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Short communication: New data support phylogeographic patterns in a marine parasite *Tristriata anatis* (Digenea: Notocotylidae)

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Short Communication

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Abstract

Intraspecific diversity in parasites with heteroxenous life cycles is guided by reproduction mode, host vagility and dispersal, transmission features and many other factors. Studies of these factors in Digenea have highlighted several important patterns. However, little is known about intraspecific variation for digeneans in the marine Arctic ecosystems. Here we analyse an extended dataset of partial *cox1* and *nadh1* sequences for *Tristriata anatis* (Notocotylidae) and confirm the preliminary findings on its distribution across Eurasia. Haplotypes are not shared between Europe and the North Pacific, suggesting a lack of current connection between these populations. Periwinkle distribution and anatid migration routes are consistent with such a structure of haplotype network. The North Pacific population appears ancestral, with later expansion of *T. anatis* to the North Atlantic. Here the parasite circulates widely, but the direction of haplotype transfer from the north-east to the south-west is more likely than the opposite. In the eastern Barents Sea, the local transmission hotspot is favoured.

Introduction

Intraspecific diversity is an element of biodiversity that links ecology and evolution. In parasitic organisms, especially those with heteroxenous life cycles, the patterns of intraspecific diversity are very complicated.

The genetic structure of a parasite population may depend on a number of factors (Nadler, 1995; Criscione *et al.*, 2005; Huyse *et al.*, 2005; Vázquez-Prieto *et al.*, 2015; Cole & Viney, 2018). One of them is the dispersal ability of the most vagile host in the life cycle (Prugnolle *et al.*, 2005; Keeney *et al.*, 2009; Louhi *et al.*, 2010; Blasco-Costa *et al.*, 2012; Enabulele *et al.*, 2018). When the life cycle is restricted to the aquatic environment (autogenic life cycle), parasite dispersal and gene flow are lower compared to life cycles where non-aquatic hosts are also involved (allogenic) (Esch *et al.*, 1988; Criscione & Blouin, 2004). This idea was formally tested for digeneans (Blasco-Costa & Poulin, 2013), and updated based on a larger dataset (Mazé-Guilmo *et al.*, 2016). Birds are able to travel long distances for feeding and migrations, and thus contribute significantly to the dispersal of their digenean parasites.

We have recently elucidated the life cycle of *Tristriata anatis* Belopolskaia, 1953 (Notocotylidae) – a common waterfowl parasite in the subarctic and low Arctic (Gonchar & Galaktionov, 2017). Its two-host life cycle is adapted for the transmission in harsh environmental conditions (Galaktionov, 2017; Galaktionov *et al.*, 2018), and the free-swimming cercaria may somewhat increase the dispersal. The first intermediate hosts – periwinkles *Littorina saxatilis* (Olivi, 1792) and *Littorina sitkana* Philippi, 1846 – are relatively site attached, and the dispersal of *T. anatis* relies on the movements of the definitive hosts, anatid birds. This dispersal has historically been enough to retain a single species concept across Eurasia. However, currently the connectivity between the North Atlantic and the North Pacific populations of *T. anatis* is limited.

Here we expand the *cox1* sequence dataset for *T. anatis* from distant parts of its range and supplement it with the sequences of another gene, *nadh1*. We provide robust support for the earlier ideas on the intraspecific variation in this species (Gonchar & Galaktionov, 2017) and develop the reasoning on the formation and current features of its transarctic range.

Materials and methods

Samples of *T. anatis* from snails and from birds were collected at the Pechora Sea in August 2017 during the expedition of the Zoological Institute of the Russian Academy of Sciences on the RV 'Professor Vladimir Kuznetsov'. We picked periwinkles *L. saxatilis* during low tide, kept them in the containers and rinsed with sea water daily until the end of the expedition. In the laboratory, the snails were dissected and screened for trematode infection, identifying

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T. anatis rediae and cercariae. We obtained common eiders *Somateria mollissima* (Linnaeus, 1758) in accordance with local regulations, euthanized them, dissected them within 24 h and picked *T. anatis* adults from the intestine. We also dissected snails from the Babushkin Bay (Sea of Okhotsk) and from Tromsø (provided by Kira V. Regel and Egor Repkin) and got additional *T. anatis* isolates. Samples were preserved in 96% ethanol. In addition to these, we used tissues, DNA and GenBank sequences from the earlier study (Gonchar & Galaktionov, 2017). Supplementary table S1 contains information on the origins of all samples.

The Sea of Okhotsk is located in the north-west of the Pacific Ocean, to the north of the Sea of Japan. The sampling in this region covered around 80 km of the Babushkin Bay and Kekurny Bay shoreline, from the Astronomicheskaya Bight in the west to the Promezhutochnaya Bay in the east. The Pechora Sea is a water body in the south-eastern edge of the Barents Sea. Here the sampling spanned around 130 km: Gulf of Sedov in the north of Vaygach Island, Lyamchina Bay in its south-western part and Dolgy Island further south. Other sampling locations are restricted to the shoreline within 1 km. Their coordinates, except for the spot in Tromsø (Norwegian Sea), are given in Gonchar & Galaktionov (2017, p. 47).

DNA was extracted from a single redia for each sample from a snail; and from one or several adult worms for each sample from a bird, using the oral sucker region and keeping the rest of the worm as a voucher (Chelex® 100 protocol; e.g. Gonchar *et al.*, 2019). Sample series 110, 111 and 112 correspond to replicate isolates from the three birds. Samples 59a, 60a, 63a and 87a correspond to the new redia individuals from the same infected molluscs, as in Gonchar & Galaktionov (2017).

To amplify the partial *cox1* gene we used primers JB3 and JB4.5 (Bowles *et al.*, 1993). For the *nadh1* gene fragment we used the newly designed primers eND1_f1 (AAGGGBCCTAA YAAGGTTG) and eND1_r (ACMAYACATAAARCAMGCYT CAA). The 25 µl reaction mixture contained 5 µl ScreenMix (Evrogen), 0.5 µl of each primer, 2 µl of the DNA and MilliQ water. Polymerase chain reactions (PCRs) were run on the Veriti thermal cycler (Applied Biosystems, Waltham, MA, USA) using the following temperature profile: 95°C for 5 min; 35 cycles with 30 s at 95°C, 30 s at 55°C (*cox1*) or 54°C (*nadh1*) and 1 min at 72°C; 72°C for 10 min; and cooling to 4°C. PCR products were stained with SYBR Green and visualized following an electrophoresis in a 1% agarose gel.

Sequencing was performed on the ABI PRISM 3130 (Applied Biosystems, Waltham, MA, USA) using forward and reverse primers. We analysed chromatograms, edited and aligned sequences in Geneious® 11.1.4 (https://www.geneious.com). Previous data on *T. anatis* from GenBank were included into our alignments. For haplotype visualization we submitted an alignment file to PopART 1.7 (Leigh & Bryant, 2015) to build an integer neighbour-joining network (reticulation tolerance 0.5). Measures of genetic diversity were calculated in DnaSP6 (Rozas *et al.*, 2017) and Arlequin suite version 3.5 (Excoffier & Lischer, 2010). Significance of differences in genetic diversity between populations was tested using the R script 'genetic_diversity_diffs v1.0.6' (R Core Team, 2015; Alexander *et al.*, 2016).

Results and discussion

We analysed marker DNA sequences from 90 individuals of *T. anatis* coming from the north of the Atlantic and the Pacific coasts of Eurasia. The dataset included 77 new partial *nadh1* gene sequences, and 88 (65 new and 23 previously obtained) partial *cox1* gene sequences. The *nadh1* sequences were fewer because for some older samples neither tissue nor DNA was available. Information about the sequences is presented in the supplementary table S1.

The *cox1* alignment, once trimmed to the shortest sequence, was 384 bp long and revealed 12 haplotypes (cH1–cH12, see fig. 1 and supplementary tables S1 and S2). We confirmed the five positions that had been previously found to differentiate the European and the North Pacific isolates, and found two more in the 5′-region (alignment positions 23 and 32). Nucleotide substitutions were synonymous except for the replacement of threonine with glycine in the position 97 of the amino acid alignment (sample 57). (Isolates 110-4 and 111-6 had ambiguous positions (K); T would mean no substitution and G would mean a non-synonymous substitution). In the *nadh1* alignment (275 bp long), the European and the North Pacific isolates also differed consistently in particular positions (17, 70, 106, 127, 148, 162, 178 and 226). The total number of haplotypes was 21 (nH1–nH21; see fig. 1 and supplementary tables S1 and S2). Nine substitutions were non-synonymous; one of them was specific for the North Pacific isolates (for details, see supplementary table S3).

Data obtained from the *cox1* and *nadh1* sequences are consistent and support our previous findings. The lack of *T. anatis* haplotypes shared between Europe and the North Pacific (fig. 1) and the statistics of genetic diversity (supplementary table S4) indicate the lack of current connection between the respective populations of this species. The reason for this stems from the distribution of periwinkles (absent along the coasts of the Siberian seas) and the migrations of definitive hosts – waterfowl and shorebirds. The North Pacific and the North Atlantic bird populations currently split at Taymyr Peninsula (Dau *et al.*, 2000; Bustnes *et al.*, 2010). However, it has not always been this way.

The initial arrival of *T. anatis* to the North Atlantic could only happen following the arrival of periwinkles, and, thus, after the opening of the Bering Strait *ca.* 3.5 Myr BP (Reid, 1996). Later, during multiple warm interglacials in the Pleistocene, transarctic bird flights became possible and periwinkles from the North Atlantic and the North Pacific advanced along the Arctic Ocean coast. In these periods, exchange between the European and the North Pacific populations of *T. anatis* apparently happened (Gonchar & Galaktionov, 2017). This parasite's life span of one to several months is long enough for transfer during the bird host migrations. Probably as a result of this former exchange, *T. anatis* represents a single species today.

The reasons to consider all our samples one species are identity of their morphometric and morphological features, and no difference in the variable D2 domain of the 28S rDNA (Gonchar & Galaktionov, 2017). Thus, the observed *cox1* and *nadh1* variations are now regarded as intraspecific. Speciation could be the next step, but, in fact, climate warming makes modern transarctic bird flights possible (as already reported; e.g. Able *et al.*, 2014). So, gene exchange may resume and sustain the single *T. anatis* species. The preliminary phylogeographic analysis for this species (Gonchar & Galaktionov, 2017) was based on limited data. In this study, we increased the sample size more than threefold, and the *nad1* marker was used in addition to *cox1*.

The new dataset reveals the most frequent *cox1* haplotype from the Sea of Okhotsk (cH2), which is consistently central in the network (fig. 1). The added *nadh1* sequences are fewer and the

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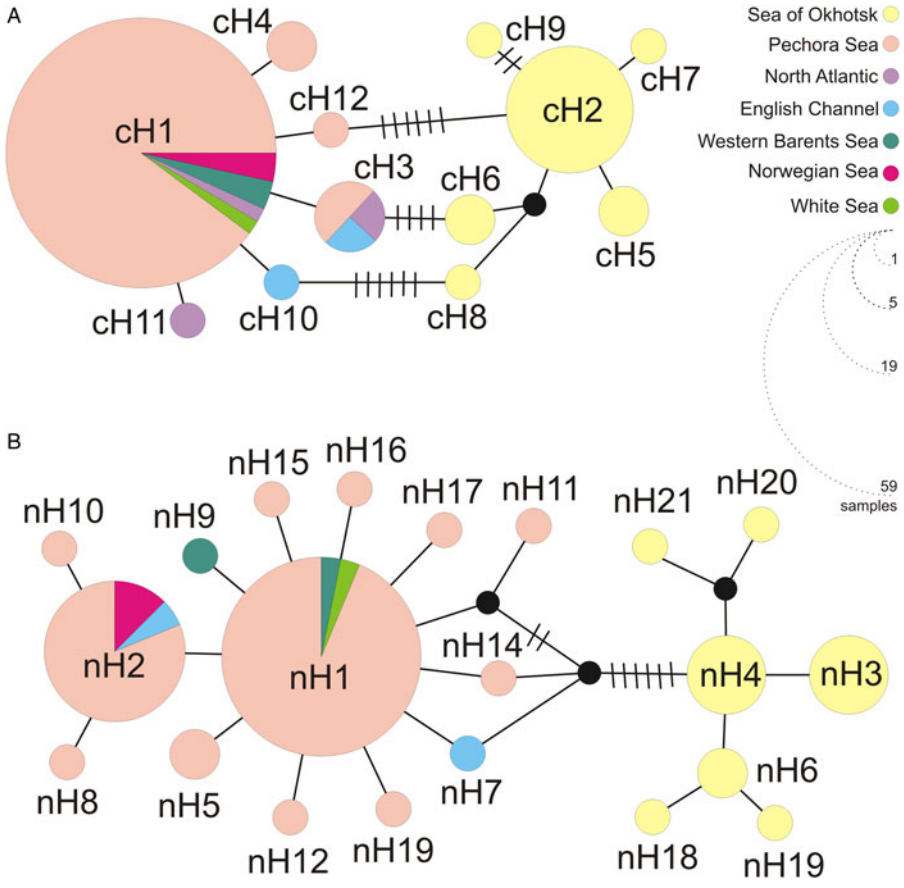

Fig. 1. Haplotype networks for *Tristriata anatis* based on partial *cox1* (a) and *nadh1* (b) sequences. Circle size represents the haplotype frequency; hatch marks represent missing haplotypes.

alignment is shorter, but this fragment turned out to be more variable (see fig. 1 and supplementary table S2). One consequence of the smaller *nadh1* sample size is that Iceland is not represented in the *nadh1* network; another is that four Pacific isolates out of 20 are missing, which we discuss in the following paragraph.

We previously assumed that the species *T. anatis* might be of Pacific origin (Gonchar & Galaktionov, 2017). Our new data and analyses support this idea. Higher haplotype diversity (Hd) is expected in the ancestral population (Crandall & Templeton, 1993; Duran *et al.*, 2004; Miura *et al.*, 2011). Sample groups from the Sea of Okhotsk and the Pechora Sea are best suited for comparison because they cover a similar geographic scale (see 'Materials and methods' section). However, the sample size (N) in the Sea of Okhotsk is smaller, so Hd there could be underestimated. We found that for the *cox1* marker, the Hd in the Sea of Okhotsk ($N = 20$, Hd = 0.579) is significantly higher than in the Pechora Sea ($N = 58$, Hd = 0.165; $P < 0.05$). For the *nadh1* marker, it does not differ significantly ($N = 16$, Hd = 0.825 vs. $N = 54$, Hd = 0.641; $P = 0.11$). The lack of statistic support in the case of *nadh1* may be an effect of missing four isolates. It is noteworthy that three of them (48, 49 and 57) represent unique *cox1* haplotypes and, thus, would likely add haplotypes to the *nadh1* network as well. Even with this sampling shortcoming, Hd in the Sea of Okhotsk is relatively high.

Nucleotide diversity (π) is quite low for all sampling locations (supplementary table S2). Haplotypes differ by one or two substitutions, except for divergence between the European and the Pacific population (fig. 1). The star-like patterns in the European part of the haplotype network suggest low geographic structure. Central dominant haplotypes are not a result of accidental expansion by one parasite clone in a certain year – they include samples collected between 2006 and 2017. This suggests that the life cycle of *T. anatis* runs steadily in the Pechora Sea region, forming a transmission hotspot.

Birds are vagile hosts that can accumulate various genotypes of the same parasite. We sequenced the marker fragments for co-parasites (adult worms) from the same bird host. Replicate sequences (22) from the three common eiders from Dolgy Island belong to the same *cox1* haplotype (cH1), except for the isolate 112-6 (cH12). Within-host diversity is higher for the *nadh1* marker: four haplotypes from eight co-parasites (110), two haplotypes from eight co-parasites (111) and five haplotypes from seven co-parasites (112). These three birds were juvenile, so were infected locally, and the haplotypes discovered from them should also be present in local snails. Moreover, all the worms from adult king eiders *Somateria spectabilis* (Linnaeus, 1758) at Dolgy Island (isolates 14 and 92) were young, with egg production at early stages, also suggesting a recent infection. Certain factors favour the *T. anatis* local transmission hotspot in the Pechora Sea. There is no second intermediate host which, if vagile, could enhance dispersal; and ducks stay in the region long enough to get infected, and for mature worms to develop and release eggs that serve as infection agents for snails. In addition to this local circulation, the haplotype network suggests that there is a transfer of haplotypes along the 5000-km shoreline between the English Channel and the eastern Barents Sea. The direction from the north-east to the south-west most likely prevails, according to data on the migration and feeding of birds.

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Common eiders keep to the shore during breeding – this is when they can encounter the *T. anatis* metacercariae in the intertidal. In winter they feed in the subtidal and would normally not get infected (Bustnes & Erikstad, 1988). King eiders and other ducks that may serve as definitive hosts for *T. anatis* nest in the Siberian tundra. They fly there directly in late May and June from the Baltic and Northern seas (long-tailed ducks *Clangula hyemalis* (Linnaeus, 1758), black scoters *Melanitta nigra* (Linnaeus, 1758), velvet scoters *Melanitta fusca* (Linnaeus, 1758)), and from the shores of the Kola peninsula and northern Norway (king eiders and Steller's eiders *Polysticta stelleri* (Pallas, 1769)) (Anker-Nilssen *et al.*, 2000; Krasnov *et al.*, 2019). Long-tailed ducks feed on crustaceans; king eiders and scoters feed on benthic molluscs (e.g. blue mussels); in winter, Steller's eider feed on amphipods and isopods near the shore (Bustnes & Erikstad, 1988; Anker-Nilssen *et al.*, 2000; Bustnes & Systad, 2001; Krasnov *et al.*, 2019). It is unlikely that they carry *T. anatis* from the wintering grounds. If some do, they probably lose the parasite by the time they return to the Pechora Sea for moulting in the end of July and for a stopover on their way to the wintering grounds in August–September. So, the major direction of *T. anatis* transfer is from Vaygach and Dolgy Islands to the Baltic and south-west of the Barents Sea and Norwegian Sea. Parasites can reach further south with other ducks, e.g. mallards (*Anas platyrhynchos* Linnaeus, 1758) that migrate to the coast of western Europe up to Great Britain and France.

In this study, we assessed intraspecific diversity in a common waterfowl digenean species *T. anatis* at a large geographic scale with two genetic markers, and linked it to previous and current transmission features. In the face of the changing Arctic, this is an important reference point to track how bird helminths respond to these changes.

Supplementary material. To view supplementary material for this article, please visit https://doi.org/10.1017/S0022149X19000786

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Conflicts of interest. None.

Ethical standards. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guides on the care and use of laboratory animals.

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