The diversity of *Trichinella* in natural habitats of the Russian Far East

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Summary. The juveniles of *Trichinella* nematodes were collected from the muscular tissue of different predaceous mammals in the natural habitats of the Russian Far East (Primorsky Region of the Russian Federation). Identification of *Trichinella* nematodes up to the species level was based on the analysis of nucleotide sequences of mitochondrial DNA. The amplified sequence contained *Cox*I gene and adjacent tRNA sequences. Three species of trichinellids were detected in the studied material: *T. spiralis, T. pseudospiralis* and *T. nativa*. The latter species demonstrated the strongest genetical diversity as five haplotypes of *T. nativa* were discovered in the studied material. New hosts were reported for *Trichinella* nematodes in the Russian Far East including a leopard cat (*Prionailurus bengalensis*). Other mammal hosts included an Asian badger (*Meles leucurus*), a brown bear (*Ursus arctos*), a lynx (*Lynx lynx*), a red fox (*Vulpes vulpes*) and a sable (*Martes zibellina*).

Key words: CoxI mtDNA, predaceous mammal hosts, *Trichinella*, *Trichinella spiralis, Trichinella pseudospiralis, Trichinella nativa.*

The nematodes of the genus *Trichinella* pose a significant threat to human health, as in most acute cases the consumption of meat containing the juveniles of these nematodes can be fatal. The ability of *Trichinella* nematodes to complete their life cycle with the use of different vertebrate animals and ability to survive in the organism of invertebrates makes the task of the veterinary control very complicated. Usually, the cases of human infection with *Trichinella* are related to the consumption of pork as a product of animal husbandry or the meat of wild game from natural habitats. In Russian Federation, the outbreaks of trichinosis are often related to the consumption of a bear's meat or meat of other predaceous mammals, either wild or kept on fur farms (Odoyevskaya *et al*., 2013; Uspensky *et al*., 2018). Although the circulation of *Trichinella* larvae in anthropogenic ecosystems has been studied quite intensively, the circumstances of these parasitic nematodes circulation in the natural habitats remain little studied. The last two decades have been a time of significant progress in understanding the taxonomic structure of the genus *Trichinella* (Pozio & Murrell, 2006; Pozio & Zarlenga, 2013). According to modern concepts, this genus includes 12 species, which are genetically characterised and demonstrate a certain level of uniformity throughout the areal of distribution (Pozio *et al*., 2009). Nine of these 12 species correspond to the described species, while three species yet have to be characterised morphologically and named. Two widely distributed species of *Trichinella* (*T. nativa* Britov & Boev, 1972 and *T. pseudospiralis* Garkavi, 1972) have been described based on material collected in the Russian Federation (Britov & Boev, 1972; Garkavi, 1972). The findings of *T. nativa* are usually associated with wild sylvatic carnivores; the second species is associated with sylvatic mammals and birds, but was also reported from domestic pigs

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(Gottstein *et al*., 2009). In recent years, intensive collections of samples of the muscle tissue of dead animals were carried out in the Primorsky Region by one of the authors (I.V.S.). It made possible returning once more to the problem of the taxonomic diversity of *Trichinella* in natural ecosystems of the Russian Far East.

MATERIAL AND METHODS

Fragments of muscular tissue were collected from wild fallen predatory mammals in Primorsky Region of the Russian Federation. The list of studied samples is given in Table 1. The tissue samples were examined by 'the compression method'. Samples positive for *Trichinella* juveniles were then digested in artificial gastric juice. The juveniles were isolated by sedimentation in a Baermann funnel and frozen. After thawing, 5–12 first-stage juveniles from each sample were collected with a needle and transferred to Eppendorf 0.5 ml tubes with transparent walls in a 2-3 µl drop of sterile water on the inner surface of a tube. It was possible to check the presence and calculate the number of collected juveniles. Then, 22 µl of sterile water and 25 µl of lysis buffer containing proteinase K and mercaptoethanol were added (Holterman *et al.*, 2006). After incubation for 1.5 hours at 65 ° C, followed by heating at 99° C for 5 minutes, 1-1.6 µl of the obtained homogenate was used as template for PCR. The PCR protocol corresponded to that described earlier (Odoyevskaya & Spiridonov, 2016). The primer pair 37F_Tri GCA GTA AAT TTA GAA TTT AAA C and 42R_Tri-CCA AAT ATT CAT GGT GTT CATT was used. A 1400 bp long portion of mitochondrial genome containing Ala-tRNA, Cys-tRNA, Tyr-tRNA and *Cox*I mtDNA gene was amplified. The obtained PCR products were purified by electrophoresis in 0.8% agarose gel, isolated from the gel using a Wizard SV Gel and PCR Clean-Up System (Promega, Madison, USA) and finally cleaned by precipitation with 70% ethanol in the presence of ammonium acetate. Amplified DNA fragments were sequenced with the same primers used for PCR. Chromatograms were analysed using the Chromas 1.45 program (www.technelysium.com.au/ chromas.html). Obtained sequences were saved as FASTA format. The closest matches for the obtained sequences were found in NCBI GenBank using the BLAST algorithm (Altshul *et al.*, 1990). The resulting set of sequences containing newly obtained ones and down-loaded from GenBank were aligned in Clustal X (Thompson *et al.*, 1997). The flanking parts of different

Table 1. Origin of the studied *Trichinella* isolates and accession numbers in NCBI GenBank.

Locality	Host	Isolate designation	Species	NCBI GenBank accession number
Primorsky Region, Russian Far East	Sable	Tri113	*Trichinella nativa*	MK189165
Primorsky Region, Russian Far East	Leopard cat	Tri122	*Trichinella nativa*	MK189166
Primorsky Region, Russian Far East	Asian badger	Tri126	*Trichinella nativa*	MK189167
Primorsky Region, Russian Far East	Sable	Tri132	*Trichinella nativa*	MK189168
Primorsky Region, Russian Far East	Sable	TrN2	*Trichinella spiralis*	MK257742
Primorsky Region, Russian Far East	Leopard cat	TrN3	*Trichinella nativa*	MK032471
Primorsky Region, Russian Far East	Leopard cat	TrN12	*Trichinella pseudospiralis*	MK257739
Primorsky Region, Russian Far East	Red fox	TrN13	*Trichinella nativa*	MK034472
Primorsky Region, Russian Far East	Lynx	TrN17	*Trichinella nativa*	MK257736
Primorsky Region, Russian Far East	Brown bear	TrN24	*Trichinella nativa*	MK032473
Primorsky Region, Russian Far East	Red fox	TrN26	*Trichinella nativa*	MK257738
Kamchatka Peninsula, Russian Far East	Domestic pig	Tri79	*Trichinella pseudospiralis*	MK257740

length were removed in Genedoc 2.7. (Nicholas *et al*., 1997) to obtain the rectangular matrix of data. This data matrix was used for phylogenetic analysis in the MEGA7 program (Kumar *et al*., 2016). Three methods of analysis were used: maximum parsimony (MP), neighbour joining (NJ) with 1000 bootstrap pseudo-repeats and maximum likelihood (ML) under Tamura-Nei model with gamma-distributed sites (TN93+G), Nearest-Neighbor-Interchange as tree inference option and with rapid bootstrapping under 500 replicates. The *Trichinella* sequences representing separate haplotypes were deposited with NCBI GenBank (accession numbers in Table 1).

RESULTS

The obtained rectangular matrix with mismatching flanking parts removed had a length of 1135 bp. Among these positions of the alignment, 808 positions were constant, 67 positions were non-informative and 260 positions were informative. The topologies of the trees obtained by all the methods (MP, NJ and ML) were similar and values of bootstrap support for clades were presented on Fig. 1. The maximal (100%) level of support was observed for two species (*T. spiralis* and *T. pseudospiralis*) and for a clade consisting of *T. nativa* and sequence of *Trichinella* sp. T6 genotype. Bootstrap support for isolates of *T. nativa* was quite low (70-77% depending on the method of analysis).

The obtained phylogram (Fig. 1) also showed that the level of nucleotide variability within the studied species differed significantly. For the species *T. pseudospiralis*, the differences between distant intra-specific groups were 58 bp.

The sequence of the same DNA locus obtained for *T. pseudospiralis* isolated from domestic pigs in Kamchatka peninsula was also included in this analysis. This isolate *T. pseudospiralis* differed from the isolate of this species from Primorsky Region by one nucleotide. Although both of these Russian isolates showed significant proximity to the previously studied isolates 'ISS176', 'ISS13' and 'ISS588', the Kamchatka isolate can be classified as a separate intra-specific haplotype. Within the studied species *T. spiralis* and *T. nativa*, the limits of nucleotide variability for this locus are less significant and amount, respectively, to 2-4 bp and 1-6 bp. The level of differences in the studied DNA region between *T. nativa* and *Trichinella* sp. 'T6' is 7-8 bp. At least two haplotypes can be distinguished within nematodes of the species *T. spiralis* from Primorsky Region. The nematodes of laboratory samples Tri117 and TrN2 differ in two nucleotide substitutions from the isolate Tri109. This latter with non-complete labelling was provided by Prof. V.A. Britov and, as seems, also originated from Far East of the Russian Federation. The isolates of *T. nativa* from Primorsky Region belong to several haplotypes (Table 2).

Table 2. Nucleotide differences between *Trichinella nativa* isolates in the partial *Cox*1 mtDNA sequence

	Trichinella species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	*T. nativa* Tri122	–														
2	*T. nativa* Tri100	3	–													
3	*T. nativa* Tri108	6	3	–												
4	*T. nativa* ISS10 NC025752	5	2	1	–											
5	*T. nativa* Tri107	5	2	1	0	–										
6	*T. nativa* TrN17	2	1	4	3	3	–									
7	*T. nativa* TrN24	1	2	5	4	4	1	–								
8	*T. nativa* Tri102	1	2	5	4	4	1	0	–							
9	*T. nativa* TrN23	1	2	5	4	4	1	0	0	–						
10	*T. nativa* Tri126	1	2	5	4	4	1	0	0	0	–					
11	*T. nativa* TrN13	1	2	5	4	4	1	0	0	0	0	–				
12	*T. nativa* Tri132	1	2	5	4	4	1	0	0	0	0	0	–			
13	*T. nativa* Tri113	2	3	6	5	5	2	1	1	1	1	1	1	–		
14	*T. nativa* TrN3	2	3	6	5	5	2	1	1	1	1	1	1	0	–	
15	*Trichinella* sp. T6	8	7	8	7	7	6	7	7	7	7	7	7	8	8	–

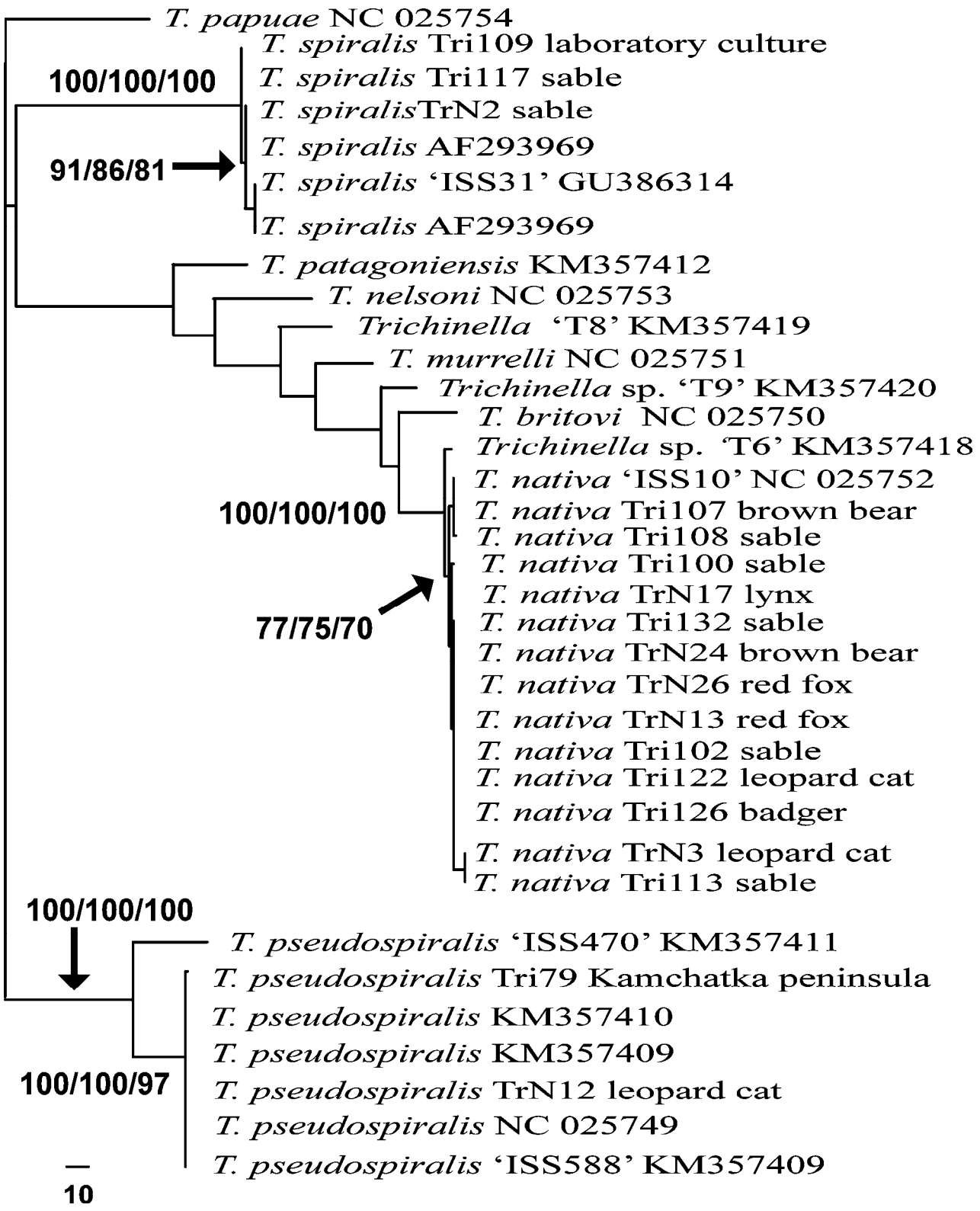

Fig. 1. The taxonomic position of the *Trichinella* isolates from Russian Far East. The bootstrap support values are presented in the format MP/NJ/ML.

DISCUSSION

The nematodes of the genus *Trichinella* were intensively studied in the last 10 years with all available methods of molecular genetics. Quite recently full mitochondrial genomes of all 12 known species were published together with the phylogeny inferred from these data (Mohandas *et al*., 2014). In this study the mitochondrial genomes of five intraspecific forms of *T. pseudospiralis* were also published. Complete nuclear genomes of all species of *Trichinella* were partially annotated (Korhonen *et al*., 2016). For each of the species, from 11 to 16 thousand genes were identified, where more than a thousand ones were single-copy genes. The genus phylogeny inferred from the analysis of these single copy genes was proposed. In the main features, the phylogeny inferred from the *Trichinella* nuclear genome was similar to mitochondrial ones. Analysis of the data obtained on the basis of the molecular clock model allowed to determine the lifetime of the common ancestor of the nematodes of the genus *Trichinella* and *Trichuris suis* as 384-204 million years ago. The time of *Trichinella* division into two evolutionary lines with the species able or unable for incapsulation in the host muscle tissues was defined as 28-15 million years ago. The authors of this comprehensive analysis (Korhonen *et al*., 2016) concluded from their phylogeographic analysis that the *Trichinella* nematodes evolutionary origin is in the prehistoric areas that now constitutes Eurasia. It means that the territory of the Russian Federation was an area for expansion of species and intraspecific groups of trichinellids. The haplotypes of e.g. *T. pseudospiralis* isolated in the Primorsky Region of the Russian Federation were similar to three haplotypes isolated in Eurasia (T4.1-3), and significantly differed from the North American (T4.4.) and Australian (T4.5) haplotypes. As reported before, the haplotype of *T. spiralis* from Primorsky Region differed from the haplotypes widespread in the Western Europe and North America (Spiridonov *et al*., 2016). A study of *T. nativa* isolates from different regions of the Russian Federation showed a complex haplotype structure of this species (Odoyevskaya & Spiridonov, 2016), but the comparable level of haplotype diversity was found between isolates originated from Primorsky Region alone.

The diversity in the nucleotide sequences of the studied part of mitochondrial genome in three *Trichinella* species found in the Russian Far East demonstrated three different patterns. *Trichinella spiralis* genetic uniformity throughout Western Europe, Americas and Australia was reported before (Rosenthal *et al*., 2008), with only East Asian isolates being different and probably representing the basal form for this species. The isolates found in the Russian Far East also differ from West Eurasian ones and probably represent the same East Asian intraspecific form as Chinese isolates. *Trichinella pseudospiralis* is quite uniform in Eurasia with only North American and Australian isolates being prominently different. It can be speculated that genetic uniformity of *T. pseudospiralis* can be explained by their ability to travel over long distances in the muscular tissues of their bird hosts. It is the *T. nativa* that demonstrates haplotype diversity in the material from Russian Far East and throughout the Russian territory (Odoyevskaya & Spiridonov, 2016). Rosenthal *et al*. (2008) demonstrated that *Trichinella* species with wildlife hosts demonstrate the highest level of genetic variability compared with species associated with domestic animals. This genetic variability can be considered as a manifestation of the fragmentation of the trichinellid species inside its area. Such a fragmentation might have been a result of association with only local food chains significantly limiting the gene flow.

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REFERENCES

ALTSCHUL, S.F., GISH, W., MILLER, W., MYERS, E.W. & LIPMAN, D.J. 1990. Basic local alignment search tool. *Journal of Molecular Biology* 215: 403-410.

BRITOV, V.A. & BOEV, S.N. 1972. Taxonomic rank of various strains of Trichinella and their circulation in nature. *Vestnik Akademii Nauk Kazakhskoi SSR* 28: 27-32 [in Russian].

GARKAVI, B.L. 1972. Species of *Trichinella* isolated from wild animals. *Veterinariya* 10: 90-91 [in Russian].

GOTTSTEIN, B., POZIO, E. & NOCKLER, K. 2009. Epidemiology, diagnosis, treatment, and control of trichinellosis. *Clinical Microbiology Reviews* 22:127-145 DOI:10. 1128/ CMR.00026-08

HOLTERMAN, M., VAN DER WURFF, A., VAN DEN ELSEN, S., VAN MEGEN, H., BONGERS, T., HOLOVACHOV, O., BAKKER, & J., HELDER. 2006. Phylum-wide analysis of SSU rDNA reveals deep phylogenetic relationships among nematodes and accelerated evolution towards crown clades. *Molecular Biology and Evolution* 23: 1792-1800. DOI: 10.1093/molbev/msl044

ISOMURSU, M. & KUNNASRANTA, M. 2011. *Trichinella nativa* in Grey Seal *Halichoerus grypus*: spill-over from a highly endemic terrestrial ecosystem. *Journal of Parasitology* 97: 735-736 DOI:10.1645/ GE2717.1

MOHANDAS, N., POZIO, E., LA ROSA, G., KORHONEN, P. K., YOUNG, N. D., KOEHLER, A. V., HALL, R. S., STERNBERG, P. W., BOAG, P. R., JEX, A. R., CHANG, B. & GASSER, R. B. 2014. Mitochondrial genomes of *Trichinella* species and genotypes – a basis for diagnosis, and systematic and epidemiological explorations. *International Journal for Parasitology* 44: 1073-1080. http://dx.doi.org/10.1016 /j.ijpara. 2014.08.010

KORHONEN, P. K., POZIO, E., LA ROSA, G., CHANG, B. C.H., KOEHLER, A. V., HOBERG, E. P., BOAG, P. R, TAN, P., JEX A. R., HOFMANN, A., STERNBERG, P. W., YOUNG, N. D., & GASSER, R. B. 2016. Phylogenomic and biogeographic reconstruction of the *Trichinella* complex. *Nature Communications,* 7: article number N 10513, doi:10.1038/ncomms10513

KUMAR, S, STECHER, G. & TAMURA, K. 2016. MEGA7: Molecular evolutionary genetics analysis. Version 7.0 for bigger datasets. *Molecular Biology and Evolution* 33: 1870-1874. https://doi.org/10.1093/ molbev/msw054

NICHOLAS, K.B., NICHOLAS, H.B. JR. & DEERFIELD, D.W. 1997. Multiple Sequence Alignment Editor and Shading Utility, Version 2.7.000. http://www.psc.edu/biomed/genedoc

ODOEVSKAYA, I.M. & SPIRIDONOV, S.E. 2016. *Trichinella nativa* haplotypes in Russia show diversity in cytochrome oxidase mtDNA gene. *Veterinary Parasitology,* 231: 39-42.

POZIO, E., HOBERG, E P., LA ROSA, G. & ZARLENGA, D. S. 2009. Molecular taxonomy, phylogeny and biogeography of nematodes belonging to the *Trichinella* genus. *Infection, Genetics and Evolution* 9: 606-616.

POZIO, E. & MURRELL, K. D. 2006. Systematics and epidemiology of *Trichinella*. *Advances in Parasitology* 63: 367–439.

POZIO, E. & ZARLENGA, D. S. 2013. New pieces of the *Trichinella* puzzle. *International Journal of Parasitology* 43: 983–997.

ROSENTHAL, B.M., LAROSA, G., ZARLENGA, D., DUNAMS, D., CHUNYU, Y., MINGYUAN, L., & POZIO, E. 2008. Human dispersal of *Trichinella spiralis* in domesticated pigs. *Infection, Genetics and Evolution* 8: 799-805. doi: 10.1016/j.meegid.2008.07

SPIRIDONOV, S.E., BUKINA, L.A., SERYODKIN, I.V., & ODOYEVSKAYA, I.M. 2016. Characteristic non-synonymous SNP in coxI mtDNA of Russian isolates of *Trichinella spiralis*. *Russian Journal of Nematology* 24: 127-129.

ZARLENGA, D. S., ROSENTHAL, B. M., LA ROSA, G., POZIO, E. & HOBERG, E. P. 2006. Post-Miocene expansion, colonization, and host switching drove speciation among extant nematodes of the archaic genus *Trichinella*. *Proceedings of the National Academy of Sciences USA* 103: 7354–7359.

И.М. Одоевская, И.В. Серёдкин, С.Э. Спиридонов. Taxonomic diversity of *Trichinella* nematodes in the natural ecosystems of Primorsky Region of the Russian Federation.

Резюме. Личинок нематод рода *Trichinella* извлекали из мышечной ткани павших хищных животных, останки которых собирали в природных экосистемах Приморского края на Дальнем Востоке Российской Федерации. Определение полученных личинок было основано на анализе нуклеотидных последовательностей *Cox*I гена и последовательностей tRNA митохондриального генома. Было выявлено три вида трихинеллид: *T. spiralis, T. pseudospiralis* и *T. nativa*. Последний вид продемонстрировал самый высокий уровень генетического разнообразия – было выявлено не менее пяти гаплотипов этого вида, отличающихся на 1-6 п.н. Амурский кот (*Prionailurus bengalensis*) отмечен как новый хозяин для *T. pseudospiralis* и *T. nativa* на россйском Дальнем Востоке. Также трихинеллы выявлены у азиатского барсука (*Meles leucurus*), бурого медведя (*Ursus arctos*), рыси (*Lynx lynx*), лисицы (*Vulpes vulpes*) и соболя (*Martes zibellina*).