Horizontal transmission of disseminated neoplasia in the widespread clam Limecola balthica from the Southern Baltic Sea

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Abstract

Disseminated neoplasia (DN) is one of the most challenging and unrecognised diseases occurring in aquatic fauna. It has been diagnosed in four bivalve species from the Gulf of Gdańsk (Southern Baltic Sea) with the highest frequency in Limecola balthica (formerly Macoma balthica), reaching up to 94% in some populations. The aetiology of DN in the Baltic Sea has not yet been identified, with earlier studies trying to link its occurrence with environmental pollution. Taking into account recent research providing evidence that DN is horizontally transmitted as clonal cells between individuals in some bivalve species, we aimed to test whether DN is a transmissible cancer in the population of L. balthica from the Gulf of Gdańsk highly affected with cancer. We examined mitochondrial cytochrome c oxidase I (mtCOI) and elongation factor 1α (EF1α) sequences of genomes obtained from haemolymph and tissues of neoplastic and healthy individuals. Sequence analysis resulted in detection of an independent transmissible cancer lineage occurring in 4 neoplastic clams that is not present in healthy animals. This paper describes the first case of transmissible DN in the clam L. balthica providing further insights for studies on this disease.

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Abstract

Disseminated neoplasia (DN) is one of the most challenging and unrecognised diseases occurring in aquatic fauna. It has been diagnosed in four bivalve species from the Gulf of Gdańsk (Southern Baltic Sea) with the highest frequency in *Limecola balthica* (formerly*Macoma balthica)* , reaching up to 94% in some populations.

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Introduction

Disseminated neoplasia (DN), also known as clam leukaemia, disseminated sarcoma, and haemic/systematic/haematopoietic neoplasia, is a cancerous disease observed in over 30 species of bivalves worldwide (Wołowicz et al. 2005, Carballal et al. 2015). The disease can act as a dangerous epizootic event leading to mass mortality in some populations (Farley et al. 1991, Muttray et al. 2012, Benabdelmouna & Ledu 2016) affecting commercially used species and leading to ecosystem unbalance (Barber et al. 2004). DN is a proliferative disorder of circulating cells predominantly found in the haemolymph. Neoplastic cells (NCs) are morphologically and physiologically distinct from normal haemolymph cells, which are gradually replaced with NCs during cancer progression. NCs, most likely of haemocyte origin, are characterized by high aneuploidy level, higher nucleus-cytoplasm volume ratio and increased proliferative activity resulting in nuclear and cellular hypertrophy (Thriot-Quiévreux & Wołowicz 1996, Smolarz et al. 2005ac, Smolarz et al. 2006a, Carella et al. 2013, Carella et al. 2017). Yet, alteration level in specific physiological and biochemical parameters of NCs differ between species (Barber et al. 2004, Le Grand et al. 2014, Carballal et al. 2015, Odintsova 2020). During cancer progression NCs are able to disseminate into tissues of an organism, leading to histological modifications, tissue/organ malfunction and eventually to death of an animal (Barber 2004, Carella et al. 2015, Carballal et al. 2015).

DN has been diagnosed in four bivalve species in the Gulf of Gdańsk (Southern Baltic Sea): *Limecola* (*Macoma*) *balthica* ,*Mya arenaria* , *Mytilus trossulus* and *Cerastoderma glaucum* (Thiriot-Quiévreux & Wołowicz 1996, Wołowicz et al. 2005, Smolarz et al. 2005bd, Smolarz et al. 2006b, Ogrodowczyk 2017). The frequency of cancer differs between species, population of a species, sampling sites, and sampling year, with the highest prevalence in*L. balthica* (the Baltic clam) reaching as high as 94% in populations inhabiting deeper (30-85 m) parts of the gulf (Thiriot-Quiévreux & Wołowicz 1996, Thiriot-Quiévreux & Wołowicz 2001). *L. balthica* is one the most widespread bivalve species found in the Baltic Sea area, but it is also commonly found in the temperate and arctic coastal waters of the North Atlantic and North Pacific (Budd & Rayment 2001). The Baltic clam plays a key role in various ecosystems, but due to the species' adaptive potential it is of particular importance in ecosystems characterized by changeable and unfavourable conditions such as low salinity, contamination and/or temporal/seasonal oxygen depletion, all found in the Baltic Sea (Segerstråle 1957, Janas et al. 2004, Janas et al. 2007). Earlier studies linked DN incidence with adverse environmental conditions and/or anthropogenic pollution as contaminants such as heavy metals, polycyclic hydrocarbons (WWA) and persistent organic pollutants (POPs) were previously proposed as ultimate carcinogenic factors in DN induction (reviewed in: Barber 2004, Carballal et al. 2015). However, those studies do not indicate any clear correlation between any specific environmental factor and cancer occurrence (Smolarz et al. 2005, Wołowicz et al. 2005, Smolarz et al. 2006) suggesting that DN in these clams may have a different cause.

In recent years, it was discovered that some types of cancer in animals can be explained by non-viral infectious aetiology, with cancerous cells being transmitted between individuals as an allograph that further leads to cancer development. Transmissible cancer based on clonal malignant cell transmission is generally considered to be a rare phenomenon, until now, observed only in exceptional and distinctive cases. To date there are three recognized types of cancer with transmissible aetiology: canine transmissible venereal tumour (CTVT) (Murgia et al. 2006, Rebbeck et al. 2009), devil facial tumour disease (DFTD) (Pearse et al. 2006), and bivalve transmissible neoplasia (BTN). BTN is a transmissible form of DN, and BTN lineages have been

found in seven bivalve species: soft-shell clam *M. arenaria* from Atlantic coast of North America (Metzger et al. 2015); *Cerastoderma edule* and *Polititapes aureus* from Galician coast of Spain (Metzger et al. 2016); and four species of *Mytilus* mussels from around the world, *M. trossulus* , *M. edulis* , *M. chilensis* and *M. galloprovincialis* (Yonemitsu et al. 2019, Skazina et al. 2021, Hammel et al. 2021). DN transmission in the above-mentioned bivalve species was confirmed using polymorphic microsatellite alleles of nuclear DNA and sequence analysis of mitochondrial and nuclear genes. These analyses found that the genomes of NCs were different from the genomes of the hosts, yet nearly identical to each other, indicating infectious nature of the disease.

The Gulf of Gdańsk, located in the southern part of the Baltic Sea, with its specific geomorphological structure, low salinity and low diversity constitutes an excellent biological model to study horizontal transfer of cancer cells. One of the four bivalve species occurring here,*L. balthica* , due to its widespread occurrence and high cancer prevalence, can be considered as a valuable model organism in DN and BTN research. Taking into account the unsolved basis of this commonly diagnosed cancer in *L. balthica* and the findings that DN in many species have been found transmissible (Metzger & Goff 2016), in this study we aim to use genomic analysis to test whether DN in *L. balthica* inhabiting the southern Baltic Sea (Gulf of Gdańsk) is a BTN. This will increase our knowledge of transmissible cancer and the ways in which cancer can affect animals in the environment.

2. Materials and methods

2.1. Clam collection and maintenance

Clams (approx. 100) were collected in February 2019$^{\text{th}}$from a sampling site H45 located in the Gulf of Gdańsk (southern Baltic Sea) at 45m depth. The selected area has been reported to have the highest prevalence of DN in *L. balthica* , as described in: Smolarz et al. (2005bd) and Ogrodowczyk (2017) (**Fig. 2.1**). Clams exceeding 10 mm in size (large enough for haemolymph withdrawal) were selected from the sediment samples collected with a Van Veen grabber on board of the *rv* Oceanograf. Transport and laboratory set-up were adjusted to imitate *in situ* conditions. Bivalves were kept in 15 L tanks (approximately 50 clams per one tank) filled with seawater collected at sea bottom from the sampling site for time not exceeding five days. No sediment substrate was added to tanks with animals for purification purposes.

2.2. Tissues processing and DN diagnostics

Samples for molecular analysis

Haemolymph was withdrawn directly from the adductor muscle using a Hamilton microsyringe. Syringes were thoroughly cleaned with 10% hydrochloric acid and washed in 70% ethanol and deionized water between individuals to avoid contamination. Haemolymph volume varied between specimens with range of approximately 20 – 100 µl. Equivalent volume of absolute ethanol was added to each haemolymph sample. Foot muscle tissue was dissected from each individual and put into 200 µl of absolute ethanol. Both haemolymph and soft tissue samples were stored at -20°C until the time of transport to our partner laboratory (PNRI, Seattle, USA) where they were further processed for molecular analysis. Ethanol-fixed samples were transported in the time not exceeding two days via plane in room temperature and after arrival were stored at -20°C.

Histological diagnostics

The remaining bodies of each clam were dissected and fixed with 5 ml of Davidson fixative (formaldehyde, acetic acid, ethanol) that was replaced with 4% phosphate-buffered formaldehyde after 48 hours. Fixed tissues were then dehydrated in increasing concentration of alcoholic solutions, cleaned in xylenes, and embedded in histological grade paraffin wax. Histological blocks were cut into 3 µm sections on a microtome (RM2245, Leica) and transferred onto microscopic slides that were later dried in 60°C for paraffin dissolution. Prepared sections were dyed using standard Harris' haematoxylin and alcoholic eosin stain (H&E) protocol for histological visualisation, mounted with xylene-based medium and investigated under light microscope. Only clams with infiltration of NCs observed in more than one tissue type, and prevalence of cancerous cells

>50% were classified as DN-positive (**Fig. 2.2**). Diagnosis resulted in selection of 4 neoplastic (labelled as #143, 144, 157, 158) and 2 healthy (#88, 89) individuals as references. Haemolymph and muscle samples from selected individuals were further used for molecular analysis.

2.3. Molecular analysis

DNA extraction

DNA was extracted from ethanol-fixed haemolymph and tissue samples using DNeasy Blood and Tissue Kit (Qiagen). Haemocyte pellet was obtained by spinning the cells down with 1400 × g speed for 10 min. After lysis of tissues, P3 Buffer (Qiagen) was added to precipitate out polysaccharides that inhibit PCR reactions. Further extraction was performed in accordance with the protocol provided by manufacturer.

mtCOI, *EΦ1α* ΠCR ανδ ϛλονινγ

Primers and annealing temperatures are listed in **Tab. 2.3** . PCR amplification for mitochondrial cytochrome oxidase c I (*mtCOI)*and elongation factor 1α (*EΦ1a)* loci was done using Q5 Hot Start High-Fidelity DNA Polymerase (NEB) with a 30 s extension time and annealing performed at 50°C. PCR reaction mix consisted of buffer (5x, Qiagen, 5 µl), dNTPs (0.5 µl), forward and reverse primers (1.25 µl of 10 µM for both), 0.25 µl polymerase and ddH2O (to 25 µl). 'Pan-molluscan' barcoding primers (Folmer et al. 1994) did not amplify*mtCOI* allele in *L. balthica.* Therefore, degenerate primers designed to match the same region of *L. balthica mtCOI*were used (Metzger et al. 2018), detailed in **Tab. 2.3** . We identified two transcripts with an annotation of *EΦ1a* in a published *L. balthica* transcriptome (Yurchenko et al. 2018). These transcripts aligned to one another with a high degree of polymorphism, indicating these may represent multiple copies of*EF1* -like genes in the *L. balthica* genome rather than different isoforms or haplotypes. We assumed intron placement would likely be conserved in the *EΦ1a* gene among bivalves, so we aligned these transcripts to an oyster genome (*Crassostrea_gigas* : oyster_v9 genome version) *EΦ1a*region using Geneious alignment with default settings. We then chose primer sites to target coding sequence and amplify across the 5th intron. We chose primers that would only target one of the *EΦ1a*transcripts (transcript ID: "evgsoapLocus_712298") to control for the possibility of multiple *EΦ1a* copies. In all cases, 25-50 ng of genomic DNA was amplified for 35 cycles with initial denaturation performed at 98°C for 30 s.

PCR products were gel extracted using QIAquick Gel Extraction Kit (Qiagen) and either directly sequenced, or, when multiple alleles at a locus could not be resolved by direct sequencing, were cloned using the Zero Blunt TOPO PCR Cloning Kit (Invitrogen). Plasmids were transformed into TOP10 or DH5α competent *Escherichia coli* (Invitrogen) and at least 6 clones were picked for further sequencing using M13F and M13R primers (Genewiz) using the difficult template protocol, due to the hairpin found in some of the *EΦ1a* alleles. In cases where a single clone was sequenced that was 1 SNP different from another clone found in multiple clones from the same animal, or a single clone was found to be consistent with recombination between two other clones found in multiple clones in the same animal, the single clones were assumed to be PCR artefacts. In cases where differences were found between the sequence results of haemolymph and tissue from the same individual, the alleles found more often in the haemolymph sample were called as the haemolymph alleles and those found more often in tissue were considered the tissue alleles. The primer binding regions were excluded from sequence analysis and all unique alleles were identified.

Phylogenetic analysis

Sequences were aligned manually in BioEdit (7.1 version) software (Hall 1999). Maximum likelihood phylogenetic trees were generated using PhyML (3.0 version) (Guindon et al. 2010) performing 100 bootstrap replicates with automatic model selection through Akaike Information Criterion. Trees were visualized using FigTree (1.4.4 version) software (Rambaut 2010).

3. Results

3.1. Phylogenetic analysis of *mtCOI* gene

Sequence analysis of *mtCOI* resulted in identification of six alleles present in tested samples and revealed that the haemocyte genome does not correspond to the genome of the host animal in any clams diagnosed with DN, whereas alleles in healthy animals represent the exact same DNA sequence in both sample types. These results are supported by high (>70) bootstrap values (**Fig. 3.1**).

Two *mtCOI* alleles, 'a' and 'b', consistent between host tissue and haemolymph within an individual, were identified in healthy clams. Alleles 'c', 'e', and 'f' discovered in solid tissue DNA of neoplastic animals were not found in their haemolymph equivalents. Allele 'd' present in haemolymph of neoplastic animals was identical between all four individuals diagnosed with DN and was distinct from both healthy alleles and alleles found in the DNA of hosts with neoplasia. All identified *mtCOI* alleles are listed in **Tab. 3.1** .

3.2. Phylogenetic analysis of EF1 *a* gene

Eight *EΦ1a* alleles were identified through sequence analysis and cloning with the resulting tree supporting the *mtCOI* phylogenetic analysis, showing variations between genomes of haemolymph and host tissue in neoplastic clams (**Fig. 3.2**).

EΦ1a alleles 'b', 'd', and 'h' represent healthy host alleles, as they were found to be identical between both DNA samples from healthy *L. balthica* individuals. *EΦ1a* allele 'e' was discovered to be common in haemolymph DNA of all clams diagnosed with DN and was not present or found at a much lower level in normal tissue samples. An additional *EΦ1a* 'g' allele was detected in haemolymph sample from #158 individual that was not present in the solid tissue of this specimen, although it was found in tissue DNA of another neoplastic clam. Two different alleles, 'g" and 'i', were found in two neoplastic hosts' DNA (**Tab. 3.2**).

4. Discussion

Results of phylogenetic analysis of both mitochondrial (*mtCOI*) and nuclear (*EΦ1a*) genes show that genomes of haemolymph and host tissue of neoplastic clams are different. Moreover, *mtCOI* and *EΦ1a* alleles found in haemolymph DNA of individuals diagnosed with DN are common between all of them, suggesting the existence of an independent cell line shared between these cancerous clams. Such results indicate the existence of at least one horizontally transmitted cancer lineage in the *L. balthica* population from the Baltic Sea, in accordance with original studies documenting first evidence of BTN in bivalves (Metzger et al. 2015, Metzger et al. 2016) that also formulated the basis of molecular identification of BTN. All tested neoplastic clams were discovered to share a common allele in both loci investigated, but one of the individuals was also identified with a distinct *EΦ1a* allele in the haemolymph genotype that was not present in host genome. This most likely indicates the presence of somatic mutation in the NCs and the loss of the second allele. Yet, at this stage the possible occurrence of another cancer lineage in the tested population cannot be excluded either. Genome variation between different types of tissue within the same organism can occur, especially in tissues characterized by elevated mitotic activity, such as hepatopancreas or gills, due to a potential accumulation of mutations resulting in somatic mosaicism (O'Huallachain 2012), but the chance of different individuals developing the same consistent alleles within multiple conserved genes is very low. Variations in coding sequences can be observed in cases of cancer development in higher organisms (O'Huallachain 2012, GTEx Consortium 2017). Genomic rearrangement within single individuals has not been described yet in invertebrates, although some genetic variability is reasonably expected in proliferative disorders such as DN, due to poly- and aneuploidy of cancerous cells and their high mutative potential (Diaz et al. 2010, Ruiz et al. 2013). However, taking into the account the nearly perfect similarity of alleles occurring in haemolymph of neoplastic *L. balthica* individuals we conclude that the recognized lineage originated from a single organism and are now transmitted within the gulf population of this species.

Phylogenetics also revealed natural polymorphism occurring within the population, as alleles found in both *mtCOI* and *EΦ1a* loci of healthy clams differ between individuals. *L. balthica* is generally characterized as a species of high genetic variability among all of its populations worldwide, mostly associated with its local adaptations for environmental conditions (Yurchenko et al. 2018). Such variability is also observed in populations inhabiting the Gulf of Gdańsk, where distinct genetic structures are observed between shallow

and deep sites (Becquet et al. 2013, Lasota et al. 2018) as populations inhabiting deeper areas are partially isolated by straitened water mixing, sea currents, and seasonally by thermocline and halocline (Kowalewski 1997, Kruk-Dowgiałło & Szaniawska 2008). A possible bottleneck effect that some Baltic *L. balthica* populations may have undergone (Belov 2011) can affect their susceptibility for evolving transmissible cancer lineages. These results suggest that BTN affecting the Baltic clam may also be present in other clam populations on a worldwide scale due to an extensive geographical range of the species and its evolutionary history (Väinölä 2003, Pante et al. 2012). DN has been already diagnosed in various *L. balthica* populations, e.g. from Finnish coast of Baltic Sea (Pekkarinen 1993), Wadden Sea (Dairain et al. 2020), and Chesapeake Bay (Christensen et al. 1974), although this paper is the first confirming clonal aetiology of this disease.

The *L. balthica* population chosen for our study comes from a site (H45) that is considered a relatively deep (45 m) sampling area in the gulf and is characterized by historically highest DN frequency in this species, ranging from 25 to 94%, depending on sampling year (Thriot-Quiévreux & Wołowicz 2001, Smolarz et al. 2005bd, Ogrodowczyk 2017). This population has been under investigation for many years, not only in terms of DN occurrence, but also because of adverse environmental conditions that occur in the site area, such as oxygen depletion and/or presence of toxic hydrogen sulphide, either temporal, seasonal or constant in some years, as well as anthropogenic pollution consisting of, among others, heavy metals, aromatic polycyclic hydrocarbons (WWAs), or polychlorinated biphenyls (PCBs), staying at a relevant level in water and/or sediments throughout the years (Renner et al. 1998; Pazdro et al. 2004, Kot-Wasik et al. 2004, Zaborska et al. 2019). Such challenging environmental characteristics, along with ecological, individual and genetic variations (e.g. trophic position, sex, fitness, genetic structure) were previously proposed to be carcinogenic factors in DN induction in the Baltic Sea (Wołowicz et al. 2005). Although our study confirms the transmissible character of DN in*L. balthica* , the role of potential pollution and other environmental factors on cancer development and susceptibility should not be overlooked. Chronic and acute exposure to various pollutants, temporal anoxic conditions, immunosuppression and co-occurring oxidative stress may potentially increase vulnerability of bivalves to contagious cancer cells (Metzger & Goff 2016) and/or induce the expression of genome-integrated retrovirus elements, as some studies also suggest the role of transmissible retroelements in BTN induction (Arriagada et al. 2014).

NCs isolated from *L. balthica* are described as highly aneuploid with high disseminating potential, enormous and pleomorphic nuclei, and low amount of cytoplasm (Thriot-Quiévreux & Wołowicz 1996, Thriot-Quiévreux & Wołowicz 2001, Smolarz et al. 2005ac; Smolarz et al. 2006a). These features of NCs are similar in all bivalve species (Barber et al. 2004, Carballal et al. 2015) suggesting some universal characteristics of DN within Bivalvia. Most probably, the cellular mechanism by which NCs are able to be transplanted between individuals evading immunological signalling is also common between taxa. Due to the absence of adaptive immunity in bivalves, biochemically changed NCs do not provoke effective pathogen-directed defence systems in these animals and those cells are able to clone themselves and disseminate into the tissues of other hosts (Metzger & Goff 2016, Ujvari et al. 2016). The mechanism of NCs transmission is not fully described yet, but it is believed that single clonal cancerous cells that originate in one neoplastic individual are expelled from its body in, either by direct release of DNA from heavily neoplastic animals (Giersch et al. 2021) or possibly through spawning or death events, and are then transmitted to other individual(s) via seawater uptake. This hypothesis is supported by studies showing successful inoculation of NCs and/or haemolymph from neoplastic to healthy bivalve through injection which resulted in further DN development and also transmission of NCs through cohabitation in different bivalve species (thoroughly discussed in Carballal et al. 2015 and Metzger et al. 2015). The studies of viability of NCs from*M. arenaria* shows that these cells are able to survive in the water column for several hours (Sunila & Farley 1989) or even up to 8 weeks in lower temperatures (Giersch et al. 2021). The success of implantation to another organism is determined by the water circulation and density of animals (Elston et al. 1990) and most probably with the filtrating potential of species. It was also reported that NCs from the*Mytilus* BTN cell line (MtrBTN2) can survive outside of host's organism up to 6 days (Burioli et al. 2021) giving plenty of time for potential transmission via water filtration. To date, there is no published paper considering *L. balthica* NCs viability, but in our routine lab work we observe that those cells can survive at least two hours when kept in an isotonic solution

(Czajkowska 2021). Ecological consequences of DN/BTN can be extremely severe as it was documented that some populations affected with this cancer experience increased mortality, even mass mortality in some cases (Farley et al. 1986, Farley et al. 1991, Muttray et al. 2012, Benabdelmouna & Ledu 2016) with critical impact on surrounding ecosystems. Such events are also observed in *L. balthica* from Gulf of Gdańsk, especially from the H45 population that is investigated here, which is affected by mass mortality occurring in bi- or triennial periods lead by an increase in DN prevalence (Sokołowski et al. 2004, Wołowicz et al. 2005).

As stated previously, disseminated neoplasia has also been diagnosed in three other bivalve species from the Gulf of Gdańsk, Poland, namely*M. arenaria* , *C. glaucum* , and *M. trossulus* , although at a much lower prevalence (Smolarz et al. 2005d; Smolarz et al. 2006b; Ogrodowczyk 2017). Further studies are needed to determine if DN in these species is also a BTN and if cancer contagiousness is related to intra- or interspecies transmission.

Our study presents the first evidence of transmissible aetiology of this cancer in *L. balthica* , and provides a base for further investigation of the severity of this transmissible cancer in other*L. balthica* populations as well as in other Baltic species affected with DN. Adding *L. balthica* to the list of BTN-affected species resulting in overall eight BTN-affected species and nine cancer lineages, making the transmissible cancer even more common in the biological world than it was thought earlier. The widespread occurrence of BTN in multiple genera is an interesting phenomenon in cancer biology, and this phenomenon has potential to be a model disease (Aguilera 2017, Fernández Robledo et al. 2019) for in-depth understanding of leukemic diseases in other organisms, including humans.

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Data accessibility and Benefit-Sharing Statement

Sequence alignment of *mtCOI* and *EΦ1a* loci are supplied in Supplementary Information and raw sequence reads will be deposited in the GenBank upon the time of publishing.

Benefits Generated: This research developed as part of international, multi-institutional collaboration. The research provided an opportunity for international student's internship therefore contributing to education and training. The training was also related to genetic resources with the full participation of Polish side of the research, providing new genetic data. All scientific information obtained within this study is accessible and is relevant for marine population studies. This research can provide as opportunity for institutional and professional relationships that will arise from an access and benefit-sharing agreement and subsequent collaborative activities.

Author Contributions

Alicja Michnowska – writing and editing manuscript, data acquiring and interpretation, sample collection and processing, conceptualisation

Samuel Hart – data acquiring and interpretation, manuscript editing

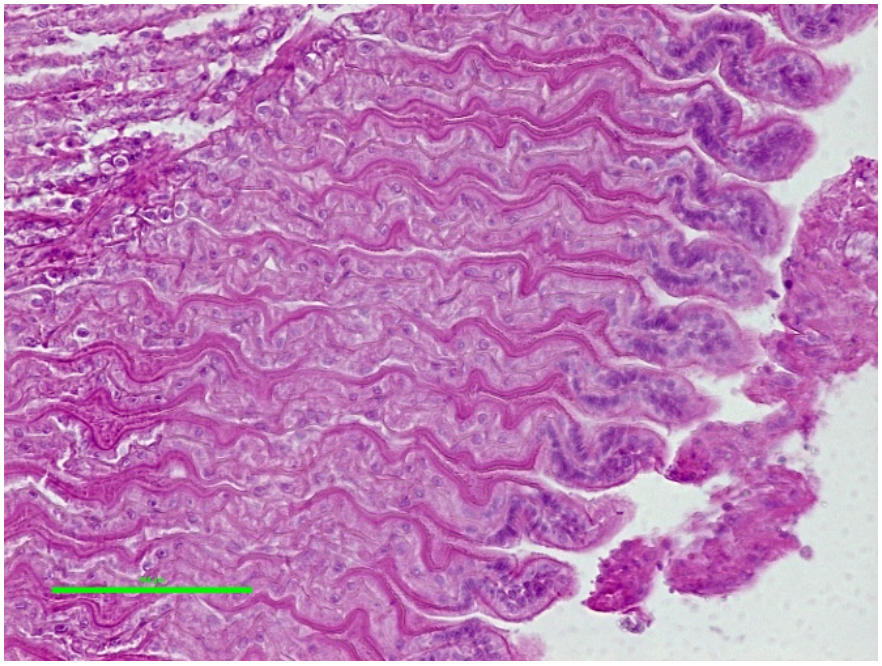
Katarzyna Smolarz – fund raiser, manuscript editing, sample collection and processing, conceptualisation

Anna Hallmann – manuscript editing, sample collection

Michael Metzger – writing and editing manuscript, data acquiring and interpretation, conceptualisation, fund raiser

Tables and Figures

Fig. 2.1. Location of *L. balthica* sampling site. Map was generated using SimpleMappr (Shorthouse 2010).



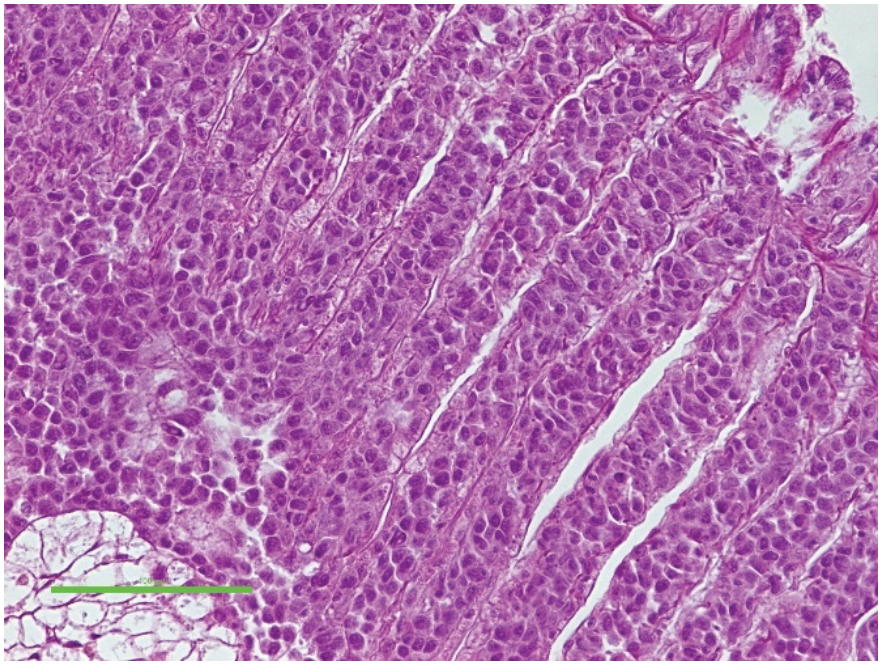


Fig. 2.2. Histological representation of gills from healthy (individual #88, left) and neoplastic (individual #158, right)*L. balthica,* H&E staining. Note the amount of highly hematoxylin-stained cells destroying tissue structure in neoplastic individual. Photographed under light microscope, x200.

Tab. 2.3. PCR primers used for amplification and sequencing.

Locus	Forward primer	Reverse primer	Annealing (°C)	Size (bp)
EΦ1α	SHO-086 _limecola_EF1_F CTTGACCTCACCAG[illegible]GGT	SHO-088 _limecola_EF1_R7 [illegible]GGTGACTCAAAGGT	50	~903-920
mtCOI	LCO1490Mba GTAGAACTAAYCAT[illegible]	HCO2198Mba [illegible]GGTGRCCAAAAAAYCA	50	~655

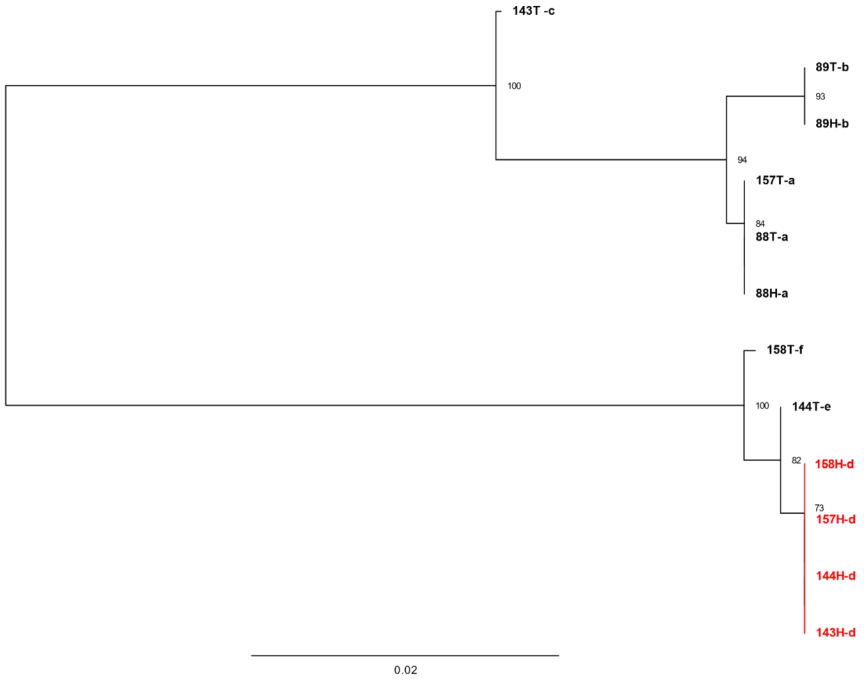


Fig. 3.1. Phylogenetic tree of *mtCOI* sequences from healthy (88, 89) and neoplastic (143, 144, 157, 158) *L. balthica*individuals. Numbers above the nodes indicate bootstrap values; values below 50 are not shown. Common cancer lineage observed in several neoplastic clams is highlighted in red. Scale bar represents genetic distance between sequences. T or H: sequence obtained from solid tissue (T) or haemolymph (H) of each clam; b-f – allele labels.

Tab. 3.1. *mtCOI* alleles associated with haemolymph (H) and host solid tissue (T) found in tested individuals, cancer-associated allele is highlighted in red.

	T	H
88 healthy	a	a
89 healthy	b	b
143 neoplastic	c	d
144 neoplastic	e	d
157 neoplastic	a	d
158 neoplastic	f	d

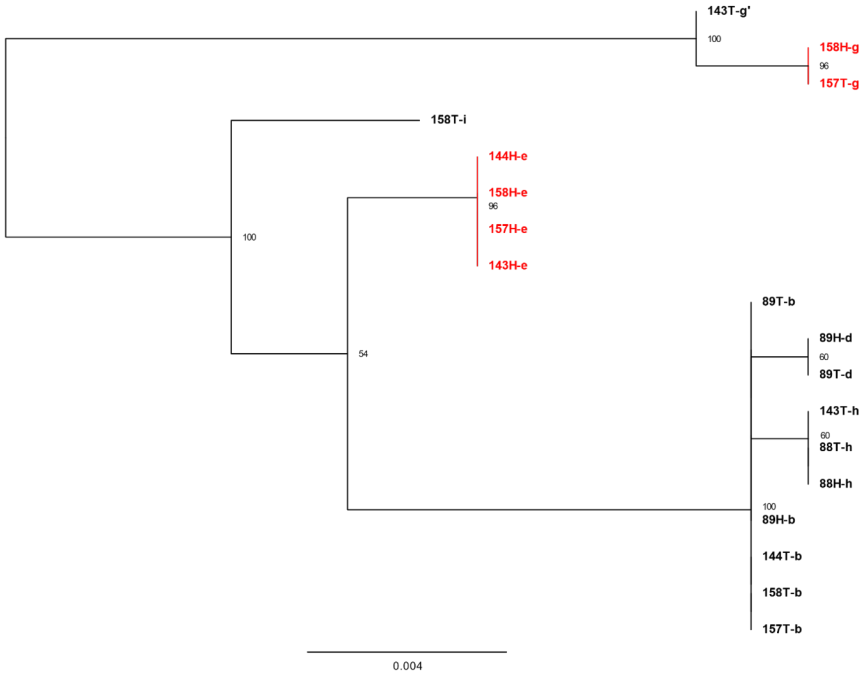


Fig. 3.2. Phylogenetic tree of *EΦ1a* sequences from selected *L. balthica* individuals. Numbers above the nodes indicate bootstrap values; values below 50 are not shown. Common cancer lineage observed in several neoplastic clams is highlighted in red. Scale bar represents genetic distance between sequences. H - haemolymph; T – host tissue; b-i – allele labels.

Tab. 3.2. EF1α alleles associated with haemolymph (H) and host's solid tissue (T) found in analyzed individuals, cancer-associated allele is highlighted in red.

	T	H
88 healthy	h	h
89 healthy	b, d	b, d
143 neoplastic	h, g'	e
144 neoplastic	b	e
157 neoplastic	g, b	e
158 neoplastic	b, i	e, g

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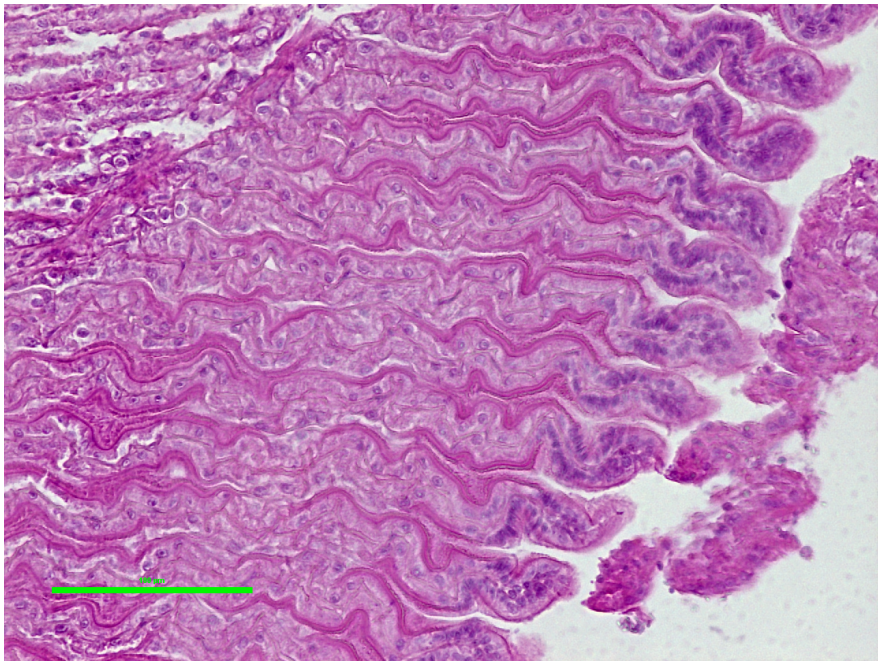

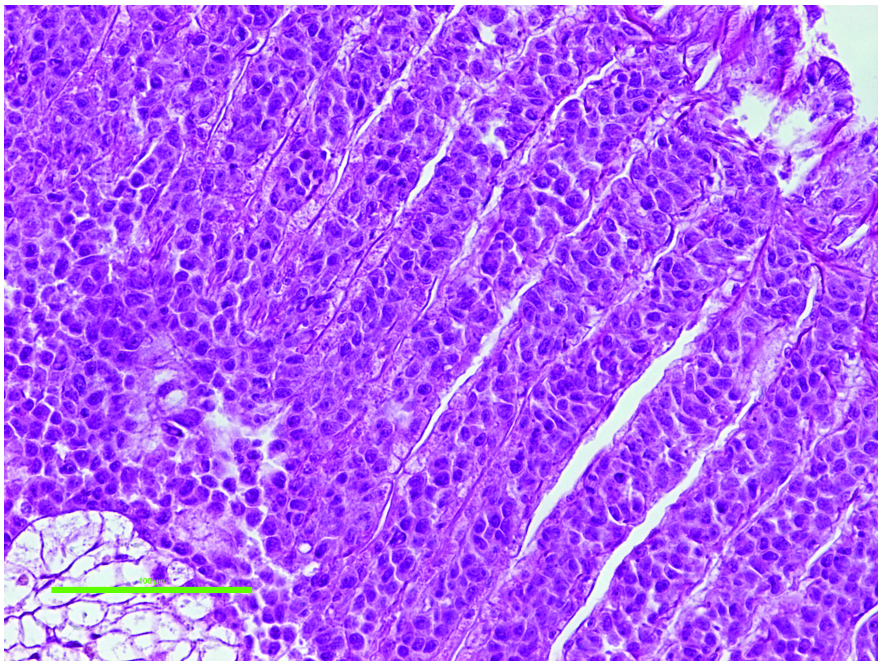

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