s at 95 °C for denaturation and 60 s at 60 °C for annealing/extension. Amplification detection occurred at the end of the 60 °C annealing/extension step. Amplification and detection were performed in a StepOnePlus Real Time PCR System (Applied Biosystems, Foster City, CA, USA).

2.4. Genotyping

Genotyping was performed using multiplex nested RFLP-PCR (Mn-PCR-RFLP) with markers including alt. SAG2, GRA6, BTUB, C-22, PK1,

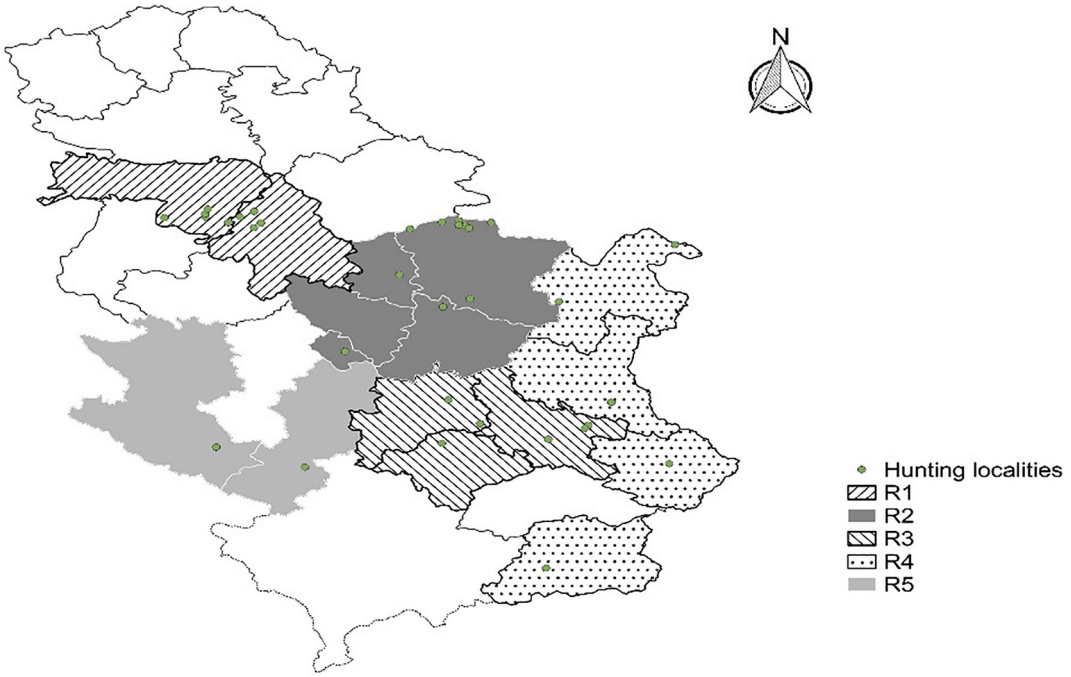

Fig. 1. Map of Serbia showing the hunting localities in the different regions (R1-R5).

CS3, L358, C29–2 and APICO, and reaction mixtures prepared as previously described [21,22]. Briefly, the multiplex PCR reaction contained 10 µl of 2× PCR MasterMix (Thermo Fischer Scientific, Waltham, MA, USA), 0.15 µM of the external forward and reverse primers for each marker and 2 µl of gDNA template. The nested PCR reaction was performed for each marker separately with the same final reaction volume, but using 0.3 µM of each internal forward and reverse primer and 2–4 µl of the multiplex reaction as template. The thermal cycling program was as follows: for the multiplex reaction 3 min at 95 °C for initial denaturation, followed by 30 cycles of 30s at 95 °C, 60s at 55 °C and 60s at 72 °C, and final extension for 5 min at 72 °C. For the nested reaction, initial denaturation and final extension remained the same, but with 35 cycles of 95 °C for 30s, 60 °C for 60s and 72 °C for 60s for all markers except APICO for which the annealing temperature was lowered to 58 °C. PCR products were first checked by electrophoresis in 2.5% agarose gels stained with ethidium bromide and subsequently RFLP was performed using appropriate restriction enzymes for each product. The digestion mixture consisted of 1–2 U of restriction enzyme, 1× Fast Digest (FD) buffer (Thermo Fisher Scientific, Waltham, MA, USA) and 5–7 µl of the Mn-PCR product in a final volume of 25 µl. Strains RH (archetype I), Me49 (archetype II) and NED (archetype III) were used as RFLP standards.

2.5. Statistical analysis

The number of *T. gondii* DNA positive animals according to species and region was analysed by the Chi-square test, with a 0.05 level of significance.

3. Results

T. gondii DNA was detected in 30 (27.3%) of the total of 110 samples (Table 1). Positive animals included 10 of the 28 red foxes (35.7%), 18 of the 72 golden jackals (25%) and two of the ten grey wolves (20%), with no significant differences between species (Chi-square$_{(df=2)}$ = 1.4601, p = .482). Moreover, despite a relatively wide range from 0% (R5) to 37.2% (R2) in the geographical distribution of positive samples (Table 2), the differences observed among the regions were not significant (Chi-square $_{(df=4)}$ = 5.388, p = .250).

Genotyping was attempted for all 30 *T. gondii* DNA positive samples. Most complete RFLP patterns and strain lineage determination are presented in Table 3. In total, 24 samples could be genotyped (nine red fox samples, 13 golden jackal and two grey wolf samples). Twenty of these belonged to lineage II (83.3%) (of which one – 075-16 – was attributed to lineage II for analysis purposes although equal number of type II and type III alleles were detected) and four to lineage III (16.7%). However, only seven were archetypes, of which four of lineage II and three of lineage III, while 16 were variant strains. One strain (126–16) could not be precisely identified as archetype or variant but was considered an archetype because the specific combination of alleles in our experience is indicative of an archetype. Interestingly, even 15 of the variant strains were lineage II, while only one was lineage III (124–16). Lineage I strains were not discovered, however, three strains (fox strains 113–16 and 061–13, as well as the jackal strain 051–16) had one type I allele each.

At the species level, lineage II strains represented 100% of the strain

Table 1
T. gondii strains genotyped per canid species.

Species	No. tested	Tg DNA positive No. (%)	Strains typed No.
Vulpes vulpes	28	10 (35.7%)	9
Canis aureus	72	18 (25%)	13
Canis lupus	10	2 (20%)	2
Total	110	30 (27.3%)	24

Table 2
Distribution of *T. gondii DNA* positive animals and genotyped samples in the examined canids according to geographical region of Serbia.

Species	R1 samples (n)			R2 samples (n)			R3 samples (n)			R4 samples (n)			R5 samples (n)		
	Total	*T.gondiiDNA* +	Genotyped	Total	*T.gondiiDNA* +	Genotyped	Total	*T.gondiiDNA* +	Genotyped	Total	*T.gondiiDNA* +	Genotyped	Total	*T.gondiiDNA* +	Genotyped
Canis aureus	31	6	5	25	10	6	8	1	1	7	2	1	1	0	0
Vulpes vulpes	5	1	1	16	6	5	7	3	3	0	0	0	0	0	0
Canis lupus	0	0	0	1	0	0	2	1	1	3	1	1	4	0	0
Total	36	7	6	42	16	11	17	5	5	10	3	2	5	0	0
Positive	19.4%			38%			29.4%			30%			0%		
Human pop. Density[a]	608/km^2			272/km^2			256/km^2			150/km^2			122/km^2		

[a] The Statistical Office of the Republic of Serbia. Available at: http://www.stat.gov.rs/en-US/oblasti/stanovnistvo, Municipalities and Regions, 2018, http://publikacije.stat.gov.rs/G2018/PdfE/G20181813045.pdf; accessed: 14 May 2019.

Table 3
PCR-RFLP genotypes of *T. gondii* in red foxes, golden jackals and grey wolves. Archetypes of lineage I (RH), lineage II (Me49) and III (NED) were used as RFLP reference strains.

Species, Strain ID, Region (R)	PCR-RFLP markers							Lineage
	Alt. SAG2	BTUB	GRA6	C22-8	PK1	Apico	CS3	
H. sapiens, RH (ref)	I	I	I	I	I	I	I	I, Arch.
O. aries, ME49 (ref)			II	II	II	II	II	II, Arch.
H. sapiens, NED (ref)	III	III	III	III	III	III	III	III, Arch.
V. vulpes 126–16, R3	II	II	II	*na*	II	*na*	*na*	II, *Arch.*[a]
V. vulpes 067–16, R2	III	III	III	III	III	*na*	*na*	III, *Arch.*
V. vulpes 068–16, R2	II	III	II	II	III	II	*na*	II, *Var.*
V. vulpes 103–16, R1	II	III	II	II	II	*na*	II	II, *Var.*
V. vulpes 121–16, R3	II	III	II	III	*na*	*na*	*na*	II, *Var.*
V. vulpes 113–16, R2	II	III	II	*na*	I	*na*	II	II, *Var.*
V. vulpes 061–13, R2	II	II	I	II	III	*na*	III	II, *Var.*
V. vulpes 124–16, R3	III	II	III	III	III	*na*	*na*	III, *Var.*
C. aureus 205–16, R1	II	II	II	II	II	II	II	II, *Arch.*
C. aureus 087–16, R1	II	II	II	II	*na*	II	II	II, *Arch.*
C. aureus 115–16, R2	II	II	II	II	II	*na*	*na*	II, *Arch.*
C. aureus 032–16, R2	III	III	III	III	III	*na*	III	III, *Arch.*
C. aureus 097–16, R4	III	III	III	III	III	III	III	III, *Arch.*
C. aureus 125–16, R3	II	II	II	II	III	III	II	II, *Var.*
C. aureus 056–13, R2	II	III	II	II	III	II	*na*	II, *Var.*
C. aureus 057–16, R1	II	III	III	II	II	II	*na*	II, *Var.*
C. aureus 075–16, R1	II	III	III	II	II	*na*	III	II or III, *Var.*
C. aureus 023–15, R1	II	III	II	II	III	*na*	*na*	II, *Var.*
C. aureus 051–16, R2	II	II	II	III	III	*na*	I	II, *Var.*
C. aureus 061–16, R2	II	III	III	II	II	*na*	*na*	II, *Var.*
C. aureus 062–16, R2	II	III	III	II	II	*na*	*na*	II, *Var.*
C. lupus 020–16, R3	II	III	II	II	III	III	*na*	II, *Var.*
C. lupus 022–16, R4	II	III	II	II	II	*na*	*na*	II, *Var.*

na: not amplified
Arch: Archetype
Var: Variant.
[a] : Uncertain call (archetype vs variant).

population in wolves, 84.6% in jackals and 77.8% in foxes. Of these, archetypes were detected in only 23%, 22% and 0% in jackals, foxes and wolves, respectively, whereas all the other lineage II strains in all three species were variants. Lineage III was only present in red foxes and golden jackals, and only in four samples. Three of these were archetypes, two of which were detected in jackals and one in foxes. The only lineage III variant strain was detected in a fox (124–16). Particularly, the *T. gondii* strain population was remarkably diverse in the red fox, with eight (88.9%) distinct RFLP patterns out of the nine identified, while the diversity was much lower in the golden jackal, with six (46.1%) distinct patterns out of 13. Both strains detected in grey wolves were distinct variants of lineage II. Diversity was observed in all regions in which *T. gondii* was detected, i.e. four out of six strains were distinct in R1, seven out of 11 in R2, four out of five in R3, and both in R4.

4. Discussion

This is the first report on the *T. gondii* population structure in wild canids from Serbia, and moreover, the first report ever regarding molecular detection and genotyping of *T. gondii* in golden jackals. Among the 24 genotyped samples, lineage II strains dominated over lineage III, but the strain populations themselves were also characterized by significant diversity, especially in foxes.

Studies on *T. gondii* infection in foxes in Europe have shown that lineage II strains are dominant and lineage III strains scarce, regardless of geographical origin of the animals [23,24]. Lineage I strains have not been documented in European foxes. The results presented here are comparable in that 78% of the strain population in Serbian foxes was of lineage II, and the rest was of lineage III. One of the strains detected in foxes, 061–13, which had a type I allele at the GRA6 locus, was identical to a strain we isolated from a free-range chicken in 2016, designated K1 (unpublished data). Although the APICO locus did not amplify for 061–13, the allele pattern at the six remaining loci matched K1. Interestingly, both animals originated from the same borough located at the urban-rural fringe on the outskirts of Belgrade (R1). If these strains proved to be identical, that would be strong evidence for the exchange of strains between the domestic and the sylvatic environment in Serbia.

A quarter of the examined golden jackals were positive for *T. gondii* DNA, making them another important reservoir species for *T. gondii* in the wild. The golden jackal has successfully established its populations and is widening its distribution range in Europe thanks in part to its status as an Annex V species [25]. The golden jackal population in Europe has been estimated to range between 97,000 and 117,000 individuals, with large breeding populations living in Bulgaria, Serbia and Hungary [26]. In addition to legal protection, the inherent ability of the jackal to adapt to various habitats and exploit a number of food sources has undoubtedly played a role in the population increase [27,28]. The golden jackal has been found to harbour even 194 parasite species [29], although *T. gondii* has been very poorly studied, and is practically limited to a single seroprevalence report in Iran [30]. The results presented here show that in Serbia, the *T. gondii* strain populations in jackals and foxes are similar in that lineage II dominates over lineage III strains, but with considerable differences in diversity; while nearly all strains in the red foxes had distinct genotypes, less than a half were distinct in the golden jackals. Of these, likely the same variant strain, which has not been previously detected in any other animal or human sample in Serbia, was present in four golden jackals (strain ID's: 057–16, 075–16, 061–16 and 062–16).

Golden jackals and red foxes, both ubiquitous omnivores with a remarkably high overlap in food choices [26], often reside in close proximity to humans and have been known to exploit anthropogenic food sources. The golden jackal is somewhat larger than the red fox and

in the wild, both species feed on small mammals, birds and carrion. All but one strain from domestic animals, and all human isolates from Serbia genotyped to date, were archetypes of either lineage II or III [9,17,19]. The fact that archetypal strains of both lineages were present in jackals and foxes is therefore probably a reflection of their exploitation of anthropogenic food sources. Indeed, an examination of the stomach contents of golden jackals in Serbia confirmed that they frequently contained offal and other waste originating from domestic animal slaughter, which is not surprising for animals known to be opportunistic foragers that exploit any easily available food sources [31]. Also, a study on *Trichinella* spp. in golden jackals showed that almost three quarters were infected with *Trichinella spiralis* and the remainder with the sylvatic *Trichinella britovi* species [31]. Taken together, the results of these studies confirm that the diet of golden jackals in Serbia is indeed very rich in anthropogenic food sources.

What may be surprising is the difference in the strain population diversity observed between red foxes and golden jackals. While both species feed on anthropogenic food sources when available, the high diversity of strains discovered in the red fox in contrast to the low diversity in the golden jackals suggests that golden jackals primarily exploit this food resource, whereas the red foxes do not. One explanation could be that the jackal as the larger mesopredator exerts dominion over anthropogenic food sources, which drives the fox as the smaller mesopredator to sustain itself primarily by hunting wild prey. The diversity of *T. gondii* strains found in foxes thus may be a result of the diversity of *T. gondii* strains in small mammals and birds, which are the fox's primary prey. Indeed, Penezić [2016, Diet of the Golden Jackal (*Canis aureus* L. 1758) in Serbia. Doctoral dissertation, University of Belgrade Faculty of Biology, Belgrade] reported that hunters in Serbia observed a reduction in the number of foxes in areas with a significant presence of jackals. The diversity of *T. gondii* strains in small mammals remains to be explored in future studies.

The grey wolf is one of the few remaining apex predators in Europe which has experienced drastic habitat loss across its distribution range and has been driven to remote areas with few human settlements [32]. In Serbia, the grey wolf population has recently been estimated at 800 individuals, which primarily reside in R4 and R5, and occasionally in R3 [33]. As the apex predator, the wolf's menu is much more extensive, and includes any animal available in the food web, domestic or wild [34]. Of the 10 wolves examined in this study, *T. gondii* has been detected in only two animals, but which harbored distinct genotypes. One of these, the lineage II variant 022–16 (R4), was likely identical to 103–16 (R1) from a red fox, while the other (from R3) was unique. A recent study showed that all *Trichinella* larvae detected in wolves in Serbia belonged to *T. britovi*, indicating that wolves tend to feed on wildlife rather than on domestic animals [33]. What is perhaps interesting however is the low number of positive animals itself. From a geographical perspective, even half of the *T. gondii* DNA negative wolves were from R5, the area with the lowest population density, which features stretches of remote, human and domestic animal free oases for wildlife. It may be speculated that in these oases, where the possible sources of *T. gondii* oocysts may only be wildcats and lynx, both exceedingly rare species in Serbia, the abundance of *T. gondii* oocysts is lower, which in turn leads to fewer infected grazers and small mammals and subsequently to fewer infected carnivores.

In conclusion, this study has shown that the golden jackal, which is quickly migrating north from the Balkans to settle in central and northern Europe, is becoming another important reservoir for *T. gondii* strains which primarily circulate in the domestic environment. The red fox however, at least in Serbia, appears to have shifted towards becoming a reservoir of *T. gondii* strains which circulate sylvatically, similarly to the grey wolf.

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Declaration of Competing Interests

None.

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