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Blood parasites (*Babesia*, *Hepatozoon* and *Trypanosoma*) of rodents, Lithuania: part I. Molecular and traditional microscopy approach

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Abstract

Wild rodents, as natural reservoir hosts carrying various species of pathogens, play an important role in the evolution and emergence of zoonotic diseases. In this study, protist parasites, namely *Babesia* sp., *Trypanosoma* sp. and *Hepatozoon* sp. were studied in rodent populations in Lithuania. Two hundred forty rodent specimens of seven species were analysed by a combined approach using polymerase chain reaction (PCR)-based techniques and traditional microscopic examination. The total prevalence of blood parasites reached 35% in rodent communities. The prevalence of *Hepatozoon* sp. reached the highest value (32%), followed by *Trypanosoma* sp. (5%) and *Babesia* sp. (3%). *Myodes glareolus* and *Microtus agrestis* were the most heavily infected rodent species. Comparison of microscopy and PCR-based methods showed that the two approaches might give different results and thus can lead to an underestimation of the actual prevalence and abundance of parasites. In our study, PCR-based assays were more sensitive and robust than traditional microscopy. However, precise molecular results for the estimation of the prevalence of *Babesia* sp. and *Hepatozoon* sp. were achieved only by using several sets of primers. To avoid inaccurate results, the improvement and detailed description of molecular and microscopy protocols are required.

Keywords Rodents · Blood parasites · *Babesia* · *Hepatozoon* · PCR · Microscopy

Introduction

Significant environmental changes such as major growth in the human population, climate change, intensification of agriculture, and constant human encroachment into wildlife habitats are some of the main contributors to the evolution and emergence of zoonotic diseases. That is why nowadays, more than 1 billion cases of human diseases are assigned as zoonotic and these numbers are rising every year (Karesh et al. 2012).

Most studies concerning zoonotic pathogens are related to humans or domestic animals, but it is extremely important to investigate wild animal populations, which can be the main drivers in the evolution of future zoonotic diseases (Ord and Lobo 2015; Solano-Gallego et al. 2016). Identifying which wildlife species are most likely to serve as reservoirs for possible zoonoses and in which regions new outbreaks are most likely to occur are the most important steps for minimising or eliminating the risk of zoonotic diseases in humans.

Wild small mammals, especially rodents, can carry a variety of pathogens—including viruses, bacteria, protists, helminths and fungi—and serve as a natural reservoir hosts for zoonoses. The dynamics of the transmission of zoonotic diseases depend on the ecology and evolutionary biology of their hosts, and these diseases might be spread when interactions between pathogens, hosts and vectors are completed. Studies should therefore be focused on investigations of the dynamics, distribution and prevalence of zoonotic pathogens in their wildlife reservoirs. Moreover, it is important to identify different parasite communities circulating in these reservoir hosts and evaluate the most appropriate screening methods that allow fast and accurate pathogen identification, because scientific papers describe different methods or protocols, which can lead to a variety of results and interpretations (Baker 1963; Bajer et al. 2001, 2014; Laakkonen et al. 2001; Karbowiak et al. 2005; Maia et al. 2014).

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In this study, we used both microscopy and PCR-based methods to investigate the diversity and prevalence of blood parasites in small mammals, namely the *Babesia*, *Hepatozoon* and *Trypanosoma* genera in Lithuania, and to evaluate the appropriate morphological and molecular protocols for the reliable determination of these parasites.

Materials and methods

The study was carried out in Lithuania in 2016–2017 (August–October). All trapping sites were located in a radius of 15 km in Vilnius District and included various habitats: natural and shrubby meadows, ecotone of bog and natural meadows, deciduous forest, re-growing clear-cutting, and ecotones of natural meadows and arable land (fields of wheat, oats and buckwheat). Rodents were trapped using Sherman live-traps baited with pieces of bread soaked in sunflower oil and bedding material. Live animals were transported to the laboratory and humanely killed by cervical dislocation. The species, sex, age and weight of rodents were identified (Pucek 1981).

Immediately after death, blood was taken from the heart using insulin syringes and three thin blood smears were prepared. Blood smears were fixed in absolute methanol for 4 min. Fixed blood smears were stained using 10% ready-to-use Giemsa solution (Carl Roth GmbH, Germany) with time correction for 1 h 10 min (Valkiūnas 2005). Every smear was examined using an Olympus CX41 light microscope equipped with Imaging MicroPublisher 3.3 RTY digital camera and QCapture Pro 6.0 image analysis system. At least 50 fields were examined at × 400 magnification and 150 fields were examined at × 1000 magnification using immersion oil (Valkiūnas 2005). After initial comparison of results based on microscopy and PCR, additional examination of smears was performed: 50 leucocytes were examined for *Hepatozoon* sp. with special attention to the edges of smears, where a higher concentration of white blood cells was typical; meanwhile, an additional 100 fields were re-examined for PCR-positive *Babesia* sp. samples.

With the use of insulin syringes, blood for molecular analyses was collected in SET buffer (Hellgren et al. 2004) and stored at − 20 °C until further analysis. Total DNA extraction was performed using standard ammonium-acetate protocol (Sambrook et al. 1989). Partial 18s rRNA gene was amplified for all investigated genera. Initially, we used universal primers BTH-1F/BTH-1R for detection of *Hepatozoon* sp., *Babesia* sp. and *Theileria* sp. (Criado-Fornelio et al. 2003a). *Trypanosoma* sp. identification was performed using primers TRY927F/TRY927R and SSU561F/SSU561R (Noyes et al. 1999). All positive samples were sequenced with forward primers. Based on obtained results from molecular and traditional microscopy approach (see details in Results), we used additional primers for identification of *Babesia* sp. with whole dataset (BabFor/BabRev (Blaschitz et al. 2008) and TheiF1/TheiR1, TheiF2/TheiR2 (Heidarpour Bami et al. 2009)). For selected samples, we also used additional primers for *Hepatozoon* sp. (HEPF/HEPR (Inokuma et al. 2002)) and *Babesia* (BT1F/BT1R/BT2R (Criado-Fornelio et al. 2003b), BJ1/BN2 (Casati et al. 2006)). All thermal protocols were used as described by other authors (Noyes et al. 1999; Inokuma et al. 2002; Criado-Fornelio et al. 2003a, 2003b; Casati et al. 2006; Blaschitz et al. 2008; Heidarpour Bami et al. 2009).

Prevalence of parasites (expressed as a percentage of positive samples) was estimated based on PCR (confirmed by sequencing) and microscopy. Intensity was counted in several ways: the number of parasites per 10,000 erythrocytes based on counts in 150 microscopic fields for *Babesia* sp. and *Trypanosoma* sp., and number of parasites in 50 leucocytes for *Hepatozoon* sp.

Fisher's exact test was used to compare prevalence among parasites and observed and expected numbers of mixed infections. A χ^2 test was used to compare sensitivity and specificity of different methods. Statistical analyses were performed using the STATISTICA 7.0 package (StatSoft, Inc. 2004)

Results

Two hundred forty specimens of seven rodent species were trapped between August and October 2016–2017 (Table 1). The highest prevalence of the investigated parasites (*Babesia* sp., *Hepatozoon* sp. and *Trypanosoma* sp.) was recorded in two rodent species, *Myodes glareolus* Schreber, 1780 and *Microtus agrestis* Linnaeus, 1761. Three more rodent species were infected with one (*Apodemus agrarius* Pallas, 1771) or two parasites (*Microtus arvalis* Pallas, 1778, *Apodemus flavicollis* Melchior, 1834). Meanwhile, *Micromys minutus* Pallas, 1771 and *Mus musculus* Linnaeus, 1758 were free of any studied blood parasites. The total prevalence of parasites in *M. glareolus* showed significantly higher values in comparison with any other species (*M. glareolus* × *M. agrestis*, Fisher's exact test: $p < 0.05$, in all other cases $p < 0.001$). The total prevalence of parasites in *M. agrestis* was also significantly higher in comparison with *M. arvalis* ($p < 0.05$), *A. flavicollis* and *A. agrarius* (in both cases, $p < 0.001$). The numbers of *Babesia* sp. in *M. agrestis* was significantly higher than they were in *A. agrarius* ($p < 0.05$) and *M. glareolus* ($p < 0.01$). Among rodents infected by *Trypanosoma* sp., the prevalence in *A. flavicollis* was significantly lower in comparison with *M. agrestis* ($p < 0.01$) and *M. glareolus* ($p < 0.05$, respectively). The numbers of *Hepatozoon* sp. in *M. glareolus* reached the highest values in comparison with *A. flavicollis* ($p < 0.001$), *M. agrestis* and *M. arvalis* (in both cases, $p < 0.05$).

Table 1 Prevalence of blood parasites in small mammals in Lithuania

Host species	Number of tested rodents	Prevalence of parasites (%)			
		Babesia sp.	*Hepatozoon* sp.	*Trypanosoma* sp.	Total
Apodemus agrarius	48	2.1	–	–	2.1
Apodemus flavicollis	77	–	5.12	1.3	6.5
Micromys minutus	6	–	–	–	–
Mus musculus	8	–	–	–	–
Microtus agrestis	10	30.0	10.0	30.0	60.0
Microtus arvalis	11	9.1	9.1	–	9.1
Myodes glareolus	80	1.3	87.5	11.3	90.0
Total	240	2.5	31.7	5.4	35.4

Mixed infections of parasites from different genera were registered in 10 individuals. The most common mixed infection was typical of *Hepatozoon* sp. and *Trypanosoma* sp. (7 cases, all in *M. glareolus*). The other options were *Babesia* sp. + *Hepatozoon* sp. (2 cases in *M. arvalis* and *M. glareolus*) and one case of *Babesia* sp. + *Trypanosoma* sp. (*M. agrestis*). In all cases, observed numbers of mixed infections were similar to expected values and did not differ significantly (in all cases, $p = 0.5$). However, all three investigated blood protists were not registered in one specimen.

The mean intensity of intraerythrocytic *Babesia* sp. was calculated in 10,000 red blood cells in two species of rodents *M. agrestis* and *M. arvalis.* We could not calculate the intensity of *Babesia* sp. in *A. agrarius* and *M. glareolus*, because we did not find any infected erythrocytes in blood smears, and *Babesia* sp. were detected only by PCR. The mean intensity of intraleukocytic *Hepatozoon* sp. was calculated in 50 white blood cells and was the highest in *M. glareolus*. Due to microscopically undetected parasites, the intensity of parasitaemia was not calculated in *A. flavicolis* and *M. arvalis*. *Hepatozoon* sp. parasites in these individuals were confirmed only by PCR. We also calculated the intensity of extracellular *Trypanosoma* sp. to 10,000 erythrocytes. This calculation was performed to unify data due to the variety in quality and thinness of blood smears. The intensity of these blood parasites varied from 3.8 to 37.8 in *M. glareolus* and *A. flavicolis*, respectively (Table 2).

Comparison of microscopy and PCR

Prevalence of parasites based on microscopy and PCR showed different results (Table 3). After the first microscopic examination (counting parasites in 150 fields), total prevalence (presence of any parasite) and prevalence of *Hepatozoon* sp. were significantly higher using PCR-based method ($\chi^2 = 16.88$, $p < 0.0001$ and $\chi 2 = 23.13$, $p < 0.0001$, respectively). The prevalence of *Babesia* sp. did not show significant differences ($\chi 2 = 3.58$, $p < 0.06$), but this could be related to the low prevalence of *Babesia* sp. in this study. The prevalence of *Trypanosoma* sp. was the only case showing identical results based on both PCR and microscopy. Thus, it was decided to re-examine smears showing positive results based on PCR. Additional examination resulted in an increase in the prevalence of *Hepatozoon* sp. up to 60.5%, and *Babesia* sp. up to 66.7% of positive samples by PCR. However, the prevalence of *Hepatozoon* sp. and the total prevalence of all parasites were still significantly higher based on PCR results ($\chi^2 = 9.19$, $p < 0.05$ and $\chi 2 = 7.26$, $p < 0.01$, respectively). Moreover, all positive samples by microscopy were also positive by PCR and no one sample vice versa.

Choice of primers

We used several sets of primers in PCR-based analyses to determine the most accurate markers. For identification of *Hepatozoon* sp., *Babesia* sp. and *Theileria* sp., universal primers BTH-1F/BTH-1R showed positive results (here and later all confirmed by sequencing) for 69 *Hepatozoon* sp. and 3 *Babesia* sp. specimens. No mixed infections between genera were found. Additionally, *Babesia* sp.–specific primers BabFor/BabRev were used for all samples. Three *Babesia* sp. and 76 *Hepatozoon* sp. cases (without any registered mixed infections) were amplified using these primers. Surprisingly, these primers identified seven additional samples of *Hepatozoon* sp. in comparison with BTH-1F/BTH-1R. Two additional PCR repeats with the latter primers for these samples still showed negative results (all these samples showed either low intensity or negative results based on microscopy). Primers TheiF1/TheiR1 and TheiF2/TheiR2 used for nested PCR were tested once more with all datasets. Based on PCR, we amplified 36 positive samples, but sequencing identified only 6 samples positive for *Babesia* sp., and the other sequences were unreadable. These samples were found as positive for *Hepatozoon* sp. with the previously mentioned primers. For further analysis of additional primers (BJ1/BN2 and BTF1/BTR1/BTR2 specific for *Babesia* sp. and HepF/

Table 2 Intensity of *Babesia* sp., *Hepatozoon* sp. and *Trypanosoma* sp. in wild rodents in Lithuania

Host species	Number of tested individuals	Mean intensity of *Babesia* sp. in 10,000 erythrocytes	Mean intensity of *Hepatozoon* sp. in 50 leucocytes	Mean intensity of *Trypanosoma* sp. to 10,000 erythrocytes
Apodemus agrarius	48	NA*	-	-
Apodemus flavicollis	77	-	NA	37.5 ($n = 1$)
Micromys minutus	6	-	-	-
Mus musculus	8	-	-	-
Microtus agrestis	10	2.0 (range 1.2–2.6; $n = 3$)	1.0 ($n = 1$)	4.7 (range 2.0–8.7; $n = 3$)
Microtus arvalis	11	1.2 ($n = 1$)	NA	-
Myodes glareolus	80	NA	7.0 (range 1–39; $n = 46$)	3.8 (range 2.0–6.8; $n = 9$)

*Presence of parasites was confirmed only by PCR

HepR specific for *Hepatozoon* sp.), we decided to analyse 26 samples (6 positive *Babesia* sp. (including 3 mixed infections *Babesia* sp. × *Hepatozoon* sp.), 10 positive *Hepatozoon* sp. and 10 negative *Hepatozoon* sp.). The specific primers for *Babesia* BJ1/BN2 identified all *Babesia* sp. positive samples (identifying only *Babesia* sp. in mixed infections), but 6 samples of *Hepatozoon* sp. also showed positive results. Primers BTF1/BTR1/BTR2 identified only 3 cases of *Babesia* sp. and 7 cases of *Hepatozoon* sp. One more primer set, HepF/HepR, was positive only for *Hepatozoon* sp. (identifying only *Hepatozoon* sp. in mixed infections); however, among positive *Hepatozoon* sp. samples, even 4 samples showed negative results. Thus, based on already published data, we could not find highly sensitive and specific primers providing reliable detection of *Babesia* sp. and *Hepatozoon* sp. parasites in the blood samples of infected animals (Tables 4 and 5).

Only one set of primers was used for *Trypanosoma* sp. detection (TRY927F/TRY927R and SSU561F/SSU561R for nested PCR). Success of amplification for the first and second nested PCR was tested on agarose gels. Results were identical for both PCR rounds, showing strong bands despite variation in the intensity of parasitaemia in different blood samples. Primer TRY927F from the first PCR was used for sequencing in order to get a longer fragment, which is more informative for species identification or phylogeny analysis. We found that use of the first primers set, TRY927F/TRY927R, is sufficient for reliable identification of *Trypanosoma* sp.

Table 3 Number of positive samples (%) based on microscopy and PCR

Parasite	Positive samples for parasites, %		
	Microscopy	Additional microscopy	PCR*
Babesia sp.	16.7	66.7	100
Hepatozoon sp.	40.8	60.5	100
Trypanosoma sp.	100	100	100
Total	52.9	68.2	100

*All positive PCR samples were confirmed by sequencing

Discussion

Nowadays, two methods for investigating small mammal blood parasites are broadly used: traditional light microscopy and polymerase chain reaction (PCR)-based analysis (Nakamoto et al. 2014, Rigó et al. 2016). Both methods have advantages and shortcomings: price (traditional parasitological methods are still cheaper), possibility to estimate the intensity of parasitaemia and sensitivity (PCR-based methods are considered to be more specific than traditional microscopy). The use of these methods to investigate blood parasites faces certain problems such as false negative samples in microscopy due to the low intensity of parasites or the broad range of different primers for PCR with unknown accuracy of amplification.

Since different protocols are used for microscopy, it is difficult to compare results of prevalence or intensity of parasites, for example, different numbers of examined fields (Healing 1981; Turner and Cox 1985; Bajer et al. 2001; Karbowiak et al. 2005, 2009; Beldomenico et al. 2009), duration of examination (Baker 1963, Laakkonen et al. 2007) and different number of examined blood cells (Turner and Cox 1985, Turner 1986, Bajer et al. 2001). Unfortunately, there are many articles with no information about the examination of blood smears (Šebek 1978, Karbowiak and Siński 1996, Duh et al. 2003, Karbowiak et al. 2004, Karbowiak and Wita 2004, Nakamoto et al. 2014, Zábojníková 2017). Moreover, in the case of low intensity of *Hepatozoon* sp. and *Babesia* sp. infections, prevalence might be underestimated, so these differences clearly necessitate a unified and detailed methodology of microscopy for blood parasites of small mammals.

There are few studies providing data on the intensity of infections, even though the evaluation of intensity might provide valuable information. Moreover, studies regarding

Table 4 Sensitivity and specificity of primers amplifying *Hepatozoon* sp. and *Babesia* sp. parasites

Parasite genus	Primers*					
	BTH-1F/ BTH-1R	BabFor/ BabRev	TheiF1/TheiR1, TheiF2/TheiR2	BJ1/BN2	BT-F1/ BTR1/BTR2	HepF/ HepR
Hepatozoon sp.	69 of 76	76 of 76	N	6 of 13	7 of 13	9 of 13
Babesia sp.	3 of 6	3 of 6	6 of 6	6 of 6	3 of 6	0 of 6

*BTH-1F/BTH-1R (Criado-Fornelio et al. 2003a), BabFor/BabRev (Blaschitz et al. 2008), TheiF1/TheiR1, TheiF2/TheiR2 (Heidarpour Bami et al. 2009), BJ1/BN2 (Casati et al. 2006), BT-F1/BTR1/BTR2 (Criado-Fornelio et al. 2003b), HepF/HepR (Inokuma et al. 2002)

information on the intensity of infections are usually incomparable because of differences in methods or lack of accurate description of methods. Bajer et al. (2014) expressed intensity as the number of parasites in 200 microscopic fields at × 1000 magnification for *Hepatozoon* sp. and *Trypanosoma* sp. and number of infected erythrocytes in 200 fields for *Babesia* sp. Beldomenico et al. (2009) suggested dividing the intensity of *Trypanosoma* sp. into 3 groups: not detected, low intensity (up to 90% of the fields with trypomastigotes) and high intensity (more than 90% fields with trypomastigotes); no data was provided on the number of examined fields. In other studies, the intensity of *Babesia* sp. was calculated as a percentage of infected erythrocytes and the intensity for *Hepatozoon* sp. and *Trypanosoma* sp. as a number of parasites per 1000 erythrocytes; however, no detailed description of calculations was provided (Karbowiak et al. 2005, Zábojníková 2017). It should be noted that the quality and thinness of smears might differ significantly depending on the experience, skills, and smear-making technique of laboratory staff (Valkiūnas et al. 2008).

In our study, the prevalence of intraerythrocytic *Babesia* sp. and extracellular *Trypanosoma* sp. was calculated after microscopy of 150 fields, and intensity was expressed as the number of parasites per 10,000 erythrocytes. After initially performing microscopy of 150 fields and comparing that with PCR results, we found that microscopy identified only 41% of *Hepatozoon* sp.–positive samples. Taking this result into account, we performed additional microscopic evaluation to search for intraleucocytic *Hepatozoon* sp. in 50 white blood cells. After the second round of microscopy, we identified 61% *Hepatozoon* sp.–positive samples in comparison with the PCR results. Moreover, all positive samples confirmed by microscopy were also positive by PCR. This indicates that the sensitivity of the PCR method is significantly higher for identifying *Hepatozoon* sp.–positive samples. There are several papers also using microscopy with screening 50 leucocytes for detection of *Hepatozoon* sp., but the authors do not use this data to estimate intensity as suggested in our study (Turner and Cox 1985, Turner 1986, Bajer et al. 2001). Unfortunately, it is impossible to compare results of parasitaemia intensity with other studies.

Different sets of primers are used for the molecular identification of blood parasites. For example, in three recent articles describing *Hepatozoon* sp. infections in small mammals, the authors used various sets of primers to amplify different sites of *Hepatozoon* sp. DNA (Bajer et al. 2014, Hamšikova et al. 2016b, Rigó et al. 2016). A similar situation was observed in *Babesia* sp. studies, where the selection of primers

Table 5 Specificity of frequently used primers to identify *Babesia* sp. in single and mixed with *Hepatozoon* sp. infections

Sample No	Parasite genus*	Primers**						Microscopy
		BTH-1F/ BTH-1R	BabFor/ BabRev	TheiF1/ TheiR1, TheiF2/TheiR2	BJ1/ BN2	BT-F1/ BTR1/ BTR2	HepF/ HepR	
LA126	H, B	H	H	B	B	H	H	H, B
LA127	B	B	B	B	B	B	N	B
LA140	B	B	B	B	B	B	N	B
LA143	B	B	B	B	B	B	N	B
LA452	H, B	H	H	B	B	H	H	N
LA466	B	N	N	B	B	N	N	N

**H*, *Hepatozoon* sp.; *B*, *Babesia* sp.; *N*, negative

**BTH-1F/BTH-1R (Criado-Fornelio et al. 2003a), BabFor/BabRev (Blaschitz et al. 2008), TheiF1/TheiR1, TheiF2/TheiR2 (Heidarpour Bami et al. 2009), BJ1/BN2 (Casati et al. 2006), BT-F1/BTR1/BTR2 (Criado-Fornelio et al. 2003b), HepF/HepR (Inokuma et al. 2002)

differs substantially (Duh et al. 2003, Beck et al. 2011, Kallio et al. 2014, Hamšikova et al. 2016a). On the contrary, the same set of primers was used in different studies for molecular identification of *Trypanosoma* sp. parasites (Noyes et al. 2002, Smith et al. 2005).

For molecular investigations, we used six different sets of primers, and our results clearly showed that the specificity and sensitivity of these primers used for detection and differentiation of genera of blood protozoans remains controversial. Special attention should be focused to mixed infections of *Babesia* sp. and *Hepatozoon* sp. parasites, which are prevalent and common in natural populations of small mammals, because the universal primers BTH-1F/BTH-1R for piroplasmids and *Hepatozoon* (Criado-Fornelio et al. 2003a) did not show any mixed infections confirmed by microscopy. Primers selectively amplified *Hepatozoon* sp. parasites and underestimated the prevalence of *Babesia* sp. in natural mixed infections of both parasites. Amplification of *Hepatozoon* sp. DNA with *Babesia* sp. specific primers (BabFor/BAbRev (Blaschitz et al. 2008), BJ/BN2 (Casati et al. 2006)) are mentioned by several authors (Oyamada et al. 2005, Najm et al. 2014, Azmi et al. 2016). Surprisingly, the amplification with BabFor/BabRev primers performed by Oyamada et al. (2005) showed different lengths of amplified products on agarose gel for *Babesia* sp. and *Hepatozoon* sp., and these results enabled to distinguish parasites using only the PCR technique. In our study however, amplification results checked on agarose gel showed identical lengths of fragments, with no possibility to identify parasites at the level of genus.

Many authors now suggest the use of qPCR as the most sensitive and specific method to provide information not only about the presence of blood parasites but also about intensity of infection, especially for parasites related to human or domestic animal diseases (*Hepatozoon* sp. of mammalian carnivores: Criado-Fornelio et al. 2007, Li et al. 2008; *Hepatozoon* sp. of lizards: Maia et al. 2014; *Trypanosoma cruzi*: Piron et al. 2007; *Babesia microti*: Hojgaard et al. 2014, Souza et al. 2016). There is, however, no data on applying these methods to parasites of rodents. It is important to test the specificity and sensitivity of qPCR on blood samples of rodents before applying this method to the screening and identification of blood parasites in small mammals.

Based on combined molecular and microscopic data, the total prevalence of all investigated blood parasites reached 35.4% in rodent communities in Lithuania. However, only two rodent species, *M. glareolus* and *M. agrestis*, were heavily infected (90.0% and 60.0%, respectively). *Hepatozoon* sp. dominated in *M. glareolus*, whereas *Trypanosoma* sp. and *Babesia* sp. were common parasites in *M. agrestis*. The prevalence of *Hepatozoon* sp. in *M. glareolus* revealed the highest value in comparison with many studies across Europe (Turner and Cox 1985, Turner 1986, Laakkonen et al. 2001, Karbowiak et al. 2005, Gimenez et al. 2009, Bajer et al. 2014, Rigo et al. 2016, Zábojníková 2017). The prevalence of *Hepatozoon* sp. in *A. flavicollis*, *M. agrestis* and *M. arvalis* also showed high values; moreover, in many studies, *Hepatozoon* sp. was not detected in these species at all (Baker 1963, Healing 1981, Turner 1986, Hamšikova et al. 2016b, Laakkonen et al. 2001). Based on the results of molecular and traditional microscopy methods, we can assume that the prevalence of wild rodent *Hepatozoon* sp. infections in other European localities might also be higher than was previously reported. However, the prevalence of *Babesia* sp. infections in *A. agrarius*, *M. agrestis*, *M. arvalis* and *M. glareolus* corresponded to other European countries (Baker 1963, Healing 1981, Turner and Cox 1985, Bajer et al. 2001, Duh et al. 2003, Karbowiak 2004, Karbowiak et al. 2009, Beck et al. 2011, Kallio et al. 2014, Obiegala et al. 2015, Hamšikova et al. 2016a). The prevalence of *Trypanosoma* sp. was also in agreement with numerous studies (Šebek 1978, Karbowiak and Siński 1996, Bajer et al. 2001, 2014, Noyes et al. 2002, Karbowiak and Wita 2004, Smith et al. 2005, Beldomenico et al. 2009). We did not however find *Trypanosoma* sp. in *A. agrarius* in contrast to a relatively high prevalence in Slovakia (Karbowiak et al. 2009).

A comparison of microscopic and molecular results revealed the advantages and shortcomings of both methods. For example, the sensitivity of traditional microscopy decreases due to low intensity of parasitaemia, and high sensitivity of PCR-based methods might be achieved only by using several sets of primers. Moreover, current primers cannot discriminate between the different genera of rodent blood parasites. Our results showed the necessity for genus-specific primers for *Babesia* sp. and *Hepatozoon* sp. when sequencing is not required, especially in long-term or large-scale studies. High variation in the use of primers provides different results, and there is no best choice of genus- or species-specific primers for blood parasites of small mammals at the moment.

The importance of studies of communities of wild small mammals as reservoirs of potentially dangerous zoonotic pathogens is undoubtedly necessary for a better understanding of the mechanisms underlying the evolution of novel diseases. Avoiding inaccuracies in results and improving molecular protocols is therefore crucial.

In the second part of the article, we intend to show the challenges related to the linkage between morphometrical measurements and genetic lineages identifying species of wild rodent blood parasites belonging to genera *Babesia*, *Hepatozoon* and *Trypanosoma*.

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Compliance with ethical standards

The research was conducted according to Lithuanian laws. The use of wild mammals for research is regulated by the Republic of Lithuania Law on Wildlife (June 22, 2010, No. XI-920) and the Order of the Director of the State Food and Veterinary Service (June 30, 2011, No. D1–533/B1–310; as last amended on April 24, 2014, No. D1–369/B1–380). According to the current procedure, no permit was required to perform investigations in the current study. Investigated species are murine rodents which are not classified as protected species in accordance with the Republic of Lithuania Law on Protected Animals, Plants and Mushrooms (Order of the Director of the State Food and Veterinary Service: Chapter III, Article 50.2).

Conflict of interest The authors declare that they have no conflict of interest.

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