

ORIGINAL ARTICLE

High prevalence of *Trypanosoma theileri*-like trypanosomes and scarcity of monoxenous trypanosomatids in tabanids of Northwestern Russia

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Summary

The hematophagous flies of the family Tabanidae are worldwide-distributed nuisance pests and at the same time vectors of various diseases for humans and livestock, including trypanosomiases. No large-scale studies dedicated to trypanosomatid infections in tabanids have been performed so far in Russia and this work intended to close this gap. Using light microscopy and molecular analyses, we screened 1180 individuals (26 species, 6 genera) of female tabanids collected in Northwestern Federal District. Our results demonstrated a high prevalence of trypanosomes (43%) belonging to the two major lineages of the *Trypanosoma theileri* species complex (TthI and TthII) and only a few cases of monoxenous trypanosomatids' presence. We performed statistical assessment of the obtained data and revealed differential prevalence of the two trypanosome lineages in different tabanid taxa. We also described the temporal dynamics of infections in tabanid populations: early start of trypanosome acquisition with the steady growth of overall prevalence during the season, but distinct trajectories for the prevalence of the two trypanosome haplotypes. We provided a detailed explanation of the observed results based on the available data on the biology of tabanids and proposed hypotheses concerning the reasons for distribution of trypanosomes in the peripheral blood of the ruminant hosts and trypanosome specificity.

Key words: horseflies, deerflies, *Trypanosoma theileri* group, vector

Introduction

Tabanidae are a worldwide distributed family of bloodsucking true flies that encompasses over 4500 described species, many of which represent

important pests for humans and livestock (Mullens, 2019; Bánki et al., 2023). The insistent attacks of a large number of female tabanids lead to significant economic losses, since affected animals lose weight and decrease milk production because of

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the stress provoked by painful bites, blood loss, and skin intoxication by saliva (Chainey, 1993). In addition, tabanids serve as mechanical and biological vectors of various infections, including filariases, as well as bacterial (tularemia, anthrax, etc.), viral (e.g., tick-borne encephalitis), and protozoan diseases (Baldacchino et al., 2014). Among the latter, trypanosomiasis occupy a special place. *Trypanosoma brucei evansi*, the agent of the fatal disease of domestic animals called surra, is predominantly transmitted by tabanids (Desquesnes et al., 2013). Although nagana (African Animal trypanosomiasis) caused by a number of related trypanosomes, such as *T. b. brucei*, *T. congolense*, *T. simiae*, and *T. vivax*, is a tsetse-transmitted disease, tabanids can also sometimes act as mechanical vectors of it (Desquesnes et al., 2013; Taioe et al., 2017; Abah et al., 2020).

One more group of trypanosomes vectored by tabanids is the *Trypanosoma theileri* species complex, which includes multiple formally described species, such as *Trypanosoma theileri*, *T. melophagium*, and *T. cervi* (Hoare, 1972). These parasites are usually regarded as nonpathogenic and therefore have been virtually neglected. However, occasionally they can cause death of infected fetuses and newborn calves or cause mild-to-severe symptoms in adult cattle (Doherty et al., 1993; Braun et al., 2002; Matsumoto et al., 2011; Sood et al., 2011; Hajihassani et al., 2020; Sukanuma et al., 2022). *Trypanosoma theileri*-like trypanosomes parasitize a wide range of ruminants (deer, sheep, cattle, antelopes, etc.), while various hematophagous dipterans (apart from tabanids, these are keds, tsetse flies, mosquitoes, and sandflies) represent known or implicated vectors (Hoare, 1972; Böse et al., 1987a, 1987b; Böse and Petersen, 1991; Votýpka et al., 2015; Calzolari et al., 2018; Werszko et al., 2020a; Brotánková et al., 2022). Molecular studies demonstrated that this group is highly diverse and that the same hosts can be infected by unrelated genotypes, which are distributed between the two major lineages – Tth I and Tth II (Rodrigues et al., 2006; Rodrigues et al., 2010; Jaimes-Dueñez et al., 2018; Sukanuma et al., 2019). In addition, one more lineage (Tth III) has been recently discovered in mosquitoes (Brotánková et al., 2022). The majority of species descriptions relied on the “one host – one parasite” paradigm, since they were made in the pre-molecular epoch. This prevents correlation of specific names and obtained sequences, with the exception of *T. melophagium* (which has a distinct host and vector – sheep and sheep ked, respectively

(Hoare, 1972)), as well as the recently described *T. trinaperronei* (Garcia et al., 2020). Therefore, a thorough taxonomic revision of this species complex is warranted (Kostygov et al., 2022a).

Data on the distribution and prevalence of *Trypanosoma theileri*-like trypanosomes in tabanid vectors are very scarce, with just a handful of studies published so far and mostly based on a limited material (Votýpka et al., 2015, 2019; Ganyukova et al., 2018; Werszko et al., 2020b; Kostygov et al., 2022a). In this work, we conducted the first large-scale multifaceted study of trypanosomatid infections in tabanids of Russia, focused on the differential prevalence of *T. theileri*-like trypanosomes associated with different tabanid taxa, geographic areas, and certain periods within summer season.

Material and methods

INSECT COLLECTION AND DISSECTION

A total of 1180 female tabanids were collected in 2021 from June to the end of July in four regions of the Northwestern Federal District of Russia: Republic of Karelia (RK), Leningrad Oblast (including St. Petersburg, LO), as well as Novgorod and Pskov Oblasts (NO and PO, respectively, Table 1). The captured insects were kept in separate plastic vials with a piece of wet cotton. Within 24 h after capture, the insects were euthanized with chloroform and dissected in a drop of normal saline. Their intestine was isolated, while the body was fixed in 96% ethanol for the subsequent morphological identification. For 711 tabanids from PO and 23 from LO, the isolated intestines were directly placed for subsequent DNA isolation into the conservation solution containing 2% SDS, 0.1 M EDTA, and 10 mM Tris. In the remaining cases, the intestinal tube was placed on a microscopic slide with a drop of normal saline, covered with a coverslip and examined under a light microscope with a 40× objective for the presence of trypanosomatids. For positive samples, the coverslip was carefully removed and the infected parts of the intestine were placed into the conservation solution as above. In addition, small parts of the intestine were preserved for the preparation of smears.

INSECT COLLECTION AND DISSECTION

Total genomic DNA was isolated from intestinal fragments of infected tabanids using the PureLink

Table 1. Collection localities.

Location	Coordinates	N
Republic of Karelia (RK)		
Matryonin peninsula, Loukhsky District	66°11' N, 33°23' E	28
Podpakhta inlet, Loukhsky District	66°11' N, 33°22' E	27
Sredniy island, Loukhsky District	66°17' N, 33°39' E	46
Total		101
Leningrad Oblast (+ St Petersburg)		
Sviritsa village, Volkhovsky District	60°28' N, 32°55' E	42
Bugry village, Vsevolozhsky District	60°05' N, 30°24' E	2
Kameshki village, Vsevolozhsky District	60°14' N, 30°03' E	8
Toksovskoye village, Vsevolozhsky District	60°09' N, 30°35' E	53
Ozerki village, Vyborgsky District	60°12' N, 29°01' E	11
Hepojarvi lake, Vsevolozhsky District	60°09' N, 30°34' E	7
Kobralovo village, Gatchinsky District	59°35' N, 30°23' E	8
Solka-4 allotment, Kingiseppsky District	59°28' N, 28°28' E	33
Sestroretsk town, Kurortny District	60°05' N, 30°02' E	22
Staraya Sloboda village, Lodeynopolsky District	60°47' N, 33°19' E	17
Total		203
Novgorod Oblast		
Oksochi village, Malovishersky District	58°40' N, 32°48' E.	130
Total		130
Pskov Oblast		
Gornyevo village, Pskovsky District	57°48' N, 28°27' E	138
Molodi village, Strugo-Krasnensky District	58°01' N, 28°42' E	581
Palitsy village, Strugo-Krasnensky District	58°02' N, 28°40' E	27
Total		746

Genomic DNA Mini Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. This DNA was used to specifically amplify the ~830 bp long fragment of the trypanosomatid 18S rRNA gene (variable regions V8, V3, V4, and V9) with the primer pair 1127F and 1958R (Ganyukova et al., 2020a) that were also used for sequencing of the obtained PCR products. The identification of species was performed using BLAST searches against the non-redundant nucleotide database of GenBank. The coinfections by two trypanosome genotypes were detected by thorough inspection of sequence chromatograms for the presence of double peaks at the variable sites. The new sequences obtained in this study were deposited in GenBank under accession numbers (PP447933-PP447939).

PHYLOGENETIC ANALYSES

The dataset of the obtained 18S rRNA gene sequences was reduced to seven unique haplotypes and combined with a representative set of 102

sequences of this gene retrieved from GenBank. The sequences were aligned in MAFFT v. 7.520 with the E-INS-i algorithm (Kato and Standley, 2013). The resulting alignment was trimmed in trimAl v. 1.2 (Capella-Gutiérrez et al., 2009) by applying the 0.2 gap threshold. The maximum likelihood phylogenetic tree was inferred in IQ-TREE v. 2.2.2.6 (Minh et al., 2020) with automatic model selection and branch support estimated using the ultrafast bootstrap method with 1000 replicates (Minh et al., 2013).

STATISTICAL ANALYSIS

The following statistics were estimated in this work: prevalence – the percentage of insects infected by a particular type of parasites among all individuals, the proportion of tabanids infected by a particular parasite species among all infected individuals, and the dominate index for an individual tabanid species – the percentage of individuals of this species among all tabanid individuals. One-

Table 2. Trypanosomatid prevalence in tabanid genera.

	<i>Hybomitra</i>	<i>Haematopota</i>	<i>Tabanus</i>	<i>Chrysops</i>	<i>Heptatoma</i>	<i>Atylotus</i>	Total
# of species	11	2	7	4	1	1	26
# of specimens	634	223	212	100	8	3	1180
total infected	332 (52.4%)	41 (18.4%)	116 (54.7%)	29 (29.0%)	2 (25.0%)	1 (33.3%)	521 (44.2%)
monoxenous single	6 (0.9%)	1 (0.4%)	1 (0.5%)	2 (2.0%)			10 (0.85%)
irresolvable mixes	5 (0.8%)	2 (0.9%)	1 (0.5%)	2 (2%)			10 (0.85%)
Tth I single	95 (15.0%)	5 (2.2%)	28 (13.2%)	11 (11.0%)			139 (11.8%)
Tth II single	12 (1.9%)	5 (2.2%)	42 (19.8%)	4 (4.0%)	1 (12.5%)		64 (5.4%)
Tth I + Tth II	214 (33.8%)	28 (12.6%)	44 (20.8%)	10 (10.0%)	1 (12.5%)	1 (33.3%)	298 (25.3%)

way ANOSIM, cluster analysis (Bray-Curtis), principal components analysis, and linear regression tests were performed using PAST 4.08 software (Hammer et al., 2001). The correlation index for a nonlinear model was calculated manually using the previously described method (Foerster and Renz, 1983). The significance of differences between proportions was calculated using the “N-1” Chi-square test (Campbell, 2007).

Results

GENERAL CHARACTERIZATION OF INFECTIONS AND MOLECULAR PHYLOGENETIC ANALYSIS

The insects collected in this work belonged to six tabanid genera, of which *Hybomitra*, *Haematopota*, *Tabanus*, and *Chrysops* were the most abundant with 100–634 specimens (from 2–11 species) per genus, while *Heptatoma* and *Atylotus* were represented by a few individuals (Table 2, Fig. 1). Our microscopic and/or molecular analyses detected trypanosomatid infections in 521 insects (44%). Among the genera with a large number of collected specimens, the highest prevalence was documented in *Tabanus* (55%) and *Hybomitra* (52%), while significantly ($p \leq 10^{-4}$) lower infection rates were documented in *Chrysops* spp. (28%) and *Haematopota* spp. (18%). The prevalence estimates for the genera *Heptatoma* and *Atylotus* (25 and 33 %, respectively) could not be considered reliable due to the low number of available specimens (Table 2, Fig. 1).

Analysis of the trypanosomatid 18S rRNA gene sequences obtained from infected tabanids allowed

identification of parasites up to a species or a genus in all cases except ten, where irresolvable mixed signals were obtained. Most infections were those by two haplotypes of the TthI and TthII clades of the *Trypanosoma theileri* species complex. Of note, the high similarity of the two haplotypes allowed their unambiguous identification in the Tth I + Tth II mixes. More specifically, we detected monoinfections by TthI in 139 specimens (11.8%), monoinfections by TthII in 64 specimens (5.4%), and the Tth I + Tth II mixture in 298 specimens (25.3%). These two haplotypes matched the sequences of the putative species Tth α and Tth β that we documented in the same region in our previous studies (Ganyukova et al., 2018; Kostygov et al., 2022a) and likely represent the same two species. However, considering the multiplicity of haplotypes in the *T. theileri* species complex, the high similarity of sequences within each major clade, and the lower resolution power of the short fragment amplified in this work compared to the full-length 18S rRNA gene (836 bp vs ~2.1 kb), it is rather possible that each of the two haplotypes corresponds to more than one trypanosome species. To avoid confusion, we decided to refer to the two haplotypes simply as Tth I and Tth II.

In addition to trypanosomes, there were 10 cases of infection by single monoxenous trypanosomatid species (0.85%, see below). Considering the quite large overall sample size, the presence of only (or predominantly) *T. theileri*-like trypanosomes and monoxenous trypanosomatids in the resolvable cases, we hypothesized that the irresolvable mixes contain at least one haplotype/species from each of the monoxenous and Tth (i.e. Tth I, Tth II or

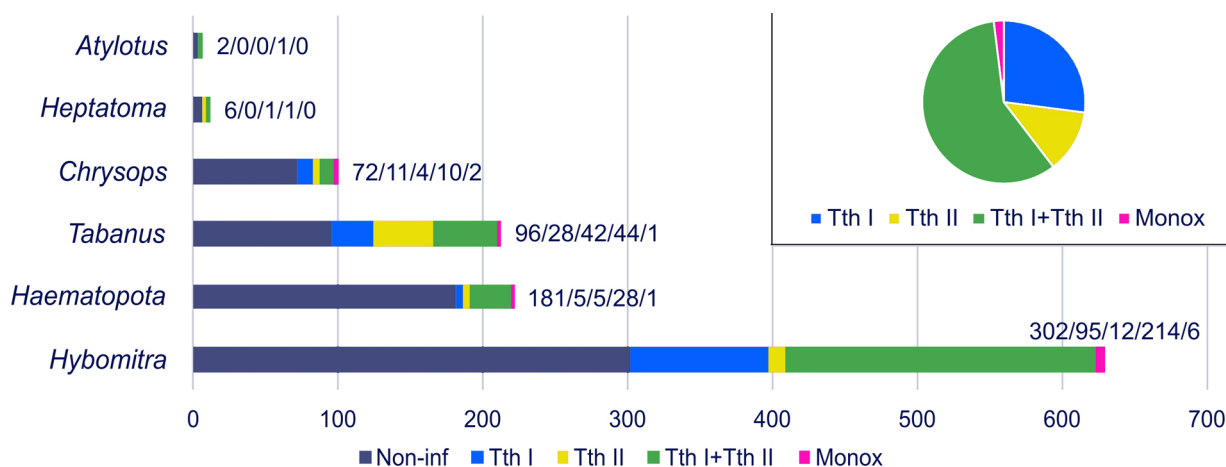


Fig. 1. Counts of trypanosomatid infections detected in tabanids from six genera. The pie chart (inset) shows the overall proportions for the main categories of infections. Irresolvable mixes are excluded.

both) groups. To test this hypothesis, we calculated presumable prevalence values for both groups as 1.69% (20/1180, single monoxenous + irresolvable mixes) and 43.3% (511/1180, all Tth + irresolvable mixes), respectively. For the mixture of these two groups, we estimated the prevalence as 0.73% (the product of the first two values), which is very close to the observed prevalence for the irresolvable mixes (0.85%) with a statistically largely insignificant difference ($p=0.7$). This is in line with our hypothesis and the assumption that the two groups of trypanosomatids infect tabanids independently. However, when we applied the same procedure to compare the observed prevalence of Tth I + Tth II mixes (25.3%) and the one predicted assuming the independent distribution of the two haplotypes (11.4%), the resulting values proved to be substantially different ($p < 10^{-4}$). Therefore, in the case of trypanosomes, the assumption of the independence is certainly wrong.

The rare infections by a single monoxenous species of trypanosomatids were detected in all four abundant tabanid genera with a prevalence of 0.4–2.0% (Table 2 and Fig.1). Expectedly, most of these infections (seven out of ten) were found in the most intensively sampled region – Pskov Oblast (Table 3). Monoxenous trypanosomatids were detected in seven species: *Hybomitra muehlfeldi*, *Hy. lundbecki*, *Hy. tarandina*, *Chrysops viduatus*, *C. caecutiens*, *Tabanus maculicornis* and *Haematopota pluvialis*, but all, except for the first one, were single cases (Table 3). *Crithidia brevicula*, a parasite with a quite wide documented host range (Kostygov et al., 2014, 2022b; Svobodová et al., 2015; Ganyukova et al., 2020b) was the most prevalent monoxenous

trypanosomatid with four of ten documented cases (Table 3). It was followed by an undescribed species of the genus *Obscuromonas*, previously reported from the biting midge *Culicoides kibunensis* (Kazak et al., 2023) and now revealed in three tabanid individuals from three different genera (Table 3, Fig. 2). Among the remaining three monoxenous species, one was associated with *Blastocrithidia nonstop*, a true bug parasite (Kachale et al., 2023), whereas the other two were previously unrecorded. These were a long branch-producing species of the genus *Herpetomonas* and a trypanosomatid, which could be tentatively assigned to *Lafontella* along with its close relative previously found in the fruit fly *Drosophila ananassae* (Chandler and James, 2013) (Table 3, Fig. 2).

CHARACTERIZATION OF TRYPANOSOME INFECTIONS IN TABANIDS AT THE LEVEL OF GENERA

In horseflies of the genera *Hybomitra* and *Haematopota*, cases of Tth I + Tth II co-infections (prevalence: 33.8 and 12.6 %, respectively) were significantly more frequent than it could be expected assuming the independent distribution of the two haplotypes (17.4% and 2.2, respectively; p -values: $>1 \times 10^{-6}$ and 2.8×10^{-5}). Individual proportions of these haplotypes were different: in *Hybomitra* spp., Tth I obviously prevailed over Tth II (total prevalence: 48.7 and 35.6%, respectively; p -value: 2×10^{-6}), while in horseflies of the genus *Haematopota* the ratio of haplotype frequencies was exactly 1:1 (14.8% prevalence in both cases). It is noteworthy that more or less the same overall picture was observed at the level of individual species, except for

Table 3. Monoxenous trypanosomatids in tabanids (to supplement).

Species	Isolate	Region	Host
<i>Crithidia brevicula</i>	A1395	Pskov Obl.	<i>Hybomitra muehlfeldi</i>
	A1437		
	F21249	Novgorod Obl.	
	A1377	Pskov Obl.	<i>Chrysops viduatus</i>
<i>Obscuromonas</i> sp.	A1165	Pskov Obl.	<i>Hybomitra lundbecki</i>
	A1204		<i>Tabanus maculicornis</i>
	A1206		<i>Chrysops caecutiens</i>
<i>Blastocrithidia nonstop</i>	M1026	Leningrad Obl.	<i>Hybomitra tarandina</i>
<i>Herpetomonas</i> sp.	F21269	Novgorod Obl.	<i>Haematopota pluvialis</i>
<i>Lafontella</i> sp.	A1098	Pskov Obl.	<i>Hybomitra muehlfeldi</i>

Hy. lapponica and *Hy. lurida*, for which sampling was too scarce (four and two specimens, respectively; Suppl. Table 1).

For deerflies of the genus *Chrysops*, the total prevalence of Tth I and Tth II haplotypes differed 1.5-fold (21 and 14%, respectively), but this difference was not statistically significant (p -value: 0.19; $N=100$). However, the prevalence of Tth I + Tth II coinfections was much higher than expected with a marginal statistical support (2.9%; p -value: 0.04; Suppl. Table 1). The situation in *Tabanus* spp. was the opposite: Tth II total prevalence was higher than that of Tth I (40.6 vs 34%; p -value: 0.012). Of note, this difference was highly significant for one of the individual species, *T. bromius* (11.4 and 50.0%, respectively; p -value: 1×10^{-6}). Despite the expected frequency of coinfections was lower than the observed one (13.8 and 20.8, respectively), this difference did not receive sufficient statistical support (p -value: 0.057). Although infections by *T. theileri*-like trypanosomes were detected in *Atylotus* and *Heptatoma*, the small sample sizes (3 and 8 specimens, respectively) did not allow for reliable interpretation of the observations (Suppl. Table 1).

TRYPANOSOME INFECTIONS IN TABANID SPECIES GROUPED BY DOMINANCE

Based on the dominance index (DI), the tabanids studied here were divided into four groups: i) dominant species ($DI > 10$, $n=3$): *Hy. bimaculata*, *Hy. muehlfeldi* and *Ha. pluvialis*; ii) subdominant species ($2.0 < DI < 10$, $n=12$): *Hy. distinguenda*, *Hy. nitidifrons confiformis*, *Hy. lundbecki*, *Hy. solstitialis*, *Hy. tarandina*, *Hy. arpadi*, *T. maculicornis*, *T. bromius*, *T. sudeticus*, *C. caecutiens*, *C. viduatus*, and *Ha. subcylindrical*; iii) minor species ($0.5 < DI < 2.0$): *Hy. kaurii*, *He. pellucens*, *T. cordiger*, *T.*

bovinus, *C. divaricatus*, and *C. relictus*; and iv) rare species ($DI < 0.5$): *Hy. lapponica*, *A. fulvus*, *Hy. lurida*, *T. glaucopsis*, and *T. miki*. Comparison of these groups showed a high variation of prevalence values for the individual trypanosome haplotypes and for their coinfections (Suppl. Table 2). Therefore, as expected, one-way ANOSIM did not reveal statistically significant differences ($R=0.07$, $p=0.02$) between the groups of dominant, subdominant and minor species (rare species were not included in the analysis due to the small sample size), suggesting that there was no relationship between the abundance of tabanid species and infection rates.

CLUSTER ANALYSIS AND PCA

We performed an attempt to classify the studied tabanid species based on prevalence parameters. To have enough data for such analyses, we considered only those species that were represented by 30 or more specimens (Table 4). For convenience, we designated the species with a total prevalence below the average (i.e., 44.2%) as rarely infected and that above the average as frequently infected.

First, we applied the hierarchical clustering algorithm using the Bray-Kurtis coefficient (Fig. 3, A). The results of this analysis demonstrated the subdivision of the total pool into four groups, with the main contribution by total trypanosomatid prevalence and that of Tth I + Tth II coinfections. The most divergent group was represented by *Chrysops caecutiens* and *Ha. pluvialis*, characterized by an extremely low percentage of infected individuals (8.7 and 15%, respectively). They were followed by *Tabanus bromius*, which had relatively high overall prevalence (52.9%), but a very low one for trypanosome coinfections (8.6%). Another group of rarely infected (25.7–32.3%) was represented by *Ha.*

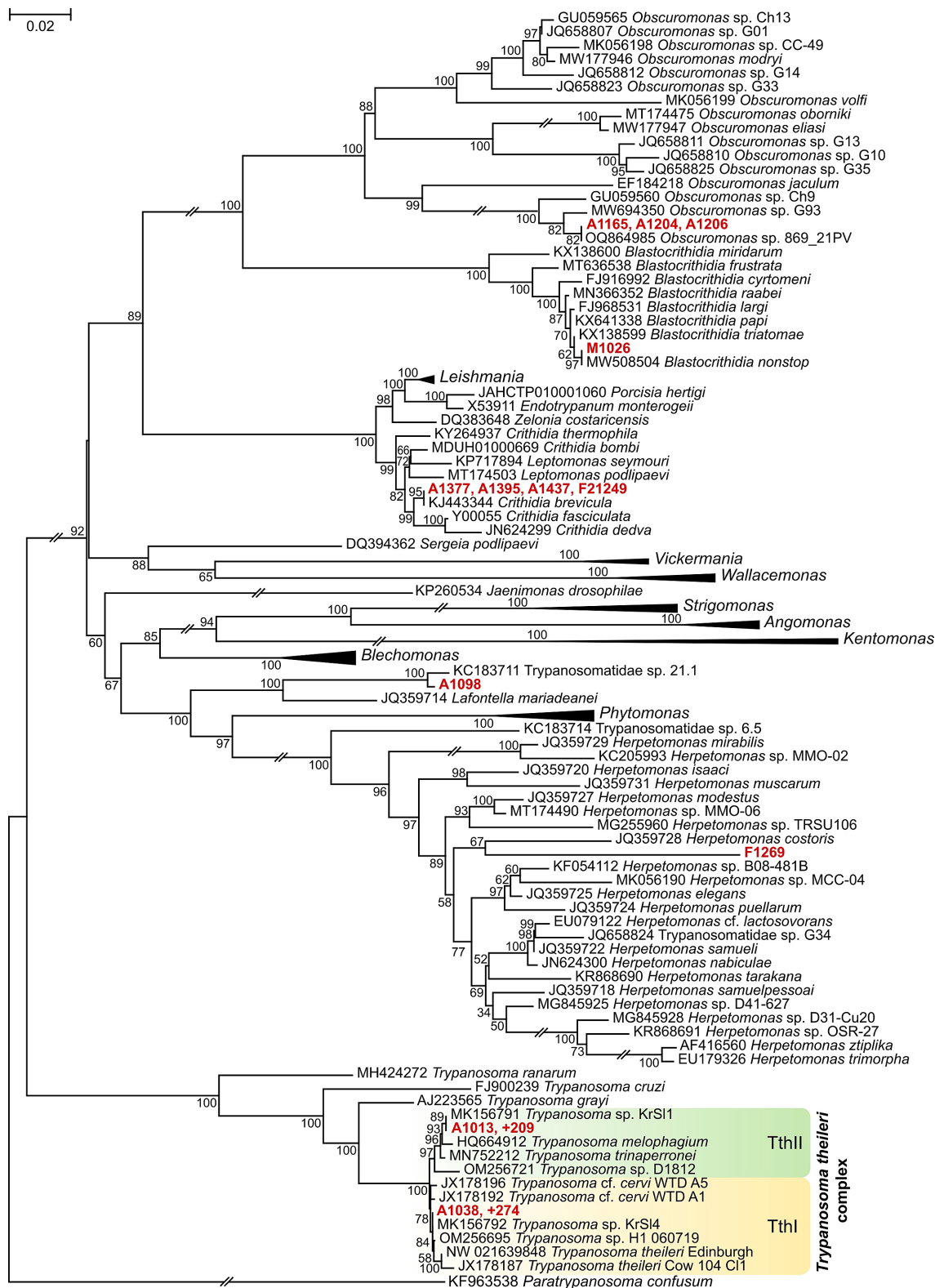


Fig. 2. Maximum likelihood phylogenetic tree of Trypanosomatidae based on the 18S rRNA gene and demonstrating the position of the isolates obtained in this work (shown in red). The clades for genera not containing such sequences are collapsed. The two major clades of the *Trypanosoma theileri* species complex are highlighted. The numbers at the nodes are ultrafast bootstrap supports. The scale bar corresponds to the number of substitutions per site.

Table 4. Infection statistics for individual tabanid species*.

Tabanid species	N	Total prevalence	Prevalence*				
			Mono-xenous	Tth I	Tth II	Tth I/II mix	Irresolvable mixes
Rarely infected species							
<i>Haematopota pluvialis</i>	153	15%	0.04	0.13	0.13	0.65	0.04
<i>Chrysops caecutiens</i>	46	9%	0.25	0.50	0.25	0	0
<i>Haematopota subcylindrica</i>	69	26%	0	0.11	0.11	0.78	0.06
<i>Hybomitra arpadi</i>	31	32%	0	0.30	0	0.60	0.1
<i>Hybomitra tarandina</i>	44	27%	0.08	0.33	0.08	0.42	0.08
Frequently infected species							
<i>Hybomitra muehlfeldi</i>	127	57%	0.06	0.38	0.01	0.56	0
<i>Hybomitra distinguenda</i>	79	61%	0	0.44	0.04	0.50	0.02
<i>Hybomitra solstitialis</i>	44	66%	0	0.41	0.03	0.55	0
<i>Tabanus sudeticus</i>	30	50%	0	0.33	0.07	0.60	0
<i>Hybomitra nitidifrons confiformis</i>	74	68%	0	0.12	0.04	0.80	0.04
<i>Hybomitra lundbecki</i>	45	62%	0.04	0.18	0.04	0.75	0
<i>Hybomitra bimaculata</i>	171	45%	0	0.21	0.04	0.75	0
<i>Tabanus maculicornis</i>	78	47%	0.03	0.30	0.22	0.43	0.03
<i>Chrysops viduatus</i>	35	54%	0.05	0.37	0.16	0.32	0.11
<i>Tabanus bromius</i>	70	53%	0	0.05	0.78	0.16	0

* For Tth I and Tth II haplotypes the total prevalence is indicated.

subcylindrica, *Hy. arpadi*, and *Hy. tarandina* (Fig. 3, A). All remaining species were united into a big cluster of frequently infected (45–67.6%) species with the prevalence of trypanosome coinfections at least twofold higher than in *T. bromius*.

In the principal component analysis (PCA), the PC1 and PC2 components were responsible for 89.8% of the variance in the multivariate data. The greatest contribution to both principal components was from total and Tth I + Tth II prevalence values. However, on top of that, PC1 had a high load from the Tth I prevalence, while PC2 – from that of Tth II (Suppl. Table 3). Traits such as the prevalence of monoxenous trypanosomatids and the index of dominance of host species did not make a significant contribution to the principal components, which agrees with previous analyses. The results of PCA supported the separation of frequently and rarely infected species, but at the same time delineated the same groups as identified in the cluster analysis, i.e. isolated position of *C. caecutiens* and *Ha. pluvialis* as well as *T. bromius* (Fig. 3, B).

Similarly to the analyses for all tabanids or their particular genera, the considered individual species also demonstrated that the observed prevalence (OP) of Tth I + Tth II coinfections was higher

than the one expected based on the frequency of individual haplotypes and their independence of each other (EP). There was a single exception, *C. caecutiens*, where the coinfections were not detected due to the low number of infected specimens. To estimate the extent of “nonindependence” between the two haplotypes, we calculated the linkage factor (LF) as $LF = (OP - EP) / EP$. This value shows how many times the excess of the observed prevalence is higher than the expected prevalence. We revealed a big split in these values between weakly and frequently infected species with the ranges of 0.5 – 1.2 and 3.0 – 6.1, respectively. This suggests that tabanids with low overall prevalence are more prone to mixed infections than the frequently infected ones.

COMPARISON OF PREVALENCE BY REGIONS

The tabanid dominance indices and species composition varied between regions. The largest number of species (24) was collected in the most intensively sampled Pskov Oblast (PO), while the smallest one (7) was from the Republic of Karelia (RK), which was sampled only during one decade. Similar numbers of species were found in the

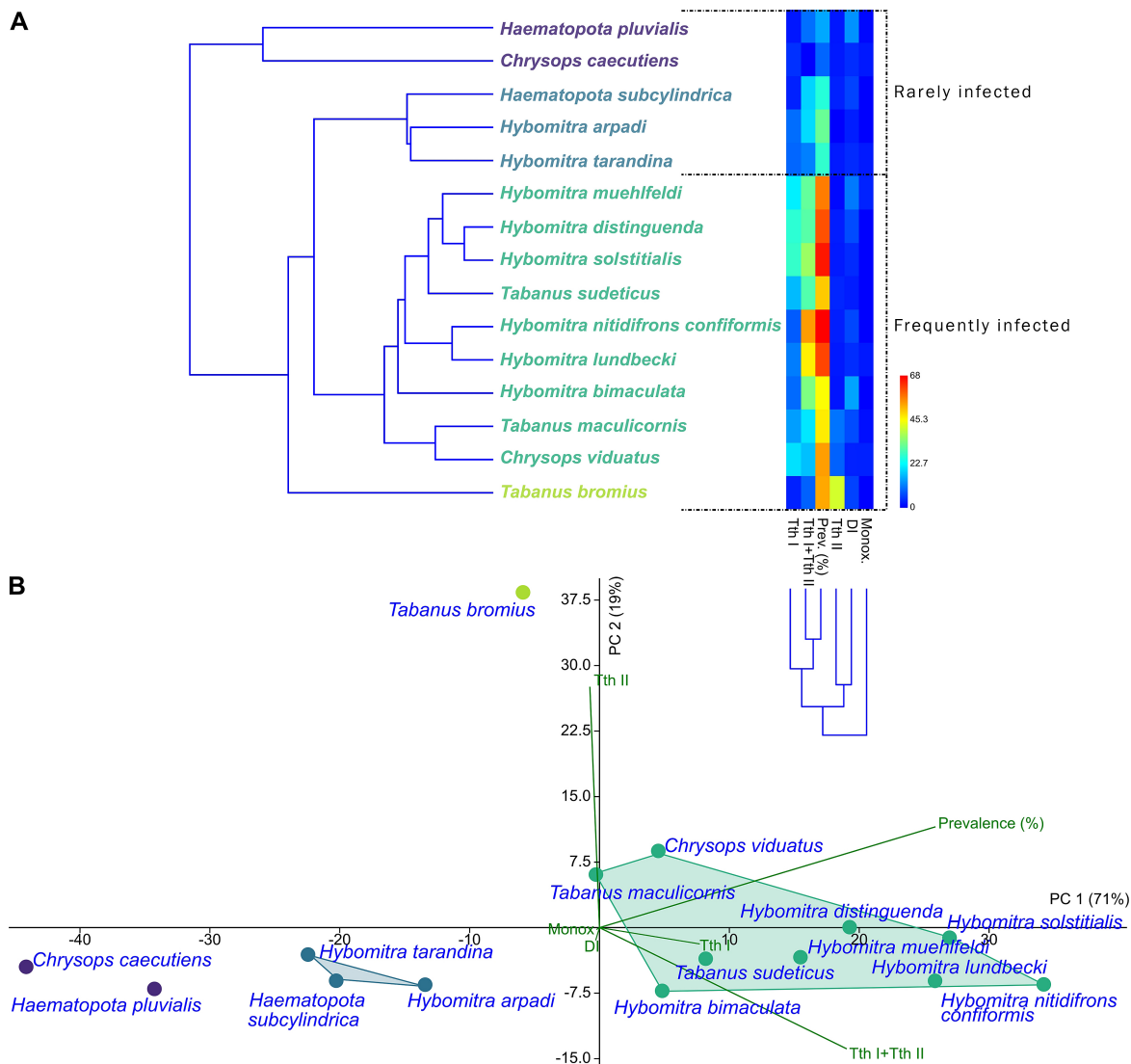


Fig. 3. Classification of tabanid species. A – cluster analysis, B – principal component analysis.

Leningrad Oblast (LO) and Novgorod Oblast (NO), respectively (17 and 16, respectively; Table 5).

The observed distribution of trypanosomatid infections varied between the studied areas. The maximum prevalence values were documented for PO and LO (52% and 41%, respectively). Less than one third of all the studied samples (30.2%) were infected in NO and only 15% – in RK (Table 5). These differences were apparently due to the abundance of particular species among collected specimens. For example, in RK the majority of collected specimens belonged to the rarely infected *Haematopota pluvialis*, whereas, in contrast, multiple frequently infected *Hybomitra* spp. and *Tabanus* spp. dominated in the PO (Suppl. Table 4).

Considering the differences in prevalence between regions, we compared the proportions of particular infection types among infected individuals. In most cases, this approach returned comparable values. Thus, the total Tth I proportions were similar in all regions (73–88%), while substantial differences were observed for Tth II proportion in tabanids from NO (46%) and the others (67–76%; p -values: 0.17, 1.1×10^{-3} , 1.4×10^{-3} for RK, LO and PO, respectively). As expected, the same pattern of differences was revealed for the proportions of mixed trypanosome infections (23% in NO versus 47–67%; p -values: 0.087, 6×10^{-6} , 5×10^{-5} for RK, LO and PO, respectively). The observed differences are likely due to the abundance of particular taxa: in NO these were

Table 5. Trypanosomatid prevalence by regions*.

	Karelia rep.	Leningrad Obl.	Novgorod Obl.	Pskov Obl.
# of species	7	17	16	26
# of specimens	101	202	129	746
Total infected	15 / 15% / -	83 / 41% / -	39 / 30% / -	385 / 52% / -
Monoxenous only	0 / 0% / 0%	1 / 0.5% / 1%	2 / 2% / 5%	7 / 1% / 2%
TthI (total)	11 / 11% / 73%	73 / 36% / 88%	31 / 24% / 79%	318 / 43% / 83%
TthII (total)	10 / 10% / 67%	63 / 31% / 76%	18 / 14% / 46%	273 / 37% / 71%
TthI + TthII mix	7 / 7% / 47%	56 / 28% / 67%	12 / 9% / 23%	220 / 29% / 57%

* Multiple values in the cells are: count / prevalence / proportion among infected samples.

members of the genera *Hybomitra* and *Chrysops*, in which Tth II has a low prevalence as compared to Tth I (see above), as well as rarely infected *Ha. pluvialis*, which does not contribute much to the frequency of trypanosome infections because of the low prevalence. In other regions, the ratio of the two haplotypes is more balanced because of the higher proportion of *Tabanus* and/or *Haematopota* spp.

SEASONAL DYNAMICS

The size and structure of our data allowed also investigating the seasonal dynamics of tabanid abundance and trypanosomatid prevalence during two months (six ten-day periods). *Hybomitra* spp. and *Chrysops* spp. were the first to appear (from the very beginning of the study period) and had abundance peak in mid-June. However, while the former could be captured until the end of July, the latter were not observable after the middle of that month (Fig. 4, A). Members of *Tabanus* and *Haematopota* emerged in the second and third ten-day periods of June, respectively. Both genera were most abundant in the beginning of July and were active until the end of the collection (Fig. 4, A).

The total abundance of tabanids demonstrated two seasonal peaks: in the second ten-day period of June and the first one of July. The lowest abundance was observed at the beginning and end of the study period (Fig. 4, A, B). Calculation of the nonlinear correlation coefficient for parabolic regression ($R = 0.73$) showed a significant relationship between seasonality and the number of captured tabanids.

The percentage of infected individuals noticeably increased for all tabanid genera reaching quite high values (up to 100%) at the end of the season (Fig. 4, A, B). *Haematopota* spp. represented a notable

exception, since they showed a low prevalence during the entire analyzed period (although with a slight increase). The overall prevalence in tabanids was as low as 16% in the beginning of June, and then from mid-June to the beginning of July it fluctuated in the range of 39–44%, reaching its maximum (64%) at the end of the study period (Fig. 4, B). The growth of the overall prevalence had only a slight deviation from a linear trend with a Pearson correlation coefficient as high as 0.91 ($p=0.012$).

The dynamics of prevalence of individual trypanosome haplotypes and their coinfections were more complex, with considerable fluctuations (Fig. 4, C), which could not be described by any of the above models. The first episode of steep growth of tabanid abundance (with a peak in mid-June) coincided in time with that of all prevalence values (Fig. 4, B, C). The subsequent abrupt decrease in abundance was also reflected in the corresponding change of all prevalence values, but with a smaller amplitude. Moreover, for Tth II and coinfections the local minimum was shifted to the next ten-day period (beginning of July), when Tth I prevalence already started growing. Interestingly, while the curves for prevalence of Tth I and coinfections reached a plateau in the middle or the end of July, those for Tth II demonstrated more or less the same growth rate throughout this month. Moreover, in the last period of observation, the prevalence of this haplotype finally exceeded that of Tth I (Fig. 4, B, C). The observed dynamics can be explained by the successive emergence of tabanid taxa, which have different relative abundance, rates of parasite acquisition (apparently, the highest in *Hybomitra* spp.) and different probabilities of acquiring one or another trypanosome haplotype (for example, the late fast growth of Tth II prevalence was due to *Tabanus* spp.).

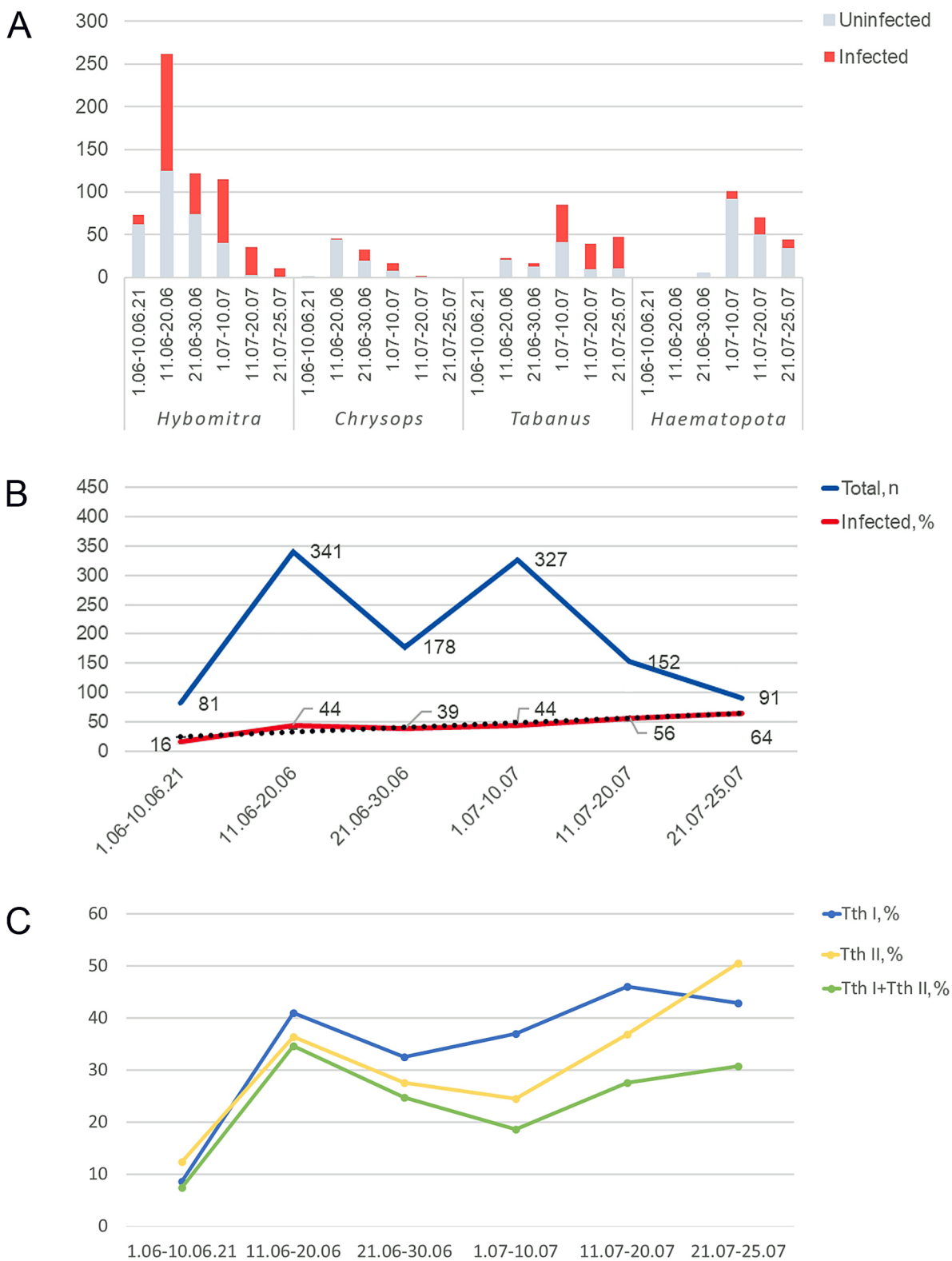


Fig. 4. Seasonal dynamics of trypanosomatid prevalence estimated for six ten-day periods in June and July. A – changes in abundance and proportion of infected individuals for particular tabanid genera, B – summarized tabanid abundance and overall trypanosomatid prevalence, C – prevalence of individual trypanosome haplotypes and their coinfections.

Discussion

The pool of specimens collected over two summer months and analyzed in this study representatively covers most of the tabanid diversity and their flight period in Northwestern Russia (Olsufjev, 1977). This allowed us conducting a comprehensive assessment of trypanosomatid infections in these insects. The majority of studied tabanids were infected with flagellates of the *Trypanosoma theileri* species complex (42.5 or 43.3%, depending on whether to count irresolvable mixes), while monoxenous trypanosomatids were very scarce (0.85% or 1.69%, respectively). Previous studies conducted in Central Europe (Poland) and West Africa likewise demonstrated a predominance of *T. theileri*-like trypanosomes, but with essentially lower prevalence values: 33.7 and 25%, respectively. As for monoxenous trypanosomatids, they were either not detected (apparently due to the low sample size in Poland – only 95 specimens) or had lower prevalence (0.18% (2 out of 1093 specimens) in West Africa) (Votýpka et al., 2019; Werszko et al., 2020b).

The high prevalence of *T. theileri*-like trypanosomes observed in tabanids of the Northwestern Russia suggests that the infection rates of ruminant hosts in this area are also high. Although the nominative species of the complex – *T. theileri* – is considered cosmopolitan (Jaimes-Duecez et al., 2018; Werszko et al., 2020b; Brotánková et al., 2022; Sukanuma et al., 2022), little is known about its prevalence in dairy cattle in Russia. However, there is indirect evidence from the beginning of the twentieth century that trypanosomiasis in cattle, caused by this flagellate, was widespread in the USSR from the Leningrad Oblast to the Trans-Urals and Transcaucasia (Olsufjev, 1937).

Interestingly, none of the examined tabanid species was documented to be free of trypanosomes, even those that were represented by a single or a few specimens. However, tabanids were not uniform in terms of parasite prevalence; they could be subdivided into rarely and frequently infected species, with several fold differences between members of these groups. This subdivision was not directly associated with taxonomic, temporal, regional, or dominance-based grouping but was probably related to the peculiarities of the biology of individual species. We believe that this phenomenon can be explained by tabanid' preferences for certain parts of the ruminant host body. Such data are available mainly for cattle and horses (Olsufjev, 1935; Skufjin, 1958; Konstantinov and Veselkin,

1989; Mullens, 2019). In our study, frequently infected tabanids were represented mainly by species that prefer the chest, lower part of the abdomen, genitals, and udder, while rarely infected species (except *Haematopota pluvialis*) feed mostly on the head, neck, and limbs (Table 6). Infections by *T. theileri*-like trypanosomes in ruminants are usually cryptic, and most aspects of their development remain obscure. We speculate that in summer, when parasitemia in the vertebrate hosts increases (Ishida et al., 2002; Sukanuma et al., 2022), trypanosomes, for some reason, are more abundant in the peripheral vessels of those body parts that are predominantly attacked by tabanids of the frequently infected group. Another factor likely influencing trypanosome prevalence in tabanids is feeding tactics. *Tabanus* spp. and *Hybomitra* spp. actively attack and pursue prey at high speed, while *Haematopota* spp. and *Chrysops* spp. lie in wait, hiding in the canopy of shrubs and trees at the edges, which results in fewer attacks and fewer (but more efficient) bites (Skufjin, 1958; Konstantinov, 1993). The lurking tactics apparently lead to the lower number of attacked hosts and, consequently, should decrease chances of trypanosome acquisition. This explains why *Ha. pluvialis*, feeding on the same body parts as frequently infected species (Skufjin, 1958), is a rarely infected tabanid. In addition, the size of tabanids also matters, since it correlates with the volume of consumed blood: from ~50 µg in small *Haematopota* spp. and *Chrysops* spp. to 180–300 µg in large *Tabanus* spp. and *Hybomitra* spp.

Seasonality of tabanid activity is quite pronounced in the studied region, with members of the four main genera appearing at different time points. It is remarkable that at the beginning of the season the trypanosomatid prevalence in these insects was already considerable (16%). This is undoubtedly associated with the dominance of frequently infected *Hybomitra* spp., as well as the presumably high prevalence of the flagellates in ruminant hosts. We observed the decrease in total abundance of tabanids in late June – early July, which was explained by the partial replacement of the early taxa by the late ones. This decline in the number of the insects was barely visible in the dynamics of the overall prevalence, but was pronounced when the frequencies of infections by the two trypanosome haplotypes were considered. Nevertheless, in all cases there was a growth in all prevalence values from the beginning to the end of the season, which is reminiscent of the situation with *Leishmania tropica* infections in the sandfly *Phlebotomus guggisbergi* (Johnson et al., 1999) or

Table 6. Biting sites of different tabanid species*.

	Species	Prevalence (%)	Biting sites									
			Head	Neck	Chest	Shoulders	Lower abdomen	Genitals and udder	Forelegs	Hind legs	Back and croup	Sides
rarely infected species	<i>Haematopota pluvialis</i>	15	++	++	+++	+	+	+			+	+
	<i>Chrysops caecutiens</i>	9	+	+++		+						+
	<i>Hybomitra arpadi</i>	32							++	++		
	<i>Hybomitra tarandina</i>	27	++						++	++	+	
	<i>Haematopota subcylindrica</i>	26	No data									
frequently infected species	<i>Hybomitra solstitialis</i>	66			+++	+	+	+++				
	<i>Tabanus sudeticus</i>	50									+	
	<i>Hybomitra lundbecki</i>	62			+++	+	+	+++				+
	<i>Hybomitra bimaculata</i>	45			+++			+++				
	<i>Tabanus maculicornis</i>	47			++		++		+	+	++	++
	<i>Chrysops viduatus</i>	54		+	++							+
	<i>Tabanus bromius</i>	53					++		++	+		
	<i>Hybomitra nit. confiformis</i>	68	No data									
	<i>Hybomitra muehlfeldi</i>	57	No data									
<i>Hybomitra distinguenda</i>	61	No data										

* Based on Olsufjev, 1935

T. theileri in the ruminant hosts (Ishida et al., 2002; Suganuma et al., 2022). Undoubtedly, the increase of prevalence in the hosts and that in the tabanid vectors contribute to each other. Such a correlation has been previously demonstrated, for example, for *T. theileri* in tabanids and cattle in Colombia (Jaimés-Dueñez et al., 2018). In this respect, the intensive growth of the Tth II prevalence in the end of the season is notable. We assume that it could be somehow related to the biology of particular vertebrate hosts. In addition, this can also be associated with the preference for certain feeders, as is probably the case of *T. bromius*, in which the Tth II haplotype is predominant.

The trypanosomes recorded in this research belonged to two haplotypes from the two major clades of the *T. theileri* species complex – Tth I and Tth II. As we stated above, they likely correspond to the putative species Tth α and Tth β that represent classical twin species, since they do not differ in morphology or details of development in the vector (Kostygov et al., 2022a). However, it is unclear whether these species (or haplotypes) somehow differ in their host affinity or specificity. At least in

the vectors both can coexist, as we demonstrated here and previously (Kostygov et al., 2022a). Moreover, the prevalence of their coinfections in tabanids at different levels (family, genera, and particular species, when a sufficient number of specimens could be statistically analyzed) was higher than the prediction based on the independence of these two haplotypes. The simplest explanation for the inferred nonindependence is their (frequent) coexistence also in (at least some of) the vertebrate hosts, allowing simultaneous acquisition by the vectors. If different hosts were able to harbor only one of these trypanosome variants, while the others could bear both, this would explain the revealed greater relative excess of observed coinfections over the predicted ones in rarely infected tabanid species. Furthermore, this would clarify the discordant prevalence dynamics of the two haplotypes.

The scarce, but at the same time more frequent than documented earlier, infections by monoxenous trypanosomatids can also shed light on some details of tabanid biology. In general, the low prevalence of such infections suggests that they can be nonspecific or even false. The latter was most

likely the case for the detection of *Blastocrithidia nonstop* and *Obscuromonas* sp. These flagellates belong to the genera that are specialized parasites of true bugs, and their detection is most likely due to the nonspecific presence of the resting stages – the cyst-like amastigotes (Frolov et al., 2021). These highly resistant cells are viable for several years and theoretically can pass intact through the gut of incompetent insects. It should be noted that one of these parasites (*Obscuromonas* sp.) has previously been detected in another haematophagous insect, the biting midge *Culicoides kibunensis* from Lithuania (Kazak et al., 2023), while a related species was reported in *Glossina tabaniformis* from the Central African Republic. Thus, such false infections are not unique: they occur in various insects and in different regions. They become revealed owing to the high sensitivity of PCR for 18S rRNA gene.

However, not all trypanosomatids that we detected in tabanids are of the same kind as the cyst-forming genera. Previously we have shown that *Wallacemonas ravinia*, originally described from an Ecuadorean flesh fly (Yurchenko et al., 2014), can undergo development in the rectum of *Hybomitra solstitialis* (Malysheva et al., 2022). Another monoxenous species, *Crithidia brevicula*, was recorded in this study as the most frequent monoxenous trypanosomatid. Considering its very wide host range, including other blood-sucking dipterans, in which it is able to efficiently develop (Kostygov et al., 2014; Svobodová et al., 2015; Ganyukova et al., 2020b; Kostygov et al., 2022b), tabanids likely represent suitable hosts for this flagellate.

The status of the two remaining monoxenous species that we detected in the current study – *Lafontella* sp. and *Herpetomonas* sp. – is uncertain. Firstly, none of these species has been previously recorded. Secondly, representatives of both genera are known exclusively (in the former case) or predominantly (the latter) from brachyceran flies (Podlipaev, 1990; Borghesan et al., 2013; Yurchenko et al., 2016; Kostygov et al., 2021), although, to the best of our knowledge, not so far recorded in tabanids. The specificity range of different members of the genus *Herpetomonas* varies, but in general, this lineage is active in exploring new hosts (Borghesan et al., 2013; Yurchenko et al., 2016; Králová et al., 2019). Meanwhile, little is known in this respect for *Lafontella*. Thus, in both cases, infections can be either specific or nonspecific, although the latter is more likely considering their uniqueness.

The acquisition of nonspecific (or false) parasites by hematophagous arthropods has been proposed to occur via consumption of water or plant sugary fluids contaminated with feces of other insects (Krige et al., 2021; Votýpka et al., 2021; Kostygov et al., 2022b). Female tabanids should not be an exception in this respect, as they are known to consume those liquids as well (Mullens, 2019), although apparently not as frequently as mosquitoes that demonstrate much higher rates of infections by monoxenous trypanosomatids (Svobodová et al., 2015; Schoener et al., 2018; Kostygov et al., 2022b). Another route for the transmission of such parasites to tabanids can be associated with the attempts of the latter to feed on carcasses of vertebrates (Olsufjev, 1937; Skufjin, 1958) that are also attractive for non-hematophagous insects. Thus, although monoxenous trypanosomatids may not play a significant role in the life of tabanids, they highlight some aspects of the biology of the latter.

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Supplementary materials

Table 1. Detailed characterization of trypanosomatid infections for tabanid species grouped by genera.

Table 2. Prevalence of trypanosomatids in tabanid species grouped based on the dominance index.

Table 3. Loadings, eigenvalues and proportions of variance for the principal components.

Table 4. Characterization of trypanosomatid infections by regions.