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GENETIC DIVERSITY IN MONOXENOUS AND TRIXENOUS DIGENEANS SHARING ONE MOLLUSCAN HOST SPECIES

© 2020 A. Gonchar[a, b, *]

[a] Department of Invertebrate Zoology, St Petersburg University,
Universitetskaya emb., 7/9, Saint Petersburg, 199034 Russia
[b] Zoological Institute Russian Academy of Sciences,
Universitetskaya emb., 1, Saint Petersburg, 199034 Russia
* e-mail: anya.gonchar@gmail.com, a.gonchar@spbu.ru

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Mudsnails *Peringia ulvae* serve as hosts for many digenean species; some of them differ a lot in their life cycle pattern. In the north of Europe two contrasting examples are *Cryptocotyle concava* (Heterophyidae) with a trixenous life cycle and *Bunocotyle progenetica* (Hemiuridae) with a monoxenous life cycle. A ~ 870 base pairs long fragment of cox1 gene sequence was used to evaluate and compare genetic structure within these two species. Our findings suggest that high dispersal of *C. concava* keeps differentiation between different locations minimal and haplotype diversity quite high. In *B. progenetica* two haplotypes dominate and have at least limited dispersal.

Keywords: Digenea, life cycle, intraspecific diversity, *Bunocotyle progenetica*, *Cryptocotyle concava*

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Monoxenous life cycles are not common in Digenea, but they occur and represent an extreme limitation of transmission (Poulin, Cribb, 2002). Such a limitation may offer some benefits, but also has significant drawbacks. One of the key concerns is the impact of one-host life cycle on the genetic diversity in a parasite. This impact may seem straightforward, but it has never been experimentally tested.

When a single host remains in the digenean life cycle, it is always a first intermediate host. Snails, the typical first intermediate hosts, usually harbour few clones of a parasite

following few infection events (Theron et al., 2004; Rauch et al., 2005; Keeney et al., 2007, 2008). Reproduction of the parthenitae – the sporocysts and rediae – does not produce genetic exchange and is in effect asexual. When the life cycle runs within one snail up until production of eggs or miracidia, these cannot be a result of mating between non-related parasites. So, in species with a monoxenous life cycle we expect lack of advantage from sexual reproduction and low genetic diversity.

Apart from sexual reproduction, intraspecific genetic diversity is also supported by spatial distribution and associated mixing opportunities. The most vagile host in the life cycle – for digeneans this generally means a vertebrate definitive host – largely determines genetic structure of a parasite. Birds and mammals provide better dispersal and less structure than fishes (Blasco-Costa, Poulin, 2013; Feis et al., 2015). Birds can even sustain connection between parasite populations that are geographically isolated (Gonchar, Galaktionov, 2020). In a monoxenous life cycle, the most vagile and the only host is a snail.

In the White Sea, the snails *Peringia ulvae* (along with their close relatives, *Ecrobia ventrosa*) serve as hosts for *Bunocotyle progenetica* (Markowski, 1936) Chabaud & Buttner, 1959 – the only monoxenous digenean in this region. These snails are also first intermediate hosts of other digeneans with more transmission events in their life cycles, for example, several species from families Microphallidae and Notocotylidae, and a heterophyid *Cryptocotyle concava* (Creplin, 1825) Lühe, 1899. The latter is an abundant parasite with a trixenous life cycle that involves fish second intermediate host and bird definitive host (a range of species with fish component in their diet, mainly gulls). It thus presents a contrasting example to *B. progenetica* regarding the expected genetic structure. Several studies have compared these two parasites of *P. ulvae* (Levakin, 2004, 2005; Levakin et al., 2013), but never in this particular aspect.

Here I for the first time assessed genetic structure of a monoxenous trematode (*B. progenetica*) and that of a trixenous one (*C. concava*) found in the same region and in the same molluscan host species.

MATERIALS AND METHODS

The mud snails *Peringia ulvae* were collected in 2018–2019 in two regions: the Chupa Inlet in the Kandalaksha Bay of the White Sea (Russia) and the Varangerfjord in the south-western Barents Sea (Norway). The distance between these regions is about 500 km directly and about 1000 km along the shore line. In Varangerfjord, all the samples were taken from the same location, the large mudflat in the head of the fjord. In the Chupa Inlet, sampling took place in four locations within 10 km from each other. Sampling sites are summarized in fig. 1 and table 1.

Mud snails were collected during low tide using a sieve with a 1 mm mesh size, taken to the laboratory and dissected under a stereomicroscope to detect infection by digeneans. I selected rediae of *Cryptocotyle concava* and *Bunocotyle progenetica* from an infected snail, rinsed them in sea water and preserved in 96 % ethanol. Prevalence and intensity were not recorded.

Table 1. Collection sites

Region	Site	Coordinates
Varangerfjord, south-western Barents Sea	Varangerbotn	70°10′19.31″N, 28°33′52.42″E
Chupa Inlet, Kandalaksha Bay, White Sea	Sukhaya Salma Bay	66°18′42.12″N, 33°39′18.58″E
	Lebyazhya Bay	66°17′39.95″N, 33°35′04.88″E
	Levin Navolok Bay	66°17′50.57″N, 33°27′37.51″E
	Krasnyi Island	66°25′06.74″N, 33°44′09.03″E

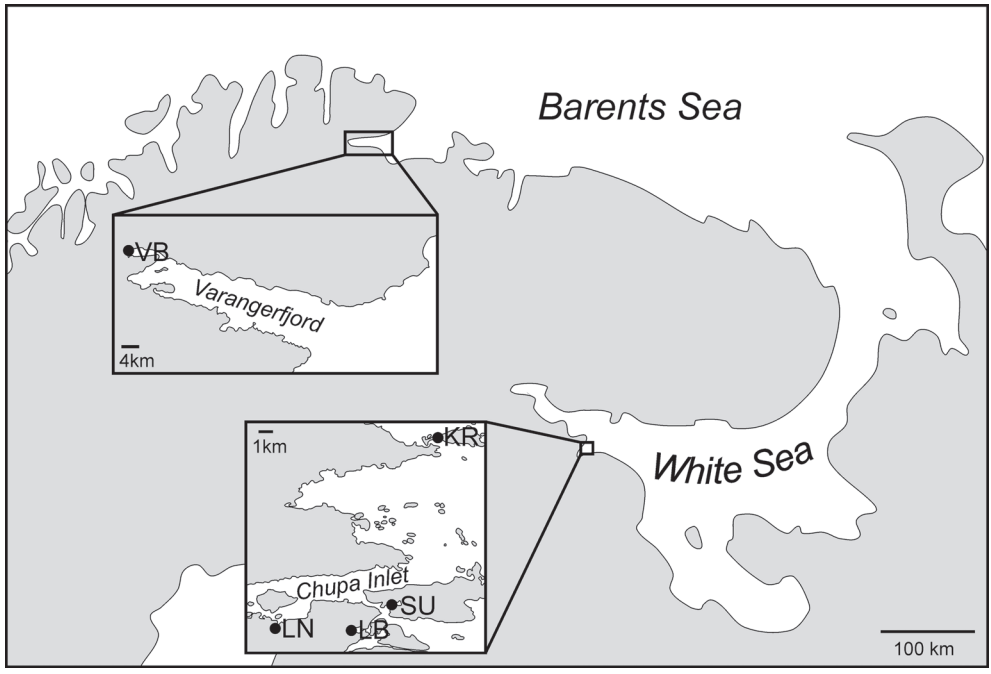

Figure 1. Schematic map showing collection sites in the Barents and White Seas.
Sampling locations are marked with circles: VB – Varangerbotn, LN − Levin Navolok Bay, LB − Lebyazhya Bay, SU − Sukhaya Salma Bay, KR − Krasnyi Island.

To extract DNA, the ion exchange resin Chelex ® 100, 200–400 mesh, molecular biology grade (Bio-Rad) was used. A single redia in a small drop of ethanol was transferred to a new 1.5 ml microtube; any remaining ethanol was evaporated by incubating an opened tube at 35 °C for 1–3 min. Then 200 µl of 5 % Chelex in Milli-Q water and 2 µl of proteinase K (20 mg/ml) were added to each tube and samples were incubated at 56 °C for 16–18 hours while mixing at 750 rpm on thermomixer (Eppendorf). Next, the samples were boiled at 90 °C for 8 min and centrifuged at 16,000 g for 10 min while cooling to 4 °C. DNA in supernatant was carefully transferred to a new tube and stored at −20 °C.

The fragment of cytochrome c oxidase subunit I (cox1) gene sequence was amplified with PCR in 20 µl reaction mixtures containing 13 µl Milli-Q water, 4 µl ScreenMix HS (Evrogen, Russia),

0.5 µl of each forward and reverse primer diluted to 10 pmol/µl and 2 µl of DNA template. I used JB3 forward primer (TTTTTTGGGCATCCTGAGGTTTAT, Bowles et al., 1993) and trem.cox1.rrnl (AATCATGATGCAAAAGGTA, Králová-Hromadová et al., 2008) or COIRtrema (CAACAAAT-CATGATGCAAAAGG, Miura et al., 2005) reverse primer. PCRs were run on a Veriti thermal cycler (Thermo Fisher Scientific) with the following thermal profile: initial denaturation at 94 °C for 3 min; 35 cycles with 40 s at 94 °C, 40 s at 48 °C (trem.cox1.rrnl) or 51 °C (COIRtrema) and 40 s at 72 °C; final elongation at 72 °C for 7 min; and cooling to 4 °C. PCR products were size-separated with electrophoresis in a 1 % agarose gel (5 min at 60 V and 40 min at 80 V), stained with SybrGREEN (Invitrogen); results were visualized and photographed using ChemiDoc MP (Bio-Rad). Sequencing of fragments was performed directly from PCR mixture and with PCR primers in both directions on the automated ABI 3500xl genetic analyzer (Applied Biosystems).

To process chromatograms I used Geneious 11.1.4 (https://www.geneious.com). Forward and reverse reads were assembled to verify resulting sequence, and the quality was checked by eye. Further quality control involved ensuring no inappropriate stop codons in a translated sequence (translation table 21), and testing for the functional effect of the amino acid substitutions using Provean with a default threshold − 2.5 (Choi et al., 2012). I also used the translated sequence to search for similar proteins using BLASTP 2.10.1 + (Altschul et al., 1997). The boundaries of cox1 gene were estimated by aligning to the annotated mitochondrial genome of *Metagonimus yokogawai* (NC_023249) as a reference. The alignment of all the obtained sequences was exported in *.nex format for further analysis. To construct a haplotype network, I used Integer NJ Net method with reticulation tolerance 0.5 in PopART 1.7 (Leigh, Bryant, 2015). To estimate sequence divergence I used MEGA7 (Kumar et al., 2016). Using DnaSP6 (Rozas et al., 2017) I calculated F_{ST} and Tajima's D; built a mismatch distribution graph using population growth-decline model and tested the fit to this model with a Harpending's raggedness index r. To test the significance of differences in haplotype diversity between the Chupa Inlet and the Varangerfjord for *C. concava* I used the R script "genetic_diversity_diffs v.1.0.6" (R Core Team, 2015; Alexander et al., 2016).

For comparison, I used partial cox1 sequences of European *Cryptocotyle lingua* isolates (Blakeslee et al., 2008; EU876333–EU876430). These were aligned and analyzed in DnaSP6 and MEGA7, as described above.

RESULTS

A total of 102 specimens were collected: 54 *Cryptocotyle concava* and 48 *Bunocotyle progenetica.* In Varangerfjord only *C. concava* were found. In Chupa Inlet, Sukhaya Salma and Lebyazhya Bays were major sampling sites, while from Levin Navolok Bay there were only five isolates and from Krasnyi Island only one.

I obtained high quality sequences covering 3′-region of the cox1 gene and adjacent tRNA-coding region for 40 *C. concava* and 32 *B. progenetica* isolates. High AT content was observed in both species: 63.5 % in *C. concava* and 67.5 % in *B. progenetica*. Stop codon was TAA in both species. All the sequences were submitted to GenBank under accession numbers MT422274–MT422345.

Sequences of *C. concava* were 872–881 base pairs (b. p.) long after trimming, and the resulting alignment contained 869 positions of which 27 were polymorphic (table 2). Average genetic divergence within the species was 0.31 ± 0.09 %. In the translated sequence, the majority of substitutions appeared synonymous, with two exceptions: Val → Ile (position 56 of the amino acid alignment) and Ser → Pro (236). None of these changes were predicted to have functional effect. Position 264 was a stop codon at the end of the cox1 gene sequence. The closest BLAST hit was *Metagonimus suifunensis* (QFS15968) with 87.6 % identity.

A total of 23 *C. concava* haplotypes were discovered; five of them were shared between Varangerfjord and Chupa Inlet. The average number of nucleotide differences between these populations was 2.885 and F_{st} was 0.12. The differences in haplotype diversity between the populations is not significant ($p = 0.195$). The cox1-based haplotype network for *C. concava* is in fig. 2*a*; it has reticulated structure and a repeated starburst pattern. Most haplotypes differed by one or two substitutions; the maximum difference between the two neighbouring haplotypes was four substitutions. Haplotype A was dominant in Varangerfjord; haplotype B occurred in all five sampling sites; 17 haplotypes were unique. Mismatch distribution was unimodal (fig. 3*a*).

For *B. progenetica* sequences were 853–860 b.p., and the alignment was 853 b.p. Average pairwise distance per site between all sequences was 0.21 ± 0.1 %. Four positions were polymorphic (table 2). In the translated sequence, three of them appeared non-synonymous: Ser → Asn (position three of the amino acid alignment), Ile → Val (4), and Thr → Met (239). None of these changes were predicted to have functional effect. Position 260 was a stop codon at the end of the cox1 gene sequence. The closest BLAST hit was *Isoparorchis eurytremum* (BAO74170) with 74 % identity.

The cox1-based haplotype network for *B. progenetica* is in fig. 2*b*. A total of three haplotypes were discovered, the neighbouring haplotypes differing by two or three substitutions. Haplotypes C (frequency 16) and D (11) were dominant, and were found in both major sampling sites (Sukhaya Salma and Lebyazhya Bays). Haplotype E (frequency 5) occurred in all the three sampling sites. Mismatch distribution was bimodal (fig. 3*b*).

I analyzed 98 cox1 sequences of *C. lingua* from GenBank. The samples originated from 16 sites in Europe: seven in southern Scandinavia, six in the UK and three along the continental coast of central Europe. The alignment was 1043 b.p. long, overlapping by 372 b. p. with the 5′-region of the cox1 alignment for *C. concava*. There were 19 non-synonymous substitutions across the whole alignment. The fragment overlapping for *C. lingua* and *C. concava* included 123 amino acids and had 10 fixed differences between the species. The total number of cox1 haplotypes for *C. lingua* was 72, with haplotype and nucleotide diversity values Hd = 0.984 and $\pi = 0.00379$.

Table 2. Genetic diversity indices based on cox1 sequences

Dataset		b. p.	N	S	H	Hd	π	K	Tajima's D
B. progenetica	all	853	32	4	3	0.627	0.00208	1.97	n.s.
C. concava	all	869	40	27	23	0.932	0.00312	2.715	−1.95**
	VB	869	20	14	12	0.879	0.00249	2.163	−1.65*
	CH	869	20	18	15	0.942	0.00335	2.911	−1.6*

VB − Varangerfjord, CH − Chupa Inlet, b.p. − length of alignment, N − number of samples,
S − Number of polymorphic sites, H − number of haplotypes, Hd − haplotype diversity, π− nucleotide diversity,
K − average number of nucleotide differences, * $p < 0.05$, ** $p < 0.005$, n.s. not significant.

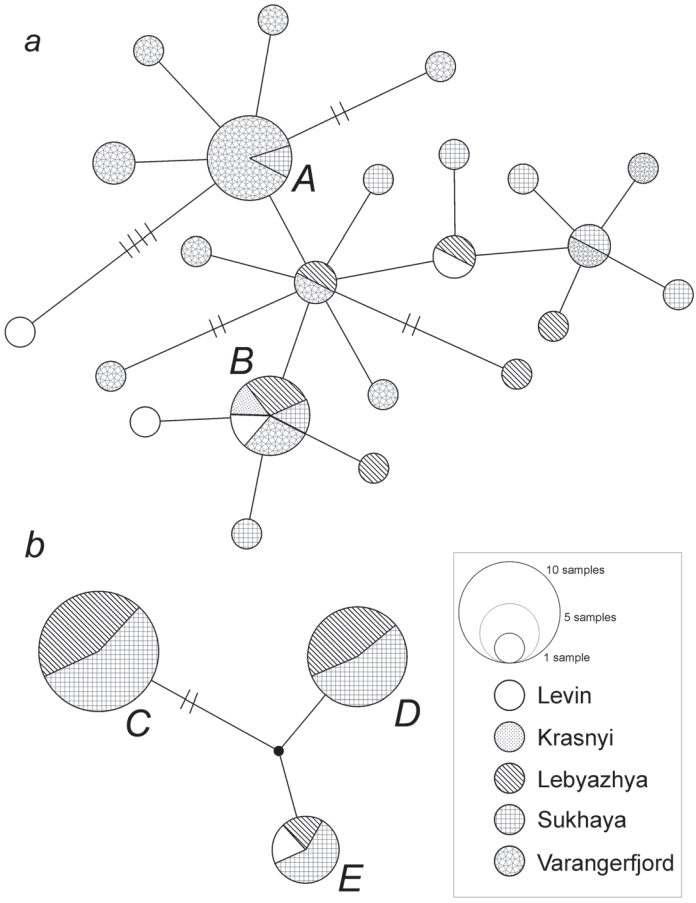

Figure 2. Haplotype networks based on cox1 sequence data for (*a*) *Cryptocotyle concava*, *n*=40; and (*b*) *Bunocotyle progenetica*, *n*=32. Circles indicate haplotypes, their frequencies correspond to circle sizes. Haplotypes connected by line differ by one (no hatch marks) or more (number of hatch marks) substitutions. Black dot illustrates a missing haplotype. Capital letters *A–E* mark haplotypes mentioned in the text. Sampling locations are represented by different fill patterns. Levin − Levin Navolok Bay, Krasnyi − Krasnyi Island, Lebyazhya − Lebyazhya Bay, Sukhaya − Sukhaya Salma Bay.

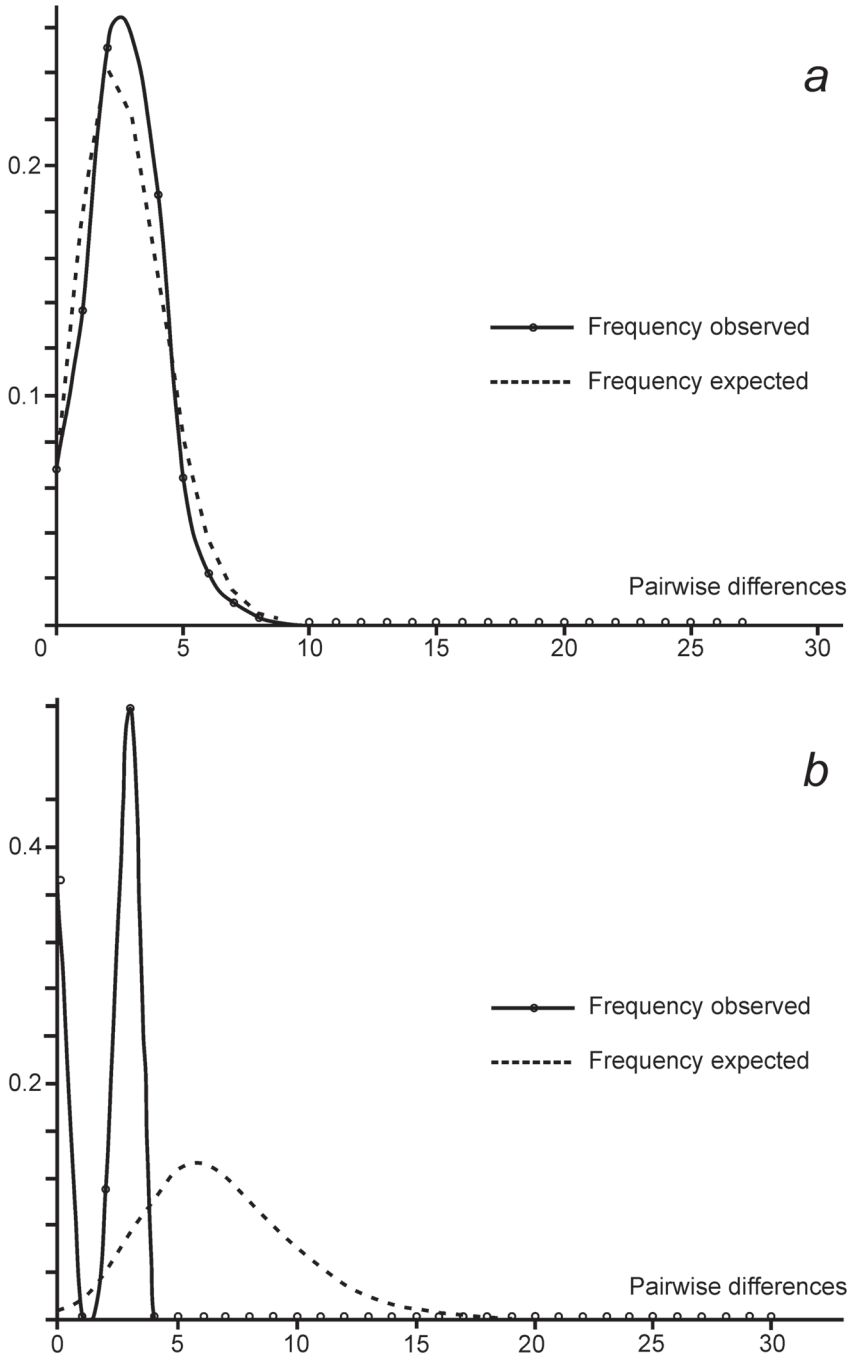

Figure 3. Mismatch distributions based on cox1 haplotypes for (*a*) *Cryptocotyle concava* and (*b*) *Bunocotyle progenetica*. Solid lines show observed frequencies, dashed lines show expected frequencies.

DISCUSSION

This study dealt with two species of trematodes that share the same first intermediate host, *Peringia ulvae*, but have a contrasting life cycle strategy. I obtained 72 sequences of a ~ 870 b.p. long fragment that included a 3′-region of cox1 gene. For *Cryptocotyle concava* this was the first molecular genetic evidence. Data on *Bunocotyle progenetica* were, to the best of our knowledge, the first intraspecific genetic diversity data for a monoxenous digenean. The study had relatively small sample sizes, relied on one DNA marker and provided non-exhaustive geographic range of sampling. The latter was especially critical for *B. progenetica* which I did not find in northern Norway. Despite these limitations, the results contribute to building a global picture of genetic diversity in digeneans.

For *C. concava*, the samples from two distant locations (Varangerfjord and Chupa Inlet) have shown lack of genetic differentiation. The haplotype network illustrates this well: five haplotypes are shared between the two locations, and haplotype pattern does not match a location pattern (fig. 2*a*). To test these observations I calculated F_{st} measure. It infers genetic differentiation from comparing genetic diversity within and among (sub)populations (Holsinger, Weir, 2009). In *C. concava*, $F_{st} = 0.12$ indicates gene flow between Varangerfjord and Chupa. This gene flow is consistent with the vagility of definitive hosts: birds can maintain long-distance dispersal of the parasite. This type of life cycle is called allogenic; the opposite is autogenic when the definitive hosts are fish, and colonization potential is smaller (Esch et al., 1988). Meta-analysis has also found that genetic structuring is lower in allogenic trematodes (Blasco-Costa, Poulin, 2013). The study of the two trixenous trematode species – autogenic and allogenic – in a marine environment gave the same result (Feis et al., 2015). Our results are thus consistent with the established ideas.

The results of this study were compared with those on three other representatives of Heterophyidae. All of them have a three-host life cycle where a definitive host is a fish-eating bird or mammal (including humans). First, I re-analyzed the published dataset for a closely related marine species *C. lingua* in Europe (Blakeslee et al., 2008), and proved no differentiation across sampling locations ($F_{st} = 0$). Then, the freshwater species *Metagonimus suifunensis* in the Russian Far East also follows this trend ($F_{st} = 0.05$, excluding the only sample from the northernmost location which had a highly diverged haplotype) (Tatonova et al., 2019). However, *Haplorchis taichui* forms several isolated populations in Vietnam (Dung et al., 2013) and, at the broader scale, in the mainland Southeast Asia (Thaenkham et al., 2017). These examples highlight that, although some trends in population genetics of trematodes have been identified, the case of every species adds new information.

Demographic history of *C. concava* inferred from sequence data suggests recent population expansion. The first evidence is the haplotype network that has star-like features, showing many unique haplotypes closely related to few central ones (structure similar to

type ii “complex star” in Jenkins et al., 2018). Population expansion can also be detected by neutrality tests, for example Tajima’s D that compares average pairwise differences with the number of segregating sites. For populations that underwent bottleneck recently it takes negative value, which is the case for *C. concava*. Further evidence is the unimodal mismatch distribution (fig. 3*a*). Probably the traces of bottleneck event that are observed in *C. concava* and *C. lingua* (Tajima’s D = –2.59) date from the last glacial maximum.

In the Chupa Inlet, *C. concava* and *Bunocotyle progenetica* were collected in the same sites, and even though sample sizes were not equal, the dataset is suitable for comparison. The number of detected haplotypes (H), haplotype diversity (Hd) and nucleotide diversity (π) were higher in *C. concava* (table 2). The difference is also evident from the haplotype network (fig. 2). Low intraspecific variability in *B. progenetica* is likely due to its life cycle that runs within one mollusc individual. This favours local expansion of single haplotypes but limits dispersal and genetic exchange.

Dispersal of *B. progenetica* relies on dispersal of its only host, the mollusc. *P. ulvae* may spread at the larval stage, but veligers cannot carry a parasite. Juveniles and adults of mud snails can float at the water surface and thus move much faster than by crawling (reviewed in Anderson, 1971; Armonies, Hartke, 1995). They may also be able to disperse once ingested by a bird, passing through the gut still alive (Haase et al., 2010; Cadée, 2011; van Leeuwen et al., 2012). At the local scale, either of these processes must be happening in the Chupa Inlet, as we find the same haplotypes of *B. progenetica* in different spots throughout the inlet (fig. 2). At the larger scale, biogeographic data on *P. ulvae* suggest that gene flow is quite high between the Baltic and the White Sea, probably due to dispersal by birds (Wilke, Davis, 2000). It is thus likely that *B. progenetica* was transferred to White Sea also from the Baltic, which is consistent with the records of this parasite there (Markowski, 1936; Reimer, 1961).

Since three *B. progenetica* haplotypes were detected at the White Sea, at least three *P. ulvae* infected with genetically distinct *B. progenetica* had arrived here at some point. This could have happened on a single occasion (for example, if snails were carried by the same bird) or as several independent events. The number of such events may be underestimated judging from present data: some parasite haplotypes may be unsampled; some could have become extinct at the White Sea; and some arriving snails could carry genetically identical parasites.

In any case, we assume that divergence of *B. progenetica* into the three haplotypes differing by 2–3 substitutions could not happen within the White Sea. It is a geologically young water body which formed and was colonized after the end of the last glacial maximum (LGM), not before 20 kya (Svendsen et al., 2004; Hughes et al., 2016). Considering the estimate of mutation rate for cox1 gene in digeneans as 2.5 % per Ma (Attwood et al., 2008),

the three haplotypes could not have formed at the White Sea. The Baltic Sea was also under ice during the LGM; the mud snails and parasites could spread here from the refugia at the Atlantic coast of Europe where *B. progenetica* is also documented now (Deblock, 1978).

Summing up, our findings on the genetic diversity in *C. concava* and *B. progenetica* illustrate some differences that would be expected between a tri- and monoxenous digenean species. Data on *C. concava* expand understanding of dispersal in species with an avian definitive host. As for *B. progenetica*, further study of intraspecific variation patterns could clarify more on its dispersal and persistence abilities. This will require sampling at a larger geographic scale, and sampling across several years to test whether the same haplotypes continue to dominate.

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ВНУТРИВИДОВАЯ ГЕНЕТИЧЕСКАЯ ИЗМЕНЧИВОСТЬ У ТРЕМАТОД С МОНО- И ТРИКСЕННЫМ ЦИКЛОМ ИЗ ОДНОГО ВИДА МОЛЛЮСКОВ-ХОЗЯЕВ

А. Г. Гончар

Ключевые слова: Digenea, трематоды, жизненный цикл, внутривидовая генетическая изменчивость, *Bunocotyle progenetica*, *Cryptocotyle concava*

РЕЗЮМЕ

Моллюски *Peringia ulvae* служат хозяевами для многих видов трематод, которые при этом могут сильно различаться по структуре своих жизненных циклов. На севере Европы два контрастных примера – это виды *Cryptocotyle concava* (Heterophyidae) с триксенным жизненным циклом и *Bunocotyle progenetica* (Hemiuridae) с моноксенным. Мы секвенировали фрагмент гена cox1 длиной ~ 870 пар нуклеотидов для оценки и сравнения генетической структуры внутри этих видов. Наши результаты свидетельствуют о том, что активное распространение *C. concava* ограничивает дифференциацию между различными географическими регионами и поддерживает довольно высокое разнообразие гаплотипов. У *B. progenetica* два гаплотипа доминируют и имеют по крайней мере некоторый потенциал для распространения.