Multiple lineages of transmissible neoplasia in the basket cockle (*Clinocardium nuttallii*) with repeated horizontal transfer of mitochondrial DNA

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ABSTRACT

Transmissible cancers are clonal lineages of neoplastic cells able to infect multiple hosts, spreading through the environment as an infectious disease. Transmissible cancers have been identified in Tasmanian devils, dogs, and bivalves. Several lineages of bivalve transmissible neoplasias (BTN) have been identified in multiple bivalve species. In 2019 in Puget Sound, disseminated neoplasia was observed in basket cockles (*Clinocardium nuttallii*), a species that is important to the culture and diet of the Suquamish Tribe as well as other tribes with traditional

access to the species. To investigate disseminated neoplasia in cockles, a nuclear locus was amplified from a collection of cockles from Agate Pass, and DNA sequences showed evidence of a single cancer lineage, termed CnuBTN1, in three cockles. We used cytology and a qPCR assay targeting sequences found in BTNs to analyze collections of cockles from eleven locations from Puget Sound and the Washington coastline to screen for BTN and identify the extent of cancer spread. Three lineages of BTN in cockles were identified, with CnuBTN1 being the most prevalent and geographically widespread and the others only found in one location each. While multiple nuclear loci show all samples of CnuBTN1 come from a single lineage, the mitochondrial alleles in each cockle with CnuBTN1 are different from each other, suggesting mitochondrial genomes of this cancer have been replaced multiple times during its evolution through horizontal transmission. The identification and analysis of these BTNs are critical for broodstock selection, management practices, and repopulation of declining cockle populations, which will enable the continued cultural connection and dietary use of the cockles by the local Tribes.

Introduction:

Cancer arises from oncogenic mutations within the cells of an organism and, despite being able to grow and metastasize, most cancers are confined to live and die with the body in which they originate. However, there are rare cases of naturally occurring transmissible cancers that have evolved to spread between individuals within or across species as an infectious allograft, causing disease long after their original host is gone (1). This phenomenon has been observed once in dogs (2, 3), twice in Tasmanian devils (4, 5), and at least eight times in several marine bivalve species (6-10).

In bivalves, disseminated neoplasia (DN) is a leukemia-like disease characterized by a massive amplification of rounded, polyploid cells in the bivalve hemolymph (circulatory fluid) that eventually progresses to cancer dissemination into host tissues and usually results in death of the individual. Outbreaks of DN in marine bivalves across the globe have historically led to massive die-offs of affected populations, posing a threat to commercial aquaculture and species conservation efforts (11-14). Of the bivalve species affected by outbreaks of DN, recent genetic analyses of DN in soft-shell clams (*Mya arenaria* (6)), mussels (*Mytilus trossulus, M. edulis, M. chilensis* (7, 9)), common cockles (*Cerastoderma edule* (7)), golden carpet shell clams (*Polititapes aureus* (7)), baltic clams (*Macoma balthica* (8)), and warty venus clams (*Venus verrucosa* (10)) have revealed the DN affecting these species to actually be cases of bivalve transmissible neoplasia (BTN), suggesting that transmissible cancer may be a more common phenomenon in marine invertebrates than previously recognized.

The basket cockle, *Clinocardium nuttallii*, is a culturally significant shellfish species for the Suquamish Tribe, as well as many other coastal tribes from Washington to Alaska, who engage in subsistence foraging of native shellfish species as a cultural and dietary practice (15). *C. nuttallii* occurs in both intertidal and subtidal habitats with a wide range along the Pacific coast from southern California up to western Alaska and Japan (16, 17). A long-term survey of Washington

C. nuttallii populations showed varied population dynamics across beaches in the southern Salish sea with decreased population abundance observed on some beaches, while other locations maintained steady or increased in population abundance (16). However, Suquamish Tribal members have notes declines in local subsistence basket cockle populations over the last two decades. The cultural importance of this species has driven efforts to investigate *C. nuttallii* aquaculture production programs to supplement wild populations.

During health assessments of potential broodstock populations, several individuals were diagnosed with DN through histological analysis (Ralph Elston, AquaTechnics), raising questions of the impact of BTN on the health and viability of *C. nuttallii* aquaculture strategies, given the negative impact of BTN on previous and current commercial shellfish farms.

While moving toward hatchery production of cockles, the Suquamish Tribe and the Puget Sound Restoration Fund simultaneously pursued research looking into whether or not genetic population structure exists in cockle populations of Washington State. Twelve collections were performed throughout Washington State with permission and assistance from multiple Tribes (18). These collections were also sampled for the presence of DN.

In this study we investigated the DN found in *C. nuttallii* to determine whether the cancer is a BTN. Combining the analysis of multiple nuclear genetic markers with cytology of cells in the cockle hemolymph, we identified at least two independent lineages of *C. nuttallii*-derived BTN (CnuBTN), CnuBTN1 and CnuBTN2. We developed a qPCR-based assay to screen cockle populations collected from beaches across the Salish Sea and Washington's Pacific coast, finding this cancer was present in populations at four of eleven locations in the northern Salish Sea. Interestingly, analysis of multiple mitochondrial DNA (mtDNA) markers showed extensive mitochondrial replacement and recombination occurring in both lineages of CnuBTN, an observation that has been made in three other transmissible cancers, including the canine transmissible venereal tumor (CTVT) in dogs (19-21) and BTNs in *M. chilensis* (9), and *C. edule* (bioRxiv ref), suggesting altered mitochondrial dynamics may be an important factor in the evolution of these cancers.

Results:

Disseminated neoplasia in cockles is a bivalve transmissible neoplasia

Due to the recent identification of DN in Puget Sound basket cockles (*C. nuttallii*), found through histological examination during health screening of potential broodstock, we set out to determine whether the DN was a transmissible cancer, as seen in other bivalves. We first examined hemolymph samples from cockles collected from Agate Pass, Washington (Figure 1A), a site where DN had previously been observed in cockles through histological analysis(22). Hemocytes of non-neoplastic cockles are highly adherent to tissue culture dishes and show a variety of cell types, many with pseudopodia (Figure 1B). In this collection of cockles from Agate Pass, we identified three individuals with DN, in which nearly all cells in the hemolymph display the rounded, uniform, non-adherent morphology typical of DN cells ((6, 7, 11, 12) Figure 1C).

115 We extracted DNA from cells in the hemolymph taken from three cockles with DN and three
116 "healthy" control animals without observed DN. We also sampled mantle tissue from each
117 animal to compare genotypes. In transmissible cancers, the genotypes of the cancer cells do not
118 match genotypes of the hosts, and instead they match those of other cancer cells from other host
119 animals (1, 6, 7). To test whether disseminated neoplasia in basket cockles is a BTN, we generated
120 a set of intron-spanning primers in a conserved gene (*α-tubulin*) that would amplify a
121 polymorphic region in all healthy and neoplastic samples. In the three healthy animals, one or
122 two alleles were identified and the same one or two alleles were identified in DNA from both
123 hemolymph and solid tissue samples (alleles are given arbitrary letter names with identical alleles
124 sharing the same letter, Figure 1D). In contrast, in neoplastic individuals, alleles found in the
125 hemolymph (mostly cancer) and the solid tissue (mostly host) were different, and nearly identical
126 alleles were found in the cancer samples for each of the three samples. The k' and k alleles differ
127 by one unique single nucleotide variant (SNV), likely due to somatic mutation within the cancer
128 (we subsequently refer to these both as the k allele). This confirms that disseminated neoplasia in
129 *C. nuttallii* is a bivalve transmissible neoplasia (termed CnuBTN).

130 Unexpectedly, we observed three alleles at this locus that were specific for the cancers instead of
131 just two (Figure 1D). This could be due to a duplicated copy of the gene in the genome of the
132 founder cockle that had the original cancer, or it could be due to horizontal transfer of nuclear
133 DNA into the cancer lineage from host cells or another cancer lineage. One of the neoplastic
134 samples (Y504) has a different third allele from the other two, (allele e instead of f), suggesting
135 that horizontal transfer of nuclear DNA may have occurred in these lineages. Based on the
136 quantitation of the allele frequencies in the cancer samples, the three alleles appear to occur in
137 roughly 1:1:1 proportions (Figure S1). This suggests the three alleles are not due to a minor
138 contamination and likely reflect the true genotype of the cancer cells at a triploid locus. Despite
139 the variable third allele, the k and c alleles are present in all three cockle cancer samples, strongly
140 suggesting that the neoplastic cells originated from a single cell lineage.

141 We designed a qPCR primer pair specific for the cancer-associated *α-tubulin* k allele, as well as a
142 control "universal" qPCR primer pair that is in a conserved region of *α-tubulin* and which
143 amplifies in all cockles. qPCR data for CnuBTN-positive animals show that the putative cancer-
144 associated k allele can be found in the solid tissue DNA samples of the neoplastic individuals,
145 likely due to dissemination of BTN into host tissues, however, it is found in a much higher
146 fraction in the DNA from the hemolymph samples, as would be expected for BTN (Figure 1E, (6,
147 7)).

148

149 **Survey of Puget Sound reveals evidence of three lineages of CnuBTN**

150 In order to determine the extent of the spread of this infectious cancer (CnuBTN) in basket cockle
151 populations, we analyzed samples from a large population survey from Puget Sound and across
152 Washington State (18). Approximately 30 individuals from 11 different locations were sampled.
153 We used both a slide-smear hemocytology method (22) to analyze cell morphology and the k-
154 specific qPCR assay to screen DNA from the hemolymph and tissue of each cockle. We first
155 analyzed samples from Semiahmoo, WA, as histological analysis of cell morphology suggested

156 that 2 individuals (SM26 and SM6) at that location had advanced DN (Figure 2A-D). qPCR
157 analysis of one of those individuals, SM26 showed a high frequency of the putative cancer-
158 associated k allele in the hemolymph and a lower frequency in the host tissues, similar to what
159 was observed in DN+ individuals from Agate Pass (Figure 2C,E). However, we also found two
160 cockles (SM21 and SM29) with normal hemocyte morphology, and with a high frequency of the
161 k allele in both hemolymph and tissue DNA (Figure 2B,E). This showed that the k allele was the
162 endogenous germline allele in those animals, and that while the k allele can be used as a sensitive
163 screen for CnuBTN, it is not specific. As such, BTN can only be confirmed if the hemolymph:tissue
164 ratio of the k allele is higher than 1.

165 Although morphological analysis of cells from cockle SM6 showed clear evidence of disseminated
166 neoplasia (Figure 2D), the k allele was not present in a high fraction of the hemolymph.
167 Amplification and sequencing of this cockle revealed that the cancer-specific alleles in SM6 were
168 different from the host, but that, instead of alleles k and c, they were both different alleles (p and
169 o), that were more closely related to alleles found in other individuals than they were to the first
170 lineage of CnuBTN. This shows that SM6 carried a different BTN lineage (CnuBTN2) that is likely
171 independent of the BTN lineage found in SM26 and the pilot Agate Pass samples (now termed
172 CnuBTN1). A p allele-specific qPCR primer pair confirms that this allele was found
173 predominantly in the hemolymph of SM6, as expected for a BTN, but it was not found in any
174 other samples (Figure 2F,G).

175 Cockles from the other 10 sites were also surveyed using morphological analysis (to identify
176 individuals with high levels of DN) and qPCR analysis (to identify individuals with the k and p
177 alleles indicative of CnuBTN1 and CnuBTN2, respectively) (Figure 3). Individuals identified as
178 likely k- or p-allele positive through qPCR screening were further verified by cloning and
179 sequencing of the *α-tubulin* locus. We confirmed the presence of CnuBTN1 in three locations in
180 Puget Sound (Semiahmoo, Agate Pass, and Penn Cove), while CnuBTN2 was only found in
181 Semiahmoo. Additionally, in Sequim Bay, one BTN-positive individual (SBg14) was identified
182 that carried the k allele and a second *α-tubulin* allele (n) different from the alleles found in other
183 CnuBTN1 samples. Although the slide-smear image was inconclusive in this animal due to a low
184 number of cells, this likely represents a third neoplasia lineage (here termed CnuBTN3) in basket
185 cockles in Washington (Figure 4).

186 Phylogenetic analysis of the *α-tubulin* alleles provides evidence supporting three independent
187 CnuBTN lineages. To confirm this finding, we used 10 microsatellite primer pairs designed to
188 identify polymorphic loci in basket cockles (Figure 5). Microsatellite alleles reveal differences
189 between the individual samples of CnuBTN1, but all CnuBTN1 microsatellite genotypes cluster
190 together, consistent with the hypothesis that all samples identified as CnuBTN1 come from a
191 single BTN lineage. Additionally, SM6 and SBg14 microsatellite genotypes are separate both from
192 the CnuBTN1 cluster and from each other, providing further evidence that these samples
193 represent additional independent lineages of BTN (CnuBTN2 and 3).

194

195 **Horizontal transfer of mitochondrial genomes in CnuBTN1**

196 Our analysis of multiple nuclear markers showed that CnuBTN1 samples form a clonal lineage;
197 however, sequencing of mitochondrial DNA revealed that every sample of BTN observed in this
198 study had a different mitogenome sequence. Sequences of both cytochrome c oxidase (*mtCOI*)
199 and cytochrome b (*mtCYTB*) show that the BTN-associated mitochondrial alleles are different
200 from host alleles, but the CnuBTN1 sequences do not support a single mitochondrial origin for
201 this cancer lineage (Figure 6). A minimum of two separate horizontal transfer events within
202 CnuBTN1 would be needed to explain the mitochondrial sequence divergence (HT1 for cockle
203 Y502 and HT2 for cockle SM26). Additionally, at least three separate mitogenome alleles were
204 observed in the case of Y504, suggesting either simultaneous infection with multiple clones, or
205 ongoing heteroplasmy and recombination (the alleles could not be confidently phased and are
206 therefore excluded from the phylogenetic tree).

207

208 **Discussion:**

209 This study provides the first report of transmissible cancer in basket cockles (*C. nuttallii*), with
210 evidence from multiple nuclear loci (*α-tubulin* alleles and 10 microsatellite loci) showing likely 3
211 independent lineages of *C. nuttallii* BTN (CnuBTN1-3).

212 One limitation of the current study is the observation that the CnuBTN1 qPCR diagnostic assay,
213 first thought to be cancer-specific, amplifies an allele later found in healthy animals at several
214 locations in Puget Sound. The cancers arise from normal cockles, so this is to be expected, but it
215 highlights the difficulty in relying on a single qPCR marker. Recently, a similar study found that
216 an *EF1-α* allele previously only found in the *Mytilus trossulus* BTN (MtrBTN1) is also present in
217 the germline of non-neoplastic animals in Northern Europe (23, 24). This suggests that the cancer
218 may have arisen from the Northern Europe mussel population, but also renders that marker non-
219 specific in animals with that allele. In the absence of better markers and more complete surveys
220 of healthy genetic diversity, researchers must use a combination of diagnostic assays, or use the
221 cancer:host tissue ratio of the marker to determine if a sample is host to a transmissible cancer
222 lineage.

223 Additionally, our finding of multiple lineages of CnuBTN relied on the identification of neoplastic
224 cells through morphological analysis, highlighting that a diagnostic approach based on genetic
225 markers alone can miss the presence of additional lineages of transmissible cancer in bivalve
226 populations.

227 Unexpectedly, while the nuclear loci strongly support a single clonal lineage of CnuBTN1,
228 mitochondrial DNA haplotypes are not consistent with a clonal origin. Horizontal transfer of
229 mitochondrial DNA has been reported in the canine transmissible venereal tumor of dogs (19,
230 21), as well as more recently in the BTNs of *Mytilus trossulus* (9) and the common cockle,
231 *Cerastoderma edule* (25). Our observations of multiple horizontal transfer events within just a few
232 samples of the CnuBTN1 lineage suggest that cockles of this specific lineage may have a
233 predisposition to mitochondrial transfer. For example, the MtrBTN2 lineage has now been
234 observed in four different species in Europe, South America, and Asia, and, to date, only one
235 horizontal transfer event has been documented. No horizontal transfers have yet been observed
236 in *Mya arenaria* BTN. It is unclear in these cases whether mitogenome replacement is due to

selection for more functional mitochondria after degradation of the mitogenome through the Muller's Ratchet of somatic mutation or due to a selfish benefit to the mitochondrial genome itself (as suggested for CTVT based on the repeated transfer of alleles with the same mutation in the mitochondrial control region (21)). The horizontal transmission of DNA is not limited to transmissible cancers. It has been suggested that this sort of parasexual recombination may be important for the evolution of human cancers (26), and human cancer cells have been shown to take up functional mitochondria from cancer-associated host cells in multiple studies (27). Based on our observation of frequent replacement of mitogenomes in cockles, these lineages are critical models for understanding the process of transfer and the selection pressures involved in cancer mitochondrial evolution.

Bivalve transmissible cancer is often a fatal disease, and in some cases has been reported to cause severe population loss of more than 90% (13, 14). It is clear that at least two lineages of CnuBTN are present in the basket cockle populations in Puget Sound, but it is not yet known whether they are causing mortality events or how they are being spread from one location to another. Further research on this newly described infectious agent will provide an important current baseline of geographic and seasonal occurrences of this disease, enable understanding of how it spreads through the environment, how future climate conditions may affect incidence and severity, how it affects the survival and selective pressures on cockles, and how aquaculture and ecological management strategies might mitigate the disease in the future.

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278 **Sequence Availability:**

279 All sequences will be available on NCBI Genbank (BioProject in progress).

280

281 **Materials and Methods:**

282 **Sample collection:** Pilot study samples were collected in April 2019 at low tide from the intertidal
283 zone of Agate Pass, WA with a shovel or clam rake. Cockles were anesthetized in 50g/L MgCl
284 ×6H_2O in artificial seawater (Instant Ocean) for 1 hr until the valves opened and did not close
285 when pressure was applied. 0.5-1mL of hemolymph was extracted from the anterior or posterior
286 adductor muscle of each animal using a 1 inch 26-gauge needle. Several drops of hemolymph
287 were placed in a well of a 96-well plate and incubated for about 1 hr at 4°C to allow normal cells
288 to adhere to the plate, and samples were screened visually for obvious disseminated neoplasia as
289 previously described (28). The remaining hemolymph was spun down at 1,000×g for 10 minutes
290 at 4°C to pellet the cells and remove cell-free hemolymph. Animals were then sacrificed and ~50
291 mg of mantle tissue was collected and frozen at -80°C either in ethanol or in RNAlater (Invitrogen)
292 until DNA extraction.

293 Additional cockles from 12 intertidal collections from across the Salish Sea and Pacific coast of
294 Washington were sampled in October 2019 to January 2020, as described (18). At Sequim Bay,
295 cockles were collected from both the open intertidal area (SB) and from within wire-enclosed
296 tubes in the intertidal area that were used for geoduck aquaculture (SBg). For hemolymph
297 collection from animals without MgCl treatment, the syringe was placed between the edges of
298 the valves, and, if needed, a small hole was bored into the anterior edge of the valves and 0.5-1
299 mL hemolymph was collected from the muscle and processed as above.

300 **Cytology:** Fixing of cells for histology was done with fresh hemolymph, as described in detail
301 previously (22). Briefly, 100 µL of the collected hemolymph was applied to poly-L-lysine slides
302 (Electron Microscopy Sciences, Hatfield, PA, USA) and allowed to rest for ten minutes at room
303 temperature on a flat surface. A "blood smear" was performed by gently removing excess
304 hemolymph using a coverslip and air drying. The adhered hemocytes were then fixed in 100%
305 methanol for 15 minutes. The slides were removed from methanol, air dried at room temperature,
306 and stained after hydration in using a Hematoxylin & Eosin fixation protocol (Leica, USA).
307 Cytology was conducted for all cockles, excluding the April 2019 cockles from Agate Pass (Y),
308 and the cockles from Hood Canal (HC), Eld Inlet (SS), the geoduck tubes at Sequim Bay (SBg),
309 and cockles 13-30 from Squaxin Island (SX).

310 **DNA extraction:** DNA was extracted from ethanol-fixed mantle and hemocyte samples using
311 DNeasy Blood & Tissue Kit (Qiagen) (18). Mantle extractions of the pilot samples were performed
312 with additional steps to remove PCR-inhibiting polysaccharides. After tissue lysis, 65 µl of P3
313 Buffer (Qiagen) was added for 5 minutes to precipitate out polysaccharides and spun down at
314 17k x g at 4°C for 5 minutes. The resulting supernatant was transferred to a new tube, 200 µl of
315 Buffer AL was added, and the manufacturer's protocol resumed.

316 ***α-tubulin, mtCOI,* and *mtCYTB* PCR and cloning:** Primers and annealing temperatures are listed
317 in Table S2. PCR amplification at the *α-tubulin* locus was done using Q5 Hot Start High-Fidelity

318 DNA Polymerase (NEB) with a 30 s extension time. Primers for *α-tubulin* were designed in exons
319 flanking an intron to amplify a ~850 bp intron-spanning region. Primers for *cytochrome B* were
320 designed to amplify a ~500 bp region. ~25 ng of genomic DNA was amplified for 35 cycles. PCR
321 products were gel extracted using spin columns (Qiagen) and sequenced, or, when multiple
322 alleles at a locus could not be resolved by direct sequencing, were cloned using the Zero Blunt
323 TOPO PCR Cloning Kit (Invitrogen). Plasmids were transformed into DH5α competent *E. coli*
324 (Invitrogen) and 6-12 clones were picked for further sequencing (Azenta/Genewiz). The primer
325 binding regions were excluded from sequence analysis. Allele-specific SNVs were identified after
326 cloning of *α-tubulin* alleles. PCR products were also sequenced using Nanopore rapid barcoding
327 chemistry, and after assembly of the consensus sequence, the number of reads supporting each
328 SNV were determined by aligning the raw reads to the consensus (sequencing and mapping done
329 by Primordium Labs, USA). For alleles with multiple unique SNVs, the frequencies of each SNV
330 corresponding to a particular allele were averaged to determine the allele frequency. One allele
331 lacked any unique SNVs (e), but it was different from the k allele by only a single SNV; In this
332 case, the e allele frequency for each sample was calculated by taking the k allele frequency in that
333 sample and subtracting the frequency of the SNV found only in k and not e (Figure S1).

334 **Trees:** All sequences were manually aligned in BioEdit (Hall, T.A., 1999). Maximum likelihood
335 phylogenies were built by PhyML 3.0 (29) using Akaike Information Criterion (30) with a
336 standard bootstrap analysis (100 repeats). The phylogenetic trees were visualized in FigTree
337 v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/).

338 ***α-tubulin* allele-specific qPCR:** Cancer-associated, allele-specific qPCR assays were developed
339 and performed in *C. nuttallii* genomic DNA samples using PowerUp SYBR Green Master Mix
340 (Applied Biosystems). Each primer set was designed to specifically amplify the cancer-associated
341 allele for CnuBTN1 and CnuBTN2, for *α-tubulin*, alleles kk (CnuBTN1) and p (CnuBTN2). A pair
342 of universal control primers were designed to amplify all possible *α-tubulin* alleles. Primers are
343 listed in Table S3. Standards for all targets consisted of a single plasmid containing the relevant,
344 cancer-associated allele target, which was linearized by NotI-HF (NEB) restriction digest before
345 qPCR. Serial dilutions (10^7-10^1 copies) of linearized standard plasmid in triplicate were used to
346 generate a standard curve for each qPCR run (quantified by qubit). In all experimental cases, 5-
347 50 ng of each genomic DNA sample was run in triplicate.

348 Allele-specific qPCR using *α-tubulin* k and *α-tubulin* universal control primers used a 3-step
349 cycling stage (95°C for 15s, 50°C for 15s, and 60°C for 30s, respectively) for 40 cycles, whereas
350 qPCR using *α-tubulin* p primers was run using a 2-step cycling stage protocol (95°C for 15s, 60°C
351 for 30s). The ratio of the cancer-associated allele to all alleles present at a given locus was
352 calculated for each sample using the average copy number of cancer-associated allele divided by
353 the average copy number of total alleles to generate an estimate of the fraction of total alleles that
354 are the cancer-associated allele. Animals were identified as potentially diseased if the ratio of
355 hemocyte and tissue cancer-associated allele fractions was at least 1.5 in CnuBTN1 (k), due to the
356 finding of animals with the k allele in the host genome, and at least 0.5 for CnuBTN2 (p). If the
357 control qPCR amplified below a threshold of 1,000 copies per reaction, the sample was excluded
358 due to low DNA extraction yield.

359 **Identification of microsatellite primers**

360 Following a microsatellite identification method used previously (31), in 2010, genomic libraries
361 were constructed from adductor muscle DNA from one individual cockle (DNeasy Tissue Kit,
362 Qiagen), from Deep Bay, British Columbia, Canada (49°27′35″N, 124°44′02″W), eluted with
363 molecular grade water. The resulting DNA was quality checked and submitted to the McGill
364 University and Genome Quebec Innovation Centre for 454 sequencing using a Roche GS-FLX
365 sequencer. Approximately 12% of 454 reads were assembled into 2,597 contigs with mean length
366 of 643 bp. A custom Perl script was used to search contigs and raw reads for uninterrupted
367 microsatellites with repeat motifs 2-10 bases long; duplicate loci were excluded. PCR primers
368 were designed using Primer3 (Rozen and Skaletsky 2000) for 89 unique microsatellites to test for
369 polymorphism that had <20 bp of flanking sequence and seven or more repeat units in 44 contigs.
370 We also found 396 di- or tri- microsatellites of 14 or more repeats and with <30 bp of flanking
371 sequence in 374 unassembled 454 reads. Sequence reads were also mined for tetranucleotide
372 repeats with at least 14 repeat units and 30 bp of flanking sequence; we found 68 of these, each in
373 a unique read. Sequences used for primer design were aligned and duplicates were excluded
374 (Sequencher v 4.8, Gene Codes Corp.). From this PCR primers were designed and used to screen
375 89 unique microsatellites.

376 For the initial testing of microsatellite primers, PCR amplifications used a DNA Engine Tetrad2
377 thermal cycler (BioRad, Hercules, California) in 5 μL volumes consisting of 0.14 U of Qiagen
378 HotStar Taq DNA polymerase, 1 μL 1:8 diluted DNA, 1× PCR buffer, 84 μM each dNTP, 0.50 μM
379 M13 fluorescently-labeled primer, 0.13 μM M13 labeled forward primer, 0.50 μM reverse primer,
380 and deionized H_2O. The thermal cycling profile included 15 min at 95°C followed by 8 cycles of
381 30 s at 94°C, 30s at 51°C and 30-40s at 72°C. Microsatellites were run on an ABI 3730, and
382 genotypes scored with ABI GeneMapper version 4.0 using an internal lane sizing standard. A
383 temperature gradient with annealing temperatures ranging from 48-59°C increasing in 1°C
384 increments was used to determine optimal annealing temperature for each locus. Initially, all 89
385 loci were tested for polymorphism on eight cockle samples from three sample sites in Canada,
386 after which a subset of 20 loci were selected for additional testing on 300 basket cockles from three
387 locations. Based on the level of polymorphism, allele size range, success of amplification, and ease
388 of scoring, 10 loci were chosen for further use. Microsatellite details, primer sequences and
389 optimal annealing temperatures for these loci are given in Table S4.

390 **Analysis of microsatellite loci:**

391 Microsatellites were amplified from DNA extracted from both hemocyte and tissue using Taq
392 polymerase (Genesee Scientific) for a select number of individuals: all possible neoplastic animals
393 based on positive qPCR screen (n=26) and one randomly selected, non-neoplastic animal from
394 each collection (n=12). Microsatellites were amplified using primers for 8 polymorphic loci:
395 Cnu55, Cnu58, Cnu63, Cnu68, Cnu72, Cnu78, and Cnu81 (Table S4). Allele sizes were identified
396 by fragment analysis using fluorescent primers (6-FAM, PET, NED, and VIC) and a 3730xl
397 Genetic Analyzer with the LIZ-500 size standard (Applied Biosystems, operated by Genewiz).
398 Alleles were called using Peak Scanner 2.0 (Applied Biosystems; see (7)), and rounded to the
399 nearest integer and binned using TANDEM (32) to account for split rounding of decimals and
400 assay variability (Table S4). One population of origin was assumed with ploidy set to two, though

401 for polyploid animals, the samples were split into two rows as if they were different samples to
402 accommodate all the alleles during integer correction.

403 After integer correction, the microsatellites were analyzed using R package, *PoppR* (33),
404 employing Bruvo's method with the infinite alleles model to build distance matrices for
405 phylogenetic tree creation. The variable "c" (repeat size) was set to 0.00001 resulting in the
406 distance between two alleles to be 0 (identical) or 1 (non-identical), adhering to the band sharing
407 model (6). The variable "sample" (number of bootstrap replicates) was set to 1000. All samples
408 were coded to be from one population. Each allele was classified as a dominant marker with a
409 total of 167 markers in 8 loci (6, 34). Each peak (allele) was coded as present (1), absent (2), or
410 ambiguous (0). In normal individuals, identical peaks were shown in the hemocyte and tissue
411 and considered present in both. In highly neoplastic animals, cancer-associated alleles were
412 present in the hemocyte to a lesser extent in the host solid tissue; occasionally the cancer-
413 associated alleles obscured possible host genotypes, resulting in an ambiguous call. The distance
414 between two samples in the distance matrix is the average of the pairwise comparison between
415 alleles at each size, excluding comparisons in which one or both individuals have an ambiguous
416 value. The phylogenetic tree generated by *PoppR* was visualized using FigTree v1.4.4.

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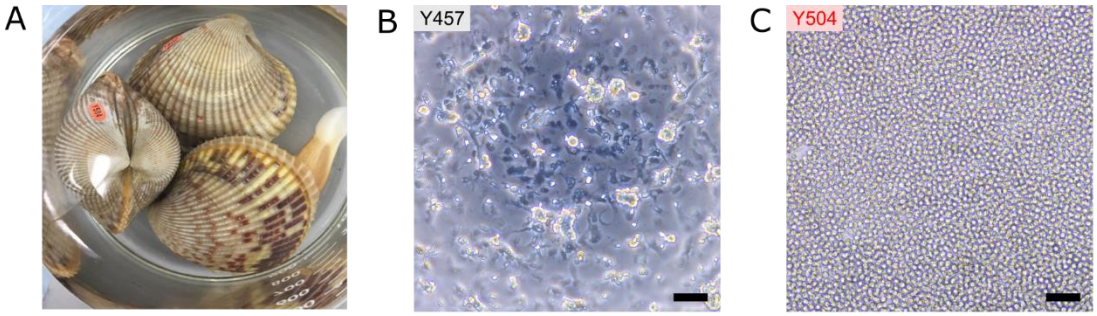

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Sample	Diagnosis	Hemocyte Allele(s)	Tissue Allele(s)
Y457	Healthy	a, c	a, c
Y466	Healthy	a	a
Y487	Healthy	c	c
Y502	Neoplastic	k, c, f	c, f
Y504	Neoplastic	k', c, e	f
Y506	Neoplastic	k, c, f	c

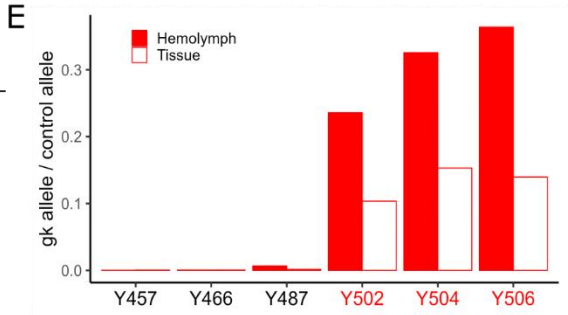
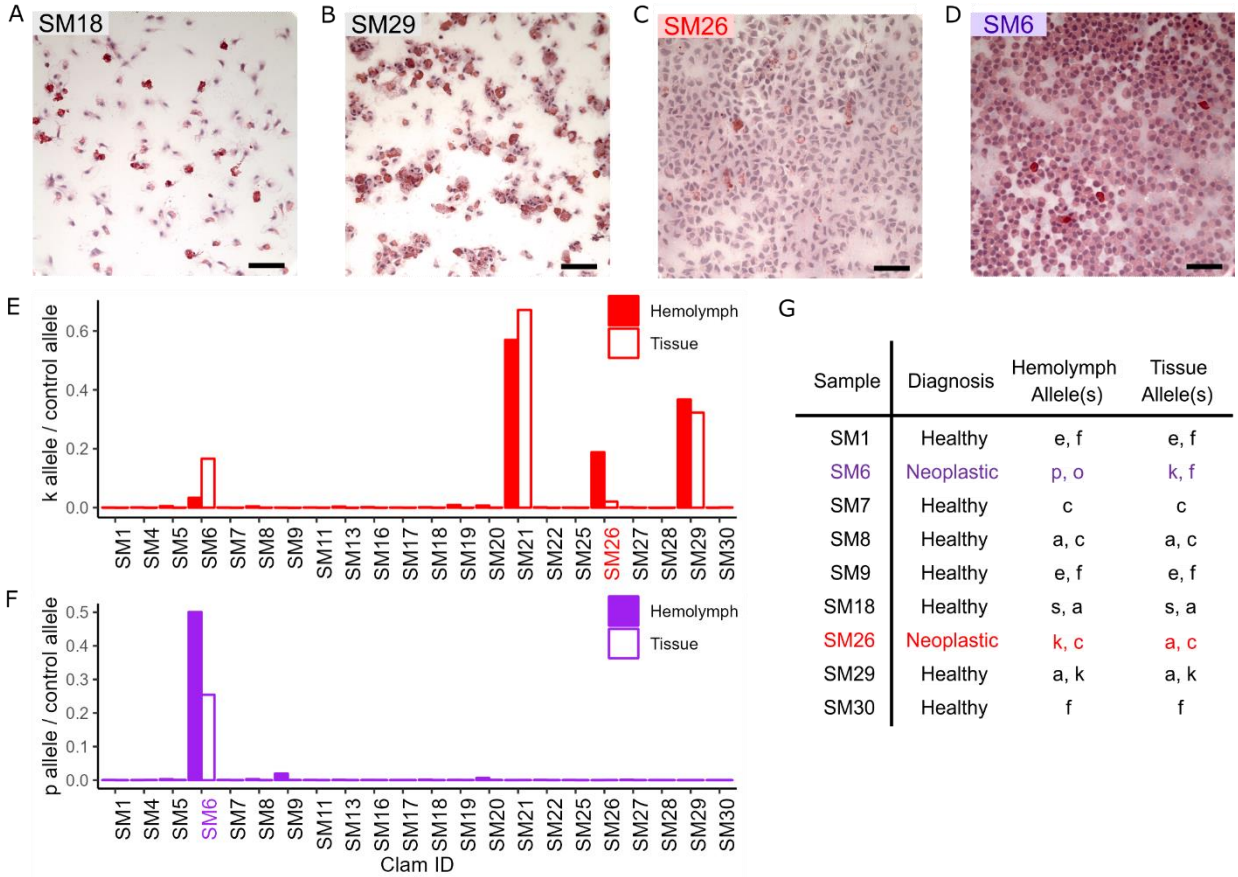


Figure 1. Identification of *Clinocardium nuttallii* bivalve transmissible neoplasia (CnuBTN1) as a transmissible cancer

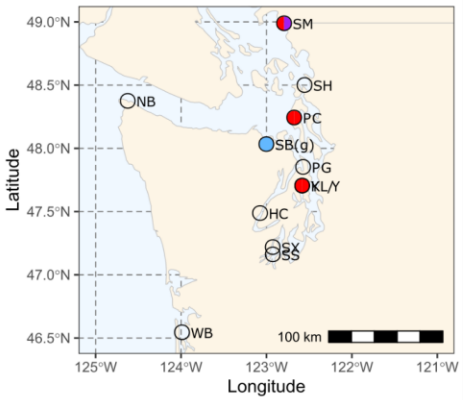
A representative image of three basket cockles (*Clinocardium nuttallii*) is shown (***A***), collected from Agate Pass, Washington, USA in April 2019, including one animal with neoplasia (Y504). Phase-contrast microscopy of cells from hemolymph shows the difference in morphology between representative healthy (***B***) and neoplastic cockles (***C***). Scale bars are 50 µm. A fragment of the nuclear *α-tubulin* was amplified and sequenced from three healthy cockles (black) and three cockles identified as neoplastic through morphological examination of cells in hemolymph (red), and the alleles identified in the hemolymph and solid tissue of each cockle are shown (***D***), showing the k and c allele in all three cases of cancer. A qPCR assay was developed to determine the fraction of the total *α-tubulin* alleles that were the cancer-associated k allele (***E***), showing a high fraction of the k allele in all three diseased animals, and a higher fraction of the k allele in the hemolymph than in the solid tissues, as expected for a BTN.



Sample	Diagnosis	Hemolymph Allele(s)	Tissue Allele(s)
SM1	Healthy	e, f	e, f
SM6	Neoplastic	p, o	k, f
SM7	Healthy	c	c
SM8	Healthy	a, c	a, c
SM9	Healthy	e, f	e, f
SM18	Healthy	s, a	s, a
SM26	Neoplastic	k, c	a, c
SM29	Healthy	a, k	a, k
SM30	Healthy	f	f

Figure 2. Screening of cockles from Semiahmoo, Washington reveals a second lineage of CnuBTN.

Cockles from Semiahmoo, Washington, USA, were analyzed by slide-smear hemocytology. Representative images from cockles diagnosed as healthy (***A***, ***B***) and neoplastic (***C***, ***D***) are shown (scale bar 50 µm). qPCR screening with the *α-tubulin* k-specific primers (***E***) showed that one neoplastic cockle was positive for the k allele in the hemolymph (SM26). The high level of k in both hemolymph and tissue in animals with normal morphology hemocytes (SM21 and SM29) show that these animals have the k allele in their germline. One cockle (SM6) was neoplastic by morphology, but negative for the k allele. The *α-tubulin* region was sequenced, and a p allele was identified that was specific for the cancer in this cockle (*F*). All alleles identified in this population are shown (***G***), with colors denoting healthy cockles (black), cockles with CnuBTN1 (red), and CnuBTN2 (purple).



Location	Code	Collected (n)	Quality DNA (n)	CnuBTN1 Verified (n)	CnuBTN2 Verified (n)	CnuBTN3 Verified (n)
Agate Pass	Y	6	6	3	0	0
Agate Pass	KL	30	30	0	0	0
Penn Cove	PC	30	29	1	0	0
Port Gamble Bay	PG	32	32	0	0	0
Squaxin Island	SX	30	28	0	0	0
Hood Canal	HC	30	30	0	0	0
Neah Bay	NB	30	29	0	0	0
Semiahmoo Spit	SM	30	22	1	1	0
Padilla Bay	SH	33	33	0	0	0
Eld Inlet geoduck	SS	30	23	0	0	0
Sequim Bay	SB	30	27	0	0	0
Sequim Bay geoduck	SBg	30	26	0	0	1
Willapa Bay	WB	30	23	0	0	0

Figure 3. Screening of 11 sites in Washington State for BTN shows widespread and patchy distribution.

Cockles collected from 11 different locations in Washington State were diagnosed for the presence of DN through cytology, and for three different lineages of transmissible cancer through qPCR and sequence analysis. Map was made with R package rnaturalearthhires (https://github.com/ropensci/rnaturalearthhires). Each site is marked as having no BTN (clear), CnuBTN1 (red), CnuBTN2 (purple), CnuBTN3 (blue), or more than one (multiple colors). The table lists the number of cockles collected, the number with extracted DNA of high enough quality to analyze, and the number of cases of each lineage of CnuBTN that were both identified through qPCR and were verified by sequencing of the *α-tubulin* locus.

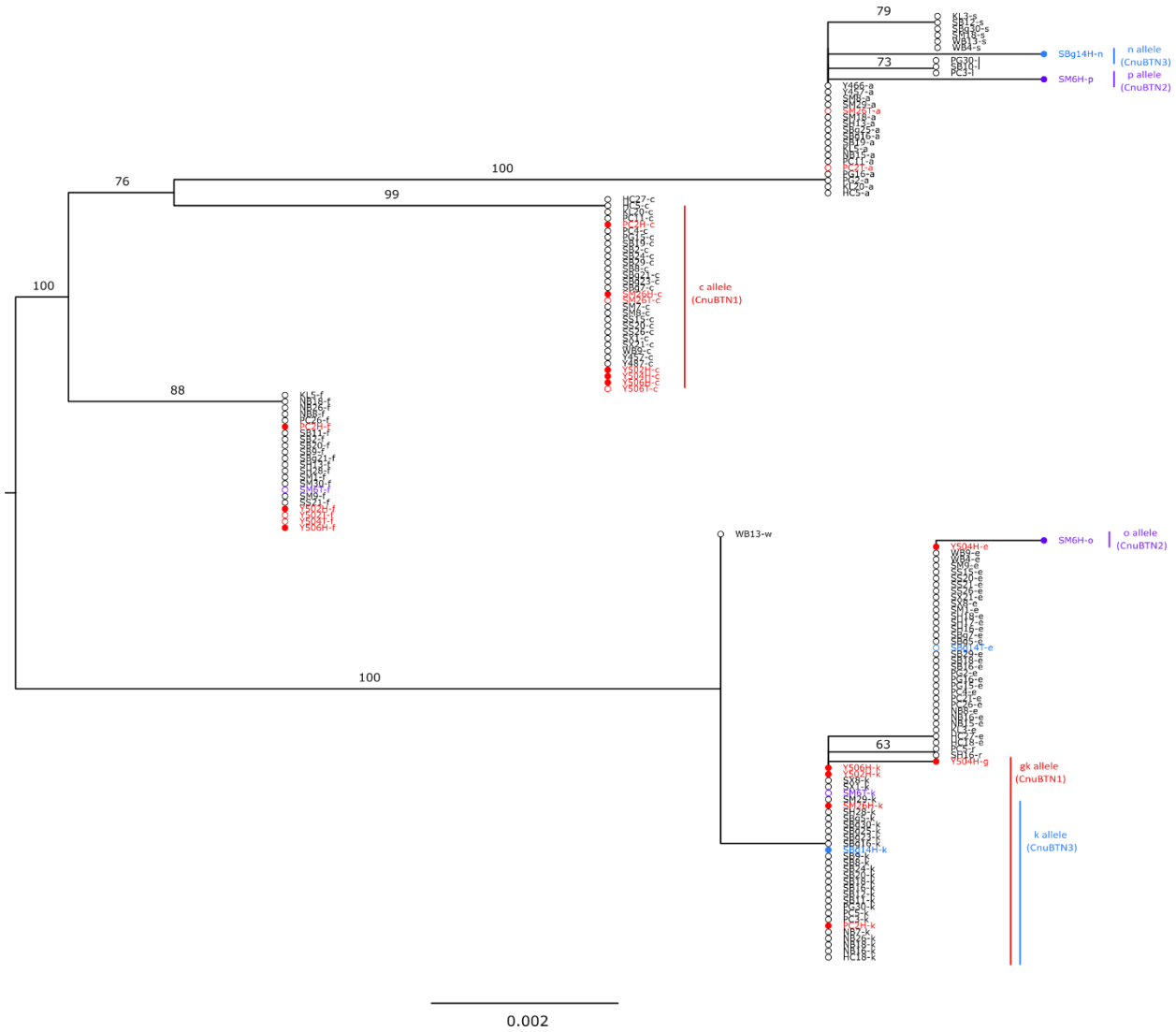


Figure 4. Phylogenetic analysis of *α-tubulin* sequence of DNA from BTN, host tissue and normal cockles.

An intron-spanning region in the *α-tubulin* region was amplified and sequenced for all cockles that were identified through qPCR screening as likely BTN and for representative healthy animals from each collection. A phylogenetic tree was made with PhyML (29, 30), rooted at the midpoint, with 100 bootstraps (values <50 not shown). Alleles are marked with cockle ID and the allele name, and healthy cockles have 1-2 alleles (black open circles). Alleles from the host tissue of neoplastic cockles (colored open circles) are marked with "T" and alleles from cancer in hemolymph (colored filled circles) are marked with "H." CnuBTN1, red; CnuBTN2, purple; CnuBTN3, blue.

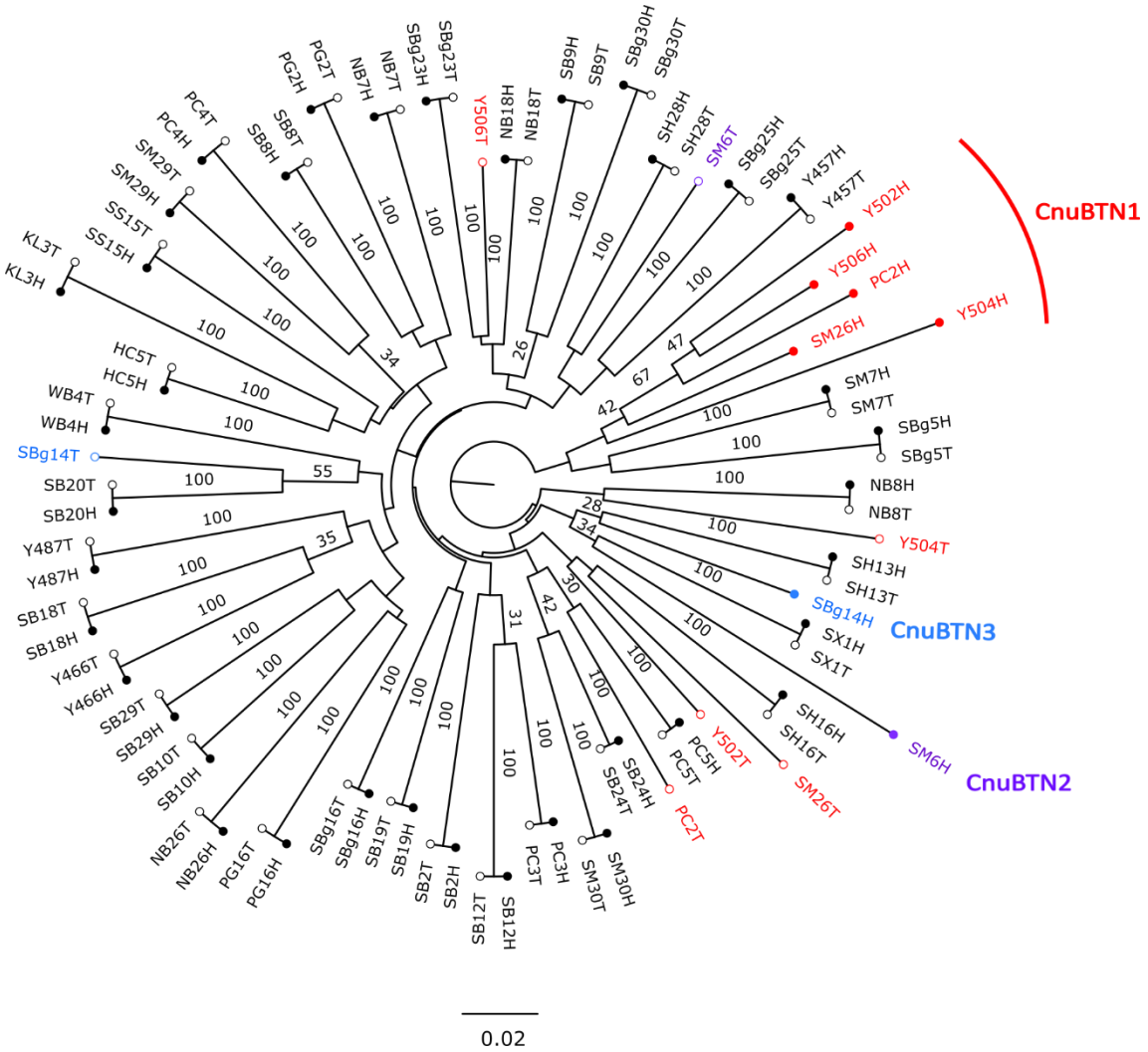


Figure 5. Microsatellite analysis of sequence of DNA from BTN, host tissue and normal cockles.

Eight microsatellite loci were sequenced, and the allele sizes were identified from all samples with BTN and representative healthy animals from each collection. A phylogenetic tree was made from the allele sizes, rooted at the midpoint, with 100 bootstraps (values <25 not shown). Genotypes are marked with cockle ID and with "H" (hemocyte genotype, filled circles) or "T" (tissue genotype, open circles). Each BTN lineage clusters separately (Healthy cockles, black; CnuBTN1, red; CnuBTN2, purple; CnuBTN3, blue).

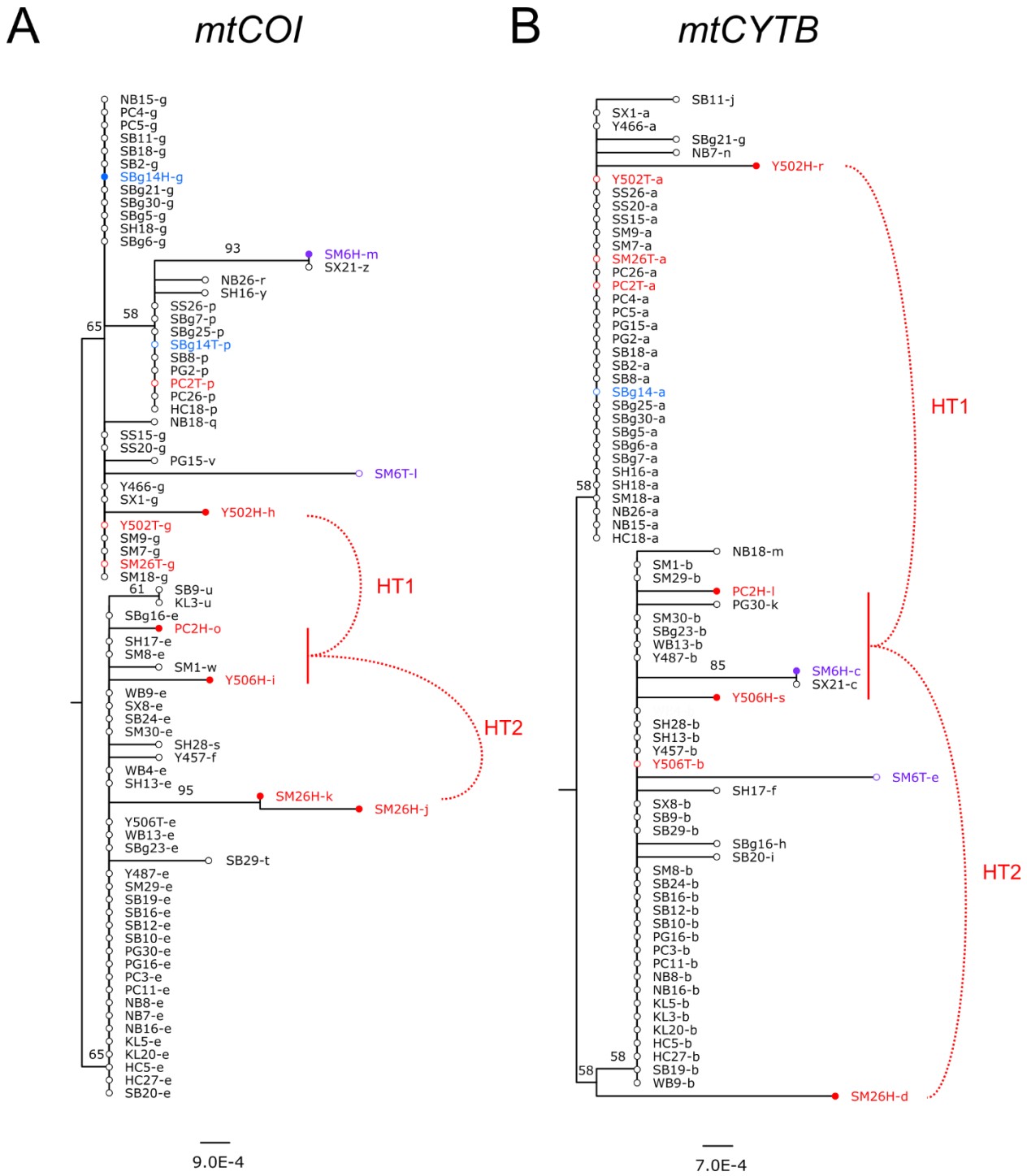


Figure 6. Phylogenetic analysis of mitochondrial loci (*mtCOI* and *mtCYTB*)

Two loci from the mitochondrial genome, *mtCOI* (***A***) and *mtCYTB* (***B***) were amplified and sequenced for each animal with BTN and representative healthy samples from each collection (healthy, black; CnuBTN1, red; CnuBTN2, purple; CnuBTN3, blue). A phylogenetic tree was made for each locus using PhyML (29, 30), rooted at the midpoint, with 100 bootstraps (values

494 <50 not shown). The mtDNA sequences of both loci from all CnuBTN1 samples (red, filled circles)
495 are different from each other, and more closely related to those sequences found in healthy
496 cockles, suggesting multiple horizontal transfer (HT) events. Some different sequences could be
497 due to HT or due to divergence from a common cancer allele (ie. the differences between PC2 and
498 Y506 could be due to the cancer originating with *mtCOI*-e & *mtCYTB*-b alleles), but for at least
499 two cases, horizontal transfer from a different healthy mitogenome is the most likely explanation
500 for the sequence divergence (HT1 to Y502 and HT2 to SM26). At least 3 alleles of each locus were
501 identified in the cancer sample from Y504, and the SNVs could not be confidently phased, so it
502 was excluded from this tree.

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