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EXON VARIABILITY OF GENE ENCODING GLYCEROL-3-PHOSPHATE DEHYDROGENASE OF *IXODES RICINUS* TICKS[1]

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Summary:

We have previously found apparent differences in *Gpdh* allele frequences between borrelia infected and uninfected *Ixodes ricinus* as revealed by native gel electrophoresis of allozyme polymorphisms. The present study deals with the genetic basis of the observed allozyme polymorphism. Multiple sequence alignment of 36 *Gpdh* open reading frames identified a total of 40 polymorphic nucleotide sites. Of the 40 polymorphic nucleotide sites, 34 were silent (did not result in amino acid residue change), while six were active causing a change in the amino acid chain. All polymorphic amino acid sites were situated within the N-terminal NAD-binding domain, whereas the C-terminal substrate-binding domain was highly conserved. Analysis of the obtained *Gpdh* sequences and GPDH allozyme polymorphisms for individual ticks pointed to amino acid changes at positions 61 (glycine-to-glutamic acid), 64 (serine-to-cysteine) and 102 (glycine-to-arginine) as a key for differential mobility of GPDH allozymes in an electric field. Our findings are discussed in the context of the molecular basis of *I. ricinus* host finding behavior.

KEY WORDS: glycerol-3-phosphate dehydrogenase, variability, *Ixodes ricinus*.

Résumé : POLYMORPHISME DES EXONS DU GÈNE CODANT POUR LA GLYCÉROL-3-PHOSPHATE DÉHYDROGENASE DES TIQUES *IXODES RICINUS*

Nous avions précédemment rapporté après analyse électrophorétique du polymorphisme des allozymes, des différences dans les fréquences alléliques de glycérol-3-phosphate déhydrogenase (GPDH) entre des tiques Ixodes ricinus *infectés ou non par* Borrelia. *Nous avons étudié ici la base génétique de ce polymorphisme. L'alignement des séquences de 36 cadres ouverts de lecture a identifié un total de 40 sites nucléotidiques polymorphes. Parmi ceux ci, 34 étaient silencieux (ne modifiant pas l'acide aminé) alors que la modification de six autres sites entraînait des changements d'acide aminé. Tous les loci polymorphes sont situés dans La partie N-terminale fixant le NAD alors que la partie C-terminale fixant le substrat de la GPDH est très conservée. L'analyse des séquences et du polymorphisme alloenzymatique de la GPDH de tiques individuelles a permis de montrer que les substitutions d'acides aminés en positions 61 (glycine remplacée par acide glutamique), 64 (sérine par cystéine) et 102 (glycine par arginine) étaient des éléments clés pour la mobilité électrophorétique différentielle des allozymes. La base moléculaire du comportement d'*Ixodes ricinus *à la recherche de son hôte est discutée en fonction de nos résultats.*

MOTS CLÉS : *glycérol-3-phosphate dehydrogenase, variabilié génétique,* Ixodes ricinus.

Ixodes ricinus (Linnaeus, 1758), the most common tick species in Europe, serves as a reservoir and a vector of many pathogens (Gray, 2002; Stanek, 2005). Assessment of the risk of contracting diseases transmitted by this species is an integral component in designing and implementing tick borne disease prevention strategies. Information on risk of exposure to Lyme disease and tick-borne encephalitis are common for European countries (Daniel *et al.*, 2006; Milutinović *et al.*, 2008; Jaenson *et al.*, 2009). Knowledge of host-seeking behaviour of *I. ricinus* ticks is very important for determining areas or seasonal periods of risk for the diseases vectored by this ectoparasite. Lees (1948) and Lees & Milne (1951) published a detailed description of *I. ricinus* host-seeking behaviour. Generally, activity of this tick species depends on humidity, temperature and day length (Gray, 1991). However, Healy (1979) indicated that activity variation in response to temperature conditions is a result of glycerol-3-phosphate dehydrogenase (GPDH, EC 1.1.1.8) variability. Electrophoretical analysis showed homodimeric structure of *I. ricinus* GPDH, coded by a single polymorphic gene locus – *Gpdh*. Four *Gpdh* alleles were detected in Irish, Swedish, Swiss and Serbian populations of *I. ricinus* by allozyme electrophoresis (Healy, 1979; Delaye *et al.*, 1997; Healy *et al.*, 2004; Radulović *et al.*, 2006). Due to the inability to detect most of the genetic variability by protein electrophoresis, the purpose of the present study was to investigate the variability of coding regions of *Gpdh* gene in *I. ricinus* ticks by amino acid sequence analysis.

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MATERIALS AND METHODS

A total of 25 adult host-seeking *I. ricinus* ticks, collected by flagging vegetation in the localities of Belgrade area in Serbia, were analysed. Individual live ticks were washed in 70 % ethanol and sterile distilled water, dried on sterile Whatman paper and cut in half. One part of each tick was used for GPDH allozyme genotyping by native polyacrylamide gel electrophoresis, as previously described (Radulović *et al.*, 2006). The other part of individual ticks was used for extraction of total RNA. Each tick part was individually homogenized on ice in 200 µl of Trizol (Chomczynski & Sacchi, 1987) by using UP50H ultrasonic homogenizer (Hielscher Ultrasonics GmbH, Teltow, Germany). Homogenates were incubated at room temperature for 5 minutes, mixed with 40 µl of chloroform and centrifuged at 11,000 × g for 15 minutes at 4 ˚C. Aqueous phase was mixed with 100 µl of isopropanol and incubated at room temperature for 10 minutes. Precipitation of RNA was performed by centrifugation at 11,000 × g for 15 minutes at 4 ˚C. Precipitated total RNA was washed once in 200 µl of 70 % ethanol, air-dried for 5 minutes and dissolved in 10 µl of DEPC-treated water. RNA samples were incubated at 55 ˚C for 10 minutes and stored at -80 ˚C.

The primers for amplification of *I. ricinus Gpdh* gene coding regions (Table I) were designed by using Vector NTI Advance 10 software (Invitrogen, Carlsbard, CA, USA). The basic sequence for primer design was sequence of *Ixodes scapularis* (Say, 1821) *Gpdh* gene (GenBank accession number - XM002434599). Primer sequences were chosen from conserved regions of *Gpdh* gene exons of *I. scapularis* and the following species: *Locusta migratoria* (Linnaeus, 1758) (AF083953), *Apis melifera* (Linnaeus, 1758) (NM001014994), *Aedes aegypti* (Linnaeus, 1762) (CH477221), *Pediculus humanus* (Linnaeus, 1758) (XM002425057), *Gadus morhua* (Linnaeus, 1758) (AY635584), *Xenopus laevis* (Daudin, 1802) (NM001090509), *Taeniopygia guttata* (Vieillot, 1817) (XM002191215) and *Rattus norvegicus* (Berkenhout, 1769) (NM022215).

Coding sequence of *I. ricinus Gpdh* gene was amplified by using following primer pairs: ZR-GPDH-N-F and ZR-GPDH-N-R, ZR-GPDH-913-F and ZR-GPDH-913-R, ZR-GPDH-C-F and ZR-GPDH-C-R. The RT-PCR assay was performed in 50 µl volume comprising 2 µl of RNA sample, 1 µl of each primer (10 µM) and adequate amounts of components of SuperScript III One-Step RT-PCR System with Platinum *Taq* High Fidelity (Invitrogen, Carlsbard, CA, USA). Reverse transcription was carried out for 30 minutes at 50 ˚C and 47 ˚C for ZR-GPDH-913-F and ZR-GPDH-913-R primer pair, followed by an initial PCR activation step for 2 minutes at 94 ˚C and then 40 cycles for 15 seconds at 94 ˚C, 30 seconds of annealing at 47 ˚C (ZR-GPDH-913-F and ZR-GPDH-913-R), 53 ˚C (ZR-GPDH-N-F and ZR-GPDH-N-R) or 57 ˚C (ZR-GPDH-C-F and ZR-GPDH-C-R) and 1 minute at 68 ˚C. A final extension step was performed at 68 ˚C for 7 minutes. The amplified cDNA was purified with a DNA Extraction Kit (Fermentas, Vilnius, Lithuania). Cloning of cDNA fragments was performed by using CloneJET PCR Cloning Kit (Fermentas, Vilnius, Lithuania) and One Shot TOP10 Competent Cells (Invitrogen, Carlsbard, CA, USA). After transformation procedure bacterial cells were spread on agar plates with ampicillin and incubated overnight at 37 ˚C. Analysis of six to eight colonies from each plate, for the presence of adequate DNA insert, was performed by colony PCR. Colonies containing a DNA of interest were inoculated into 1.25 ml of LB broth with ampicillin and incubated for 12 hours at 37 ˚C with shaking at 300 rotation per minute. Plasmid DNA from the culture was extracted using GeneJET Plasmid Miniprep Kit (Fermentas, Vilnius, Lithuania) and then sequenced via the Macrogen service center (Macrogen, Seoul, Korea). Alignment of obtained sequences were performed with the ClustalW algorithm included within the MegaAlign module of the Lasergene software package (DNASTAR, Madison, WI, USA).

Primer name	Sequence 5' → 3'	Sense
ZR-GPDH-N-F	ATGCTTTCTAGGGGATCTGC	Forward
ZR-GPDH-N-R	TTTGTCGGCGACTTCTCTGG	Reverse
ZR-GPDH-913-F	GGATCTGCTATTGCCCGCATC	Forward
ZR-GPDH-913-R	TGTCCAGCATATTCTTGTTCTTGAGC	Reverse
ZR-GPDH-C-F	GTGTCCTGATGGGGGCAAAC	Forward
ZR-GPDH-C-R	ACATGTGTTCTGGGTGATTCTTGA	Reverse

Table I. – Primer sequences used for *Ixodes ricinus* glycerol-3-phosphate dehydrogenase gene coding regions amplification.

RESULTS

A total of 36, 1023 base pairs long, coding sequences of *I. ricinus Gpdh* gene were amplified and sequenced. Nucleotide alignment of obtained sequences showed a presence of 40 polymorphic nucleotide sites (proportion of polymorphic nucleotide sites - $P_n = 0.0391$). Of this number, 34 were silent, while six polymorphic nucleotides changed the amino acid sequence of the enzyme subunit. Representative nucleotide sequences, encoding different amino acid sequences, were submitted to GenBank database (GU363538 – GU363548, Fig. 1). Table II reveals polymorphic sites along the 340 amino acids long sequence of *I. ricinus* GPDH.

GPDH allozyme genotyping showed a presence of three *Gpdh* alleles in analysed ticks, previously designated as F, S and VS (Healy, 1979). Comparison of GPDH genotype and obtained *Gpdh* coding sequences for each individual tick did not show a clear link between amino

acid substitutions and GPDH allozyme mobility in the electric field. Anyway, arginine at position 102 was detected in all sequences originating from ticks with GPDH genotype containing VS allele. Besides, the glutamic acid to glycine at position 61 and serine to cysteine at position 64 changes were detected in six sequences originating from seven ticks with GPDH genotype containing the S allele.

AA position	Consensus AA	Alternative AA
24	Histidine	Arginine
43	Isoleucine	Valine
61	Glutamic acid	Glycine
64	Serine	Cysteine
68	Glutamic acid	Aspartic acid
102	Glycine	Arginine

Table II. – Polymorphic amino acid (AA) sites in *Ixodes ricinus* glycerol-3-phosphate dehydrogenase.

DISCUSSION

Cytoplasmic GPDH catalyses the reversible conversion of glycerol 3-phosphate to dihydroxyacetone phosphate with NAD as a cofactor (Young & Pace, 1958). This reaction constitutes a link between glucose and lipid metabolism, biochemical processes which generate energy. Unfed ixodid ticks dispose with limited quantum of energy reserves, and its efficient utilisation is of great importance for the success of host-seeking activity. Barnes & Laurie-Ahlberg (1986) showed that flight ability in *Drosophila melanogaster* (Meigen, 1830) depends on interactions between GPDH allozymes and environmental temperature. Healy *et al.* (2004) presumed that the processes responsible for tick activity could be essentially similar. In this respect, polymorphism of *Gpdh* locus could represent an adaptation of *I.ricinus* to environmental temperature fluctuations (Healy *et al.*, 2004; Radulović *et al.*, 2006). As expected, the obtained results showed higher level of variability of *I. ricinus Gpdh* gene as compared to that detected by electrophoretic analysis of GPDH allozymes (Healy, 1979; Delaye *et al.*, 1997; Healy *et al.*, 2004; Radulović *et al.*, 2006). The detected proportion of polymorphic nucleotide sites is quite high. Halliburton (2004) cites that the average proportion of polymorphic sites in human genes is about 0.0033. On the other hand, Moriyama & Powell (1996) indicate proportions of polymorphic sites for gene coding regions of *D. melanogaster* (24 loci, P_n = 0.0111), *Drosophila simulans* (Sturtevant, 1919) (12 loci, P_n = 0.0166) and *Drosophila pseudoobscura* (Frolova & Astaurov, 1929) (5 loci, P_n = 0.0363), which are closer to our data. As for *Gpdh* gene, Wells (1996) reported 33 variable nucleotide positions within the 1461 nucleotides assayed for variation in *D. pseudoobscura*. Demonstrated level of nucleotide polymorphism presented in the present study indicates the possibility of using *Gpdh* gene for research of evolutionary history of *I. ricinus* species (Gillespie, 1991). Additionally, tick specimens analysed in the current research originate from a small part of *I. ricinus* areal and obtained results as for *Gpdh* gene variability cannot be considered as final.

It is interesting that polymorphic sites, detected along the *I. ricinus* GPDH amino acid sequence, are situated within N-terminal NAD-binding domain (residues 5-164) consisting of two paired Rossmann folds (Rossmann *et al.*, 1974). C-terminal substrate-binding domain (residues 184-333) is conserved within the sequences studied. Two polymorphic nucleotides and three different genetic codes, observed between 715 and 717 positions of nucleotide sequences (Fig. 1), did not affect the amino acid chain. This may point to the importance of arginine at position 239 for enzyme function. Catalytic domain (residues 193-215) is similar to amino acid sequences of other species GPDH active sites with highly conserved glycines, lysine, asparagine and alanine described by Carmon & MacIntyre (2010).

The results of previous studies, based on *I. ricinus* GPDH allozyme genotyping (Healy *et al.*, 2004; Radulović *et al.*, 2006), suggest that identification of the links between described *Gpdh* alleles and variability of GPDH amino acid sequences could be very important for the further research. An analysis of obtained sequences points that G-to-A change at the position 304 (Fig. 1) is linked with detection of VS allele in investigated ticks. This nucleotide change causes replacement of glycine, the smallest nonpolar amino acid, by larger positively charged arginine at the position 102 in the amino acid chain, resulting in a decreased mobility of this GPDH subunit in an electric field. Electrophoretically detectable differences between F and S alleles seem to result from nucleotide variability at positions 182 and 190 (Fig. 1). Features of residues of variable amino acids (Table II) suggest that F allele could be linked with the presence of negatively charged glutamic acid at the position 61 in amino acid chain. Our results are not completely consistent with this assumption. Variability at the position 64, serine-to-cysteine change, probably additionally affects GPDH allozyme mobility during electrophoresis. This may be a result of protein conformational changes caused by formation of disulfide bonds which include cysteine at the position 64. Amino acids included in changes at the positions 24, 43 and 68 are similar, and most likely do not affect GPDH allozyme mobility in an electric field, but a possible role of these changes in enzyme activity variations may not be excluded.

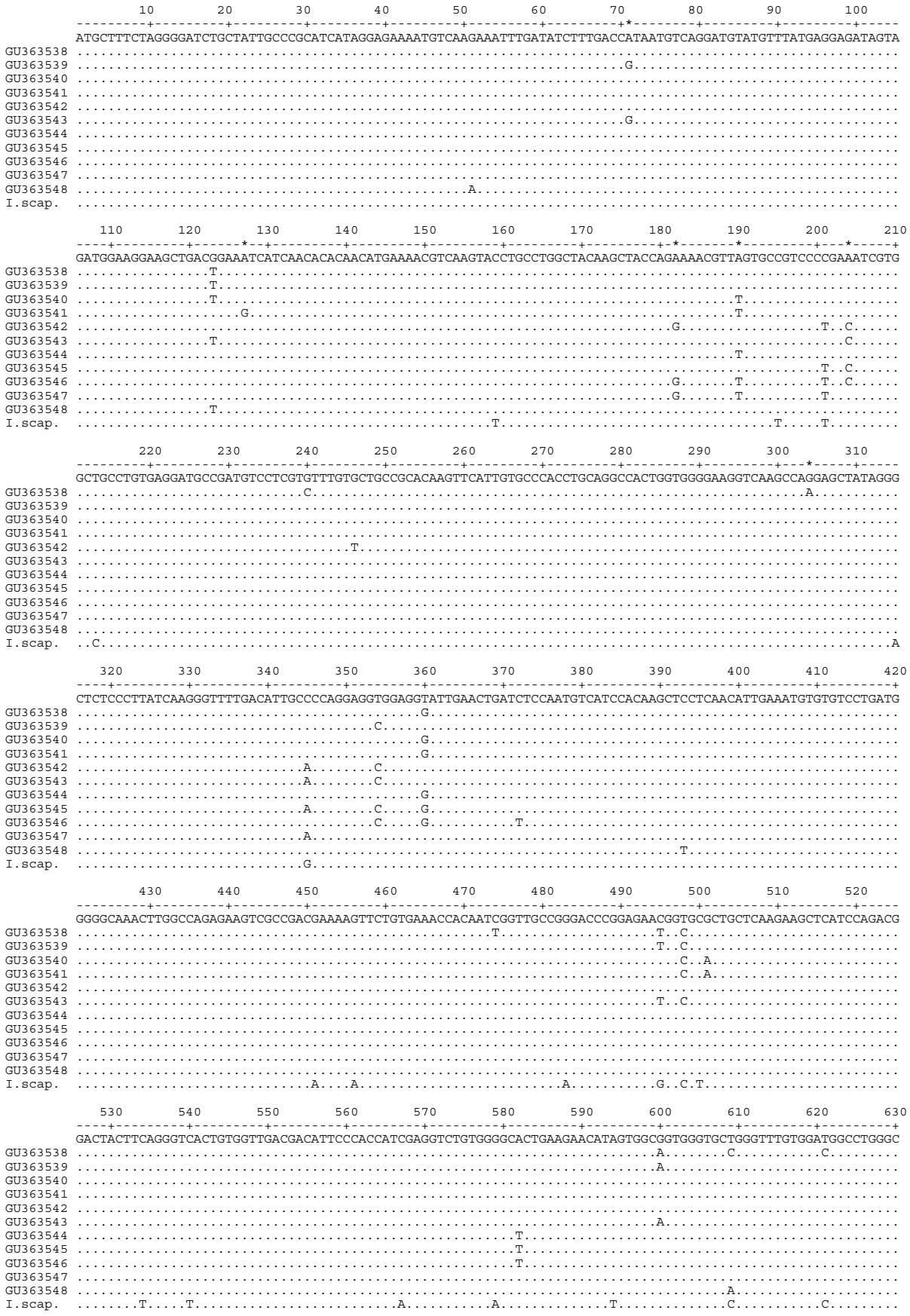

Fig. 1. – (to be continued)

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                 640       650       660       670       680       690       700       710       720       730
         ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+-----
         TACGGGGACAACACGAAGGCGGCAGTGATCCGGCTGGGGCTGATGGAGATGATCGCCTTTGCGGACACCTTCTACCCGGGCGCCCGGCTAGGCACCTTCTTCGAG
GU363538 ..............A............................................C..........................C..G...............
GU363539 ....................................................................................A.A..................
GU363540 .....................................................T...................................................
GU363541 .........................................................................................................
GU363542 .........................................................................................................
GU363543 ....................................................................................A.A..................
GU363544 .........................................................................................................
GU363545 .....................................................T...................................................
GU363546 .....................................................T............................................T......
GU363547 .........................................................................................................
GU363548 ..................................................................................................T......
I.scap.  .......................G...........A.....................................................G...............

            740       750       760       770       780       790       800       810       820       830       840
         ----+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
         AGCTGCGGGGTGGCCGACCTGGTGACCACCTGCTATGGTGGTCGGAACCGCAAGGTGGCCGAGGCCTTCGTCAGGTCCAACAAGTCCATTGAAGAGCTGGAGAGA
GU363538 ...................................C........A..............T.............................................
GU363539 ............................................A............................................................
GU363540 ........A.......................................................................................T........
GU363541 ........A.......................................................................................T........
GU363542 ........A..................................................T.............................................
GU363543 ........A................................................................................................
GU363544 ..............T..........................................................................................
GU363545 ................................................................................................T........
GU363546 ..T......................................................................................................
GU363547 ........A................................................................................................
GU363548 ........A..........................................................................A............T........
I.scap.  ...................................C...................................................................A.

                 850       860       870       880       890       900       910       920       930       940
         ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+-----
         GAAATGCTGAATGGTCAAAAGCTGCAGGGACCAGAGACAGCAGAGGAAGTGTTCACCATGCTCAAGAACAAGAATATGCTGGACAGGTTCCCTTTGTTTGTTGCC
GU363538 .......................C.................................................................................
GU363539 .........................................................................................................
GU363540 .........................................................................................................
GU363541 .........................................................................................................
GU363542 .........................................................................................................
GU363543 .........................................................................................................
GU363544 .........................................................................................................
GU363545 .........................................................................................................
GU363546 .........................................................................................................
GU363547 .........................................................................................................
GU363548 .........................................................................................................
I.scap.  .......................C.................................................................................

            950       960       970       980       990      1000      1010      1020
         ----+---------+---------+---------+---------+---------+---------+---------+---
         ATTCACCGAATATGCAGTCACGAGATTGCACCCAGCGAGATGATACAGTGCATCAAGAATCACCCAGAACACATGTAA
GU363538 ..............................................................................
GU363539 ..............................................................................
GU363540 ..............................................................................
GU363541 ..............................................................................
GU363542 ..............................................................................
GU363543 ..............................................................................
GU363544 ..............................................................................
GU363545 ..............................................................................
GU363546 ..............................................................................
GU363547 ..............................................................................
GU363548 ..............................................................................
I.scap.  ..........................C...................................................

* polymorphic nucleotides associated with changes in amino acid sequence
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Fig. 1. – Alignment of 1,023 bp long coding sequences of *Ixodes ricinus* gene encoding glycerol-3-phosphate dehydrogenase, completed with corresponding sequence of *Ixodes scapularis* (accession number – XM002434599).

It should be noted that primers for sequencing exon regions of *I. ricinus Gpdh* gene were designed on the basis of *I. scapularis Gpdh* gene sequence, which implies a possibility of errors in the obtained nucleotide sequences in the terminal primer binding regions (ZR-GPDH-N-F and ZR-GPDH-C-R). This may be important because C-terminal amino acids (glutamine, asparagine and leucine) in the flight muscle specific isoform of *D. melanogaster* GPDH are necessary for enzymatic activity (Bewley *et al.*, 1989; Sullivan *et al.*, 2003).

Alekseev & Dubinina (2000) presented the influence of temperature on host seeking activity of both *Borrelia*-infected and uninfected *Ixodes persulcatus* (Schulze, 1930) ticks. These results may be associated with the hypothesis of Healy *et al.* (2004) concerning ticks *Gpdh* gene variability and temperature fluctuations, as well as with the findings of Radulović *et al.* (2006) regarding differences in electrophoretically detected *Gpdh* allele frequencies between borreliae-infected and uninfected *I. ricinus* ticks. It may be assumed that spatio-temporal temperature variations determine daily and seasonal distribution of *Gpdh* genotypes in *I. ricinus* populations (Healy *et al.*, 2004). Overlapping of activity periods of the ticks carrying some *Gpdh* genotype and those of their hosts, which are reservoirs of borreliae, might explain differences in *Gpdh* allele frequencies between infected and uninfected ticks. These data may help in establishing correlations between *Gpdh* gene variability, temperature conditions and borreliae infection in ticks.

Our results demonstrate variability in primary structure of *I. ricinus* GPDH. Bearing in mind an adaptive role of GPDH in this tick species, our findings could contribute to the prevention of tick-borne diseases.

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