SHORT COMMUNICATION

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Neoehrlichia mikurensis: an emerging pathogen in Southeastern Poland – prevalence in *Ixodes ricinus* ticks and phylogenetic characterization

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Abstract

Introduction and Objective. *Neoehrlichia mikurensis* is an emerging pathogen increasingly detected in ticks, animals, and humans. The study aimed to investigate the presence and phylogenetic diversity of *Ixodes ricinus* ticks in the Subcarpathian Region.

Materials and Method. Of the 412 ticks collected from forest and meadow habitats, 240 specimens underwent molecular analysis for presence of *N. mikurensis* using microfluidic real-time PCR. Selected amplicons were sequenced to preform phylogenetic analysis of the *groEL* gene.

Results. *N. mikurensis* infection was confirmed in 6.35% of female and 2.38% of male ticks, with no significant differences in the prevalence between habitats. Phylogenetic analysis revealed a low genetic diversity *N. mikurensis*.

Conclusions. The findings confirm the infection of *I. ricinus* ticks with *N. mikurensis* in the studied region. The studied *N. mikurensis* groEL gene sequences showed low genetic variation, identical to other populations in Poland and Europe.

Key words

Ixodes ricinus, ticks, tick-borne diseases, *Neoehrlichia mikurensis*

INTRODUCTION AND OBJECTIVE

Neoehrlichia mikurensis, originally referred to as *Candidatus* Neoehrlichia mikurensis, is a Gram-negative, intracellular bacterium of the family Anaplasmataceae. It was first identified in 2004 in wild *Rattus norvegicus* and *Ixodes ovatus* ticks in Japan [1]. This pathogen is broadly distributed across the Northern Hemisphere, particularly in temperate regions. Unlike other tick-borne pathogens (TBPs), such as *Borreliella* spp., *N. mikurensis* has a relatively limited range of hosts and vectors. [2, 3].

Because of its eco-epidemiology, *N. mikurensis*, similar to *Borrelia miyamotoi*, *Bartonella* spp., and *Orthonairovirus hemorrhagiae* (i.e., Crimean–Congo hemorrhagic fever virus), is classified as an emerging TBP [4]. The first confirmed human case of neoehrlichiosis, occurred in 2007 [5]. Estimating the prevalence and incidence of clinical *N. mikurensis* infections in humans is extremely challenging due to non-specific manifestations, such as febrile episodes, that can last up to several months (i.e. fever of unknown origin – FUO) [6].

In Europe, including Poland, *N. mikurensis* has been increasingly detected in ticks [7, 8]. The aim of the study is to investigate the prevalence and phylogenetic diversity of *N. mikurensis* in *I. ricinus* ticks collected from vegetation in southeastern Poland, an area noted for its high incidence of tick-borne diseases (TBDs) among residents, but previously not studied for the prevalence of the ticks [9].

MATERIALS AND METHOD

Study area. Field studies were conducted in southeastern Poland in the Subcarpathian Province. Three ecologically distinct sites were selected (A, B, and C) for tick collection (Fig. 1, Tab. 1). Sites A and B were in the Western Carpathians (Central Beskids), while Site C was in the Central Beskids Foothills [10].

Tick surveillance. Ticks were collected in June and July 2022, during their peak of seasonal activity [11]. Ticks were sampled using a 1 m² flannel flag dragged over vegetation.

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Figure 1. Study area. Tick collection sites against the background of the Subcarpathian Region, southeast Poland

Table 1. Occurrence and abundance of *Ixodes ricinus* ticks depending on study site and habitat

Tick collection site	No. of active ticks			
	Nymphs	*Females*	*Males*	*Total*
A (Meadow habitat in Central Beskids; located near the village of Chyrowa (49.52° N, 21.60° E)	50	50	35	135
B (Forest habitat of the Central Beskids; located near the village of Ropianka (49.48 °N, 21.60 °E)	66	51	48	165
C (Forest habitat of Central Beskids Foothills; located near the village of Kombornia (49.71 °N, 21.88° E)	24	49	39	112
Total	**140**	**150**	**122**	**412**

Each site was sampled for 30 minutes, and weather conditions (temperature and relative humidity) recorded using a data logger (R6030, Reed Instruments, Wilmington, NC, USA).

Ticks were identified to species, gender, and developmental stage using a taxonomic key [12] and a Zeiss STEMI DV4 stereo microscope (Carl Zeiss Light Microscopy, Göttingen, Germany).

Molecular analysis – DNA extraction and microfluidic real-time PCR. DNA was extracted from randomly selected ticks using the Genomic Mini AX Tissue kit. Isolates underwent preamplification followed by microfluidic real-time PCR for high-throughput pathogen detection, as per established protocol [13]. Conventional PCR was performed to amplify a 1024 bp fragment of the *groEL* gene for validation (NM-1152as: 5′-TTCTACTTTGAACATTTGAAGAATTACTAT-3′; NM-128s: 5′- AACAGGTGAAACACTAGATAAGTCCAT-3′) [14]. Selected amplicons were sequenced and submitted to the GenBank (Accession Nos. OR435839-OR435841).

Additionally, microfluidic real-time PCR was employed for molecular confirmation of tick species targeting primers, described elsewhere [13].

Phylogenetic analysis – Collection of nucleotide sequences. The NCBI BLAST tool [15] was utilized to identify *N. mikurensis groEL* sequences similar to those obtained in this study. Unique sequences were selected by aligning retrieved data using the MAFFT v7 online tool [16], excluding redundant ones.

Phylogenetic tree reconstruction. The *groEL* sequences were aligned using the ClustalW algorithm implemented in MEGA X. The Tamura 3-parameter model (T92) was selected for phylogenetic tree construction based on the lowest Bayesian Information Criterion and corrected Akaike Information Criterion. The evolutionary history was inferred using the Maximum Likelihood method with a bootstrap value of 1,000; analysis was conducted in MEGA X.

Genetic differentiation of studied populations and haplotype network. Haplotype diversity, polymorphic sites, mutations, nucleotide differences (k), nucleotide diversity (π), and Fu and Li's D test were calculated for the populations using DnaSP v5.1 (Universitat de Barcelona, Spain). Tajima's D and Fu's Fs tests were also applied to assess neutrality, alongside shared mutations, nucleotide differences, and genetic differentiation between populations. A haplotype network was generated using the Median Joining Network method in PopArt software (University of Otago, New Zealand).

Statistical analysis. The chi-square test was used to analyze the significance of differences in the prevalence of *N. mikurensis* in *I. ricinus* ticks between studied groups.

The significance level was set at $p < 0.05$. Statistical calculations were performed using GraphPad 8.4 (GraphPad Software Inc., La Jolla, CA, USA).

RESULTS

Occurrence and abundance of *Ixodes ricinus*. In total, 412 *I. ricinus* ticks (140 nymphs, 150 females, and 122 males) were collected across all surveyed sites, with species identity confirmed using molecular testing. The highest activity was recorded in the forest habitat at site B (Ropianka), where 66 nymphs, 51 females, and 48 males were collected (Tab. 1). Molecular analyses were performed on 42 females, 14 males, and 24 nymphs (pooled in groups of 6), randomly selected from each site. Tick activity occurred within a temperature range of 23.0 °C–28.3 °C and relative humidity of 65.3%–88.9%.

Prevalence of *Neoehrlichia mikurensis* in *Ixodes ricinus* ticks. Across the studied area, 6.35% of female and 2.38% of male *I. ricinus* ticks were infected with *N. mikurensis*, while no infections were detected in nymphs (Tab. 2). In the forest habitat (site C), *I. ricinus* females were significantly more frequently infected with *N. mikurensis* than males ($P = 0.0021$). Such differences were not observed between females and males collected from site A ($P = 0.7742$) or site B ($P = 0.1552$). Additionally, there were no significant differences in the prevalence of *N. mikurensis* in *I. ricinus* based on the habitat where ticks were collected ($P = 0.1919$).

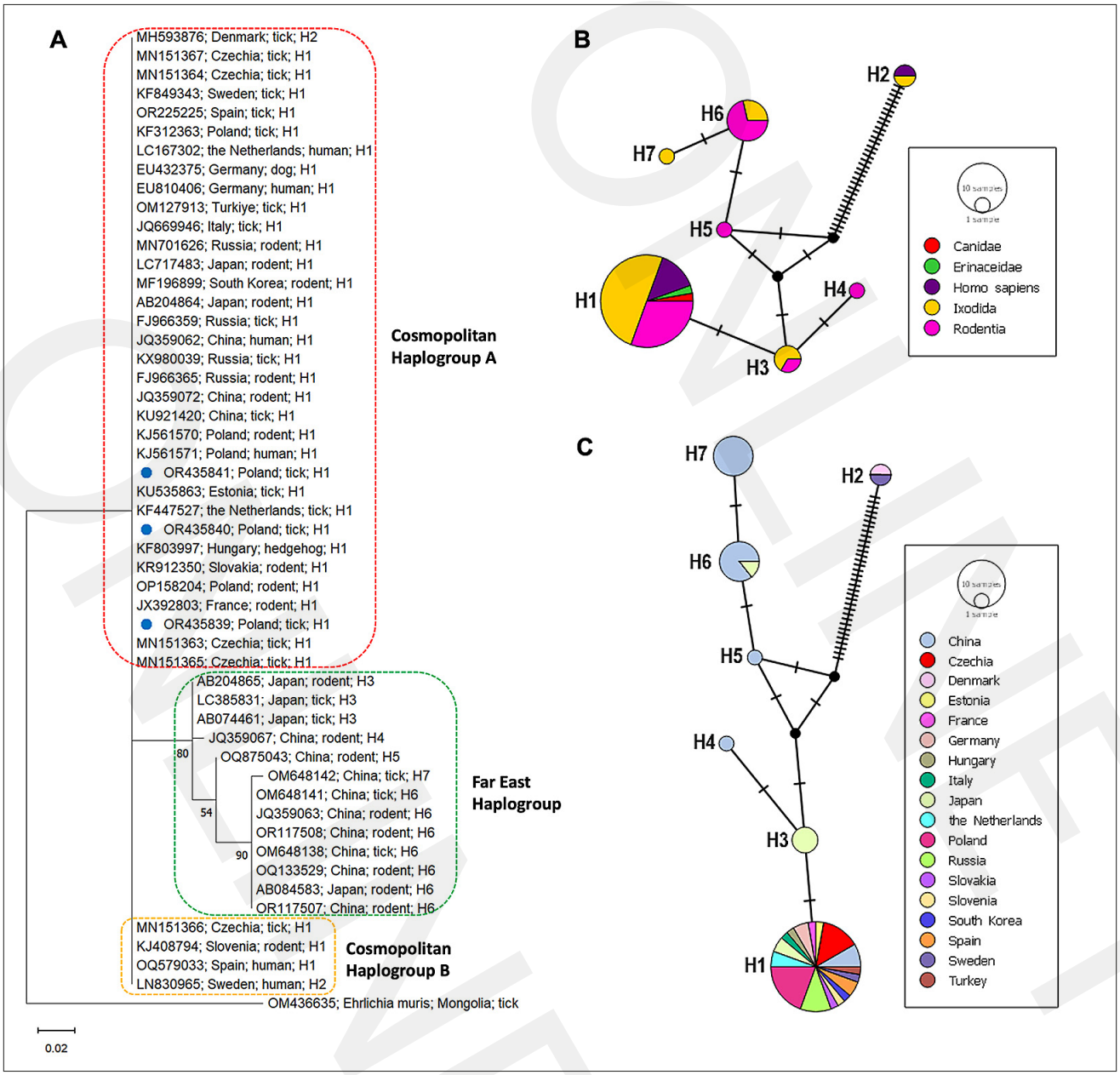

Figure 2. Phylogeny of groEL sequences of Neoehrlichia mikurensis. The evolutionary history was inferred by using the Maximum Likelihood method and Tamura 3-parameter model. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 52 nucleotide sequences. There were a total of 154 positions in the final dataset

Phylogenetic characterization of *Neoehrlichia mikurensis* populations. Phylogenetic analysis of *N. mikurensis groEL* gene sequences revealed low genetic diversity of the pathogen in Europe, identifying two haplotypes (H1-H2). The dominant European haplotype (H1) have also been detected in Japan, South Korea, and China, suggesting its cosmopolitan distribution (Fig, 2). In contrast, the Far Eastern clade contained 5 haplotypes (H3-H7) and showed a positive Tajima's D result (1.581), whereas the cosmopolitan haplogroup (H1-H2) had a negative and statistically significant result (-2.008). Genetic differentiation between clades was also statistically significant (Tab. 3).

Phylogenetic analysis confirmed that hard ticks and rodents are the primary hosts and vectors of *N. mikurensis* (Figure 2). The sequences obtained in the current study were identical to those previously reported from Poland (H1), and showed high similarity to other European sequences (Fig. 2, Tab. 4).

DISCUSSION

Ixodes ricinus is a dominant tick species in Europe, including Poland [12]. The findings of this study confirm its high abundance in the Subcarpathian Region, aligning with previous observations [17]; therefore, the region should be considered a risk for tick bites, and indirectly the transmission of TBPs.

In the studied area, infection with *N. mikurensis* was confirmed in 6.35% of females and 2.38% of males of *I. ricinus* (Table 2). A similar prevalence of *N. mikurensis* in *I. ricinus* collected from vegetation was observed in other

Table 2. Prevalence of *Neoehrlichia mikurensis* in *Ixodes ricinus* ticks collected in the studied area

Tick collection site	Tick stages	Number of positive samples and percentage rate (%)
A (Meadow habitat in Central Beskids)	Females *n* = 42	3 (7.14)
	Males *n* = 14	1 (6.67)
	Nymphs* *pn* = 4	0 (0.00)
B (Forest habitat in Central Beskids)	Females *n* = 42	1 (2.38)
	Males *n* = 14	0 (0.00)
	Nymphs* *pn* = 4	0 (0.00)
C (Forest habitat in Central Beskids Foothills)	Females *n* = 42	4 (9.52)
	Males *n* = 14	0 (0.00)
	Nymphs* *pn* = 4	0 (0.00)
All studied sites	Females *n* = 126	8 (6.35)
	Males *n* = 42	1 (2.38)
	Nymphs* *pn* = 12	0 (0.00)

*Minimum infection rate (MIR %) was applied for pooled samples; *n* - number of tested samples; *pn* - number of pools, each pool size included 6 specimens.

Table 3. Genetic diversity of *groEL* sequences of *Neoehrlichia mikurensis* used to reconstruct phylogenetic tree showed in Figure 2

Trails	Cosmopolitan haplogrups	Far East haplogroup
No. of individuals	38	13
No. of haplotypes	2	5
Haplotype diversity (*Hd*)	0.102	0.692
No. of polymorphic sites	37	5
Total No. of mutations	37	5
Average No. of nucleotide differences (*k*)	3.789	1.744
Nucleotide diversity (π)	0.054	0.025
Tajima's D test	-2.008*	1.581
Fu's test (*Fs*)	11.054	1.941
Fu and Li's D test	1.830*	0.883
No. of shared mutations	2	
Average No. of nucleotide differences between populations (*k*)	3.717	
Genetic differentiation estimates (χ^2)	51.000*	

*statistically significant ($p < 0.05$)

studies in the Central European and Balkan Region, [18, 19] as well as other regions of Poland [3, 7]. Other studies have also shown a similar prevalence of *N. mikurensis* in ticks collected from animals, e.g. in a study by Jahfar et al. [20], 2.7% of *I. ricinus* specimens collected from hedgehogs were infected with this pathogen.

The obtained results are consistent with previously recorded findings and confirm that the most common haplotype

Table 4. Genetic diversity of *groEL* seqences of *Neoehrlichia mikurensis* obtained in the current study against sequences reported from Poland and other countries. Data in the table correspond to the Figure 2

Trails	Current study	Poland	All other countries
No. of individuals	3	4	31
No. of haplotypes	1	1	2
Haplotype diversity (*Hd*)	0.000	0.000	0.125
No. of polymorphic sites	0	0	5
Total No. of mutations	0	0	5
Average No. of nucleotide differences (*k*)	0.000	0.000	4.615
Nucleotide diversity (π)	0.000	0.000	0.065
Tajima's D test	0.000	0.000	1.223
Fu's test (*Fs*)	0.000	0.000	12.093
Fu and Li's D test	0.000	0.000	1.450
No. of shared mutations	0		n/c
	n/c	0	
No. of shared mutations**	0		
Average No. of nucleotide differences between populations (*k*)	0.000		n/c
	n.c	0.000	
Average No. of nucleotide differences between populations (*k*)**	2.810		
Genetic differentation estimates (χ^2)	33.000*		

* statistically significant ($p < 0.05$); **calculations performer between sequences belonged to current study, haplogroup and other reported form Poland; n/c no calculations performed.

of *N. mikurensis* in Europe is H1 (Fig. 2). This haplotype is predominantly most often identified in *I. ricinus* ticks collected from vegetation and animals. This confirms the important role of *I. ricinus* ticks in the transmission of this pathogen. The current study also confirmed the low genetic diversity of *groEL* gene sequences of *N. mikurensis* (Fig. 2, Tab. 3). Significant genetic differences between populations from the European Region and the Far East have also been confirmed by the authors of current study (Fig. 2, Tab. 3, Tab. 4) and in previously published papers [21, 22].

CONCLUSIONS

Ixodes ricinus ticks occurring in the Subcarpathian Region are infected with *N. mikurensis*, which indirectly poses a risk of transmission of this pathogen to humans through tick bites. In terms of genetic variation of the studied *N. mikurensis groEL* gene sequence, the local population showed low diversity and was found to be identical to others from Poland and Europe.

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