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## Genetic alterations and cancer formation in a European flatfish at sites of different contaminant burdens

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1 **Genetic alterations and cancer formation in a European flatfish at sites of different**  
2 **contaminant burdens.**

3  
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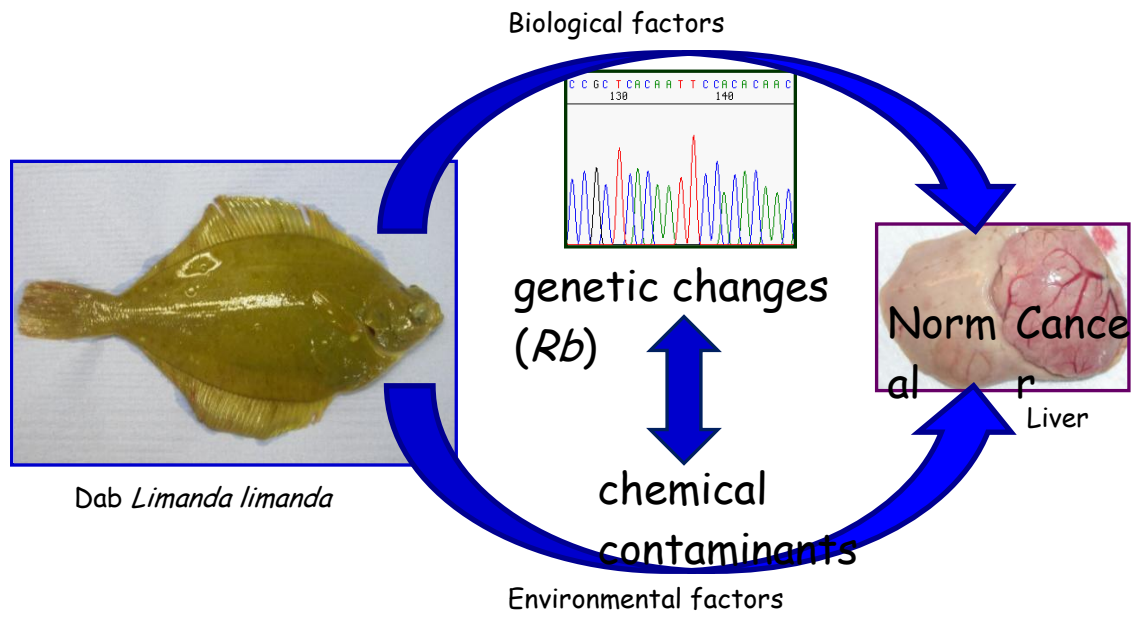
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16

17 **Running title:** Linking *Rb* genotype, tumour phenotype and contaminant exposure in the flatfish dab.



20 **Abstract**

21 Fish diseases are an indicator for marine ecosystem health since they provide a biological end-point  
22 of historical exposure to stressors. Liver cancer has been used to monitor the effects of exposure to  
23 anthropogenic pollution in flatfish for many years. The prevalence of liver cancer can exceed 20%.  
24 Despite the high prevalence and the opportunity of using flatfish to study environmentally-induced  
25 cancer, the genetic and environmental factors driving tumour prevalence across sites are poorly  
26 understood. This study aims to define the link between genetic deterioration, liver disease progression,  
27 and anthropogenic contaminant exposures in the flatfish dab (*Limanda limanda*). We assessed genetic  
28 changes in a conserved cancer gene, *Retinoblastoma (Rb)* in association with histological diagnosis of  
29 normal, pre-tumour and tumour pathologies in the livers of 165 fish from six sites in the North Sea and  
30 English Channel. The highest concentrations of metals (especially cadmium) and organic chemicals  
31 correlated with presence of tumour pathology and, with defined genetic profiles of the *Rb* gene, from  
32 these sites. Different *Rb* genetic profiles were found in liver tissue near each tumour phenotype, giving  
33 insight into the mechanistic molecular-level cause of the liver pathologies. Different *Rb* profiles were  
34 also found at sampling sites of differing contaminant burdens. Additionally, profiles indicated that  
35 histological ‘normal’ fish from Dogger sampling locations possessed *Rb* profiles associated with pre-  
36 tumour disease. This study highlights an association between *Rb* and specific contaminants (especially  
37 cadmium) in the molecular aetiology of dab liver tumourigenesis.

38

## 39 **Introduction**

40 Fish diseases represent an indicator of marine ecosystem health since they provide a biological end-  
41 point of historical exposure to stressors<sup>1</sup>. Liver pathologies of flatfish including tumours have been  
42 used to monitor the effects of exposure to pollution for many years<sup>1-4</sup>. As such they are routinely used  
43 in a number of internationally co-ordinated marine monitoring programmes and have been  
44 recommended as a key tool for assessing ecosystem health by organisations including the International  
45 Council for Exploration of the Sea (ICES) and the Oslo and Paris Convention (OSPAR) Joint  
46 Assessments and Monitoring Programme (JAMP)<sup>5</sup>.

47 A high prevalence of dab (*Limanda limanda*) liver tumours, exceeding 20% at some localities  
48 in the North Sea, has been reported<sup>6,7</sup>. This prevalence is of interest both in terms of the molecular  
49 basis of tumourigenesis, and its ecological implication. Dab is a bottom-dwelling fish particularly  
50 sensitive to environmental stressors<sup>4</sup> and can live up to 11 years making it a good indicator of the past  
51 history of contamination<sup>8</sup>. It is also widely distributed and highly abundant across the North Sea, Irish  
52 Sea and the English Channel<sup>9</sup>, facilitating population studies. The genetic structure of dab population  
53 is arguably regarded as stable over time, with a life-long residency in sampling regions proposed<sup>9</sup>. This  
54 is a fundamental criterion for sentinels of use in biomonitoring programmes. Therefore, the dab offers a  
55 unique opportunity to study environmental cancer. While there is debate among the scientific  
56 community regarding the impact of such disease on population dynamics<sup>10-13</sup>, the underlying genetic  
57 and environmental factors driving tumour prevalence across sites are still poorly documented.

58 Histopathology of tumours and pre-tumours in dab liver are currently diagnosed via a quality  
59 assured process involving histological tissue sections generated from wax-embedded samples<sup>14</sup>.  
60 Within the UK, such samples are collected and results are reported under the U.K. Clean Seas  
61 Environmental Monitoring Programme (CSEMP)<sup>6</sup>. Previous molecular studies using dab have

62 revealed differences in tumour or surrounding tumour tissues as compared to normal ones, including  
63 genetic alterations of cancer genes <sup>15-18</sup>, as well as differential gene expression <sup>6,19-21</sup>, protein synthesis  
64 <sup>22</sup>, and metabolic changes <sup>22,23</sup>. Finally, Tysklind et al. (2013), observed significant interactive effects  
65 between the genetic structuring of dab populations, environmental contaminants and certain liver  
66 pathologies from specific sites in the North and Irish Sea. While some of these studies highlight a role  
67 of chemical contaminants in the aetiology of liver pathologies, the precise mechanistic cause and effect  
68 relationship, specifically at the sub-cellular / molecular level and how chemicals may interact with  
69 genotype to influence tumour development, is still uncharacterised.

70 Cancer is a multi-factor disease, according to medical studies, resulting from gene-environment  
71 interactions. The combination of environmental stressors such as chemicals and the susceptibility of the  
72 host can result in alteration of environmentally relevant genes such as mutations in cancer genes. The  
73 development of hepatocellular carcinoma (HCC) is a multistep process of transformation of normal  
74 cells into malignant cells driven by accumulation of genetic and epigenetic alterations in such genes <sup>24-</sup>  
75 <sup>27</sup>.

76 The *Rb* gene was the first tumour suppressor gene to be characterised <sup>28</sup>. In vertebrates, the *Rb*  
77 gene product is a nuclear phosphoprotein that regulates normal cell cycle progression. In humans, *Rb*  
78 mutations have been reported in hepatocellular carcinoma (HCC) and RB protein is inactivated in the  
79 majority of human cancers <sup>29</sup>. *Rb* alterations have been detected in chemically-induced retinoblastoma  
80 in the medaka (*Oryzias latipes*), a laboratory fish model <sup>17</sup>. Dab possess both a similar  
81 histopathological liver tumour profile to humans <sup>30</sup> and homologs of human cancer genes <sup>15,16</sup>. It is  
82 likely that dab and human share downstream signalling cascades underlying HCC formation; further  
83 support for the suitability of this species as a relevant model of environmentally-induced liver cancer.

84 The present study aims at defining the link between genetic deterioration, visible disease  
85 progression and environmental contaminant burdens in a discrete population of flatfish dab <sup>9</sup>. To  
86 achieve this, the *Rb* genetic changes and histopathological diagnosis of normal, pre-tumour and tumour  
87 in liver of 165 fish collected at four sites at Dogger Bank and two sites in the east English Channel,  
88 were assessed. Concentrations of metals (cadmium, Cd; mercury, Hg; lead, Pb; zinc, Zn; copper, Cu)  
89 and organic chemicals (polybrominated diphenyl ethers, PBDEs, and polychlorinated biphenyls, PCBs)  
90 in the liver of fish from the same sites were analysed in parallel to provide contaminant burden  
91 indication.

92

## 93 **Material and Methods**

### 94 *Sample Collection*

95 Dab (*Limanda limanda*) were captured at UK CSEMP sites on the Dogger Bank (North Dogger, North  
96 East Dogger, Central Dogger and West Dogger), North Sea and the English Channel (Rye Bay and  
97 Newhaven) (Table S1) during July 2010, using 30 min tows of a standard Granton trawl aboard the RV  
98 *Cefas Endeavour*. These sites are among those used for both ICES and OSPAR statutory monitoring  
99 and have been identified as having historically high (Dogger) or low (Rye Bay/Newhaven) prevalence  
100 of liver tumours <sup>6, 31</sup>. Upon landing, fish were immediately removed from the catch and placed into  
101 flow-through tanks containing aerated seawater. The sex and size (total length) and presence of  
102 external signs of disease were noted for each fish using methodology specified by ICES <sup>14</sup>. Otoliths  
103 were sampled from each fish and processed for age determination according to Easey & Millner  
104 (2008)<sup>32</sup>. Following euthanasia, the body cavity was opened and the liver assessed for the presence of  
105 macroscopic liver tumours according to the guidelines set out by Feist et al. (2004)<sup>14</sup>. For each fish ( $n =$   
106 165), a standardised cross section was obtained for histological analysis and placed into 10% neutral

107 buffered formalin and processed as described in ‘*Histology/histopathology*’. A part of the liver from the  
108 same individual fish (and beside the previous dissected fragment) was also sampled and snap frozen in  
109 liquid nitrogen for molecular analysis as described in ‘*Total RNA, cDNA preparation and Rb cDNA*  
110 *isolation*’ below.

111

112 *Chemical concentrations and biomarkers of exposure to polycyclic aromatic hydrocarbons (PAHs) in*  
113 *bile, liver or flesh from fish*

114 Data pertaining to chemical and biomarker analysis was collated from the Marine Environment  
115 Monitoring and Assessment National database ([www.bodc.ac.uk/projects/uk/merman/](http://www.bodc.ac.uk/projects/uk/merman/)), which holds  
116 UK data collected to fulfill the UK’s mandatory monitoring requirements under the OSPAR Joint  
117 Assessments and Monitoring Programme (JAMP). In brief, the measurement of metals, PBDEs and  
118 PCBs was performed on 5 pools of livers (flesh for Hg) from 5 fish (representing 25 fish in total) for  
119 each site. The fish were from the same trawl as the fish used in the molecular and histology analyses.  
120 Chemical analyses were processed using standardised protocols as previously described for metals <sup>33</sup>,  
121 PBDEs <sup>34</sup>, and PCBs <sup>35</sup>. For an indication of exposure to PAHs, bile hydroxypyrene levels and  
122 ethoxyresorufin *O*-deethylase (EROD) activities were obtained from a subset of twenty fish (10 males  
123 and 10 females) sampled during the same trawls at each site. The livers and gall bladders were  
124 collected and analyzed for both EROD and bile measurements following standard protocols published  
125 in the ICES Techniques in Marine Environmental Sciences Series (ICES TIMES). EROD activity was  
126 determined in liver tissue using a fluorescent assay <sup>36</sup>. Bile samples were analyzed for fluorescent bile  
127 metabolites using synchronous fluorescence spectrometry (SFS)<sup>37</sup>.

128

129 *Histology/histopathology*



130 Fish were assessed for grossly visible tumours and histopathological assessment of liver samples from  
131 flatfish populations collected under CSEMP. The lesions recorded include those thought to precede the  
132 development of benign and malignant lesions such as foci of cellular alteration, non-neoplastic  
133 toxicopathic lesions (such as nuclear and cellular polymorphism) and lesions associated with cell death,  
134 inflammation and regeneration. Currently, 32 categories of liver lesion are classified under the  
135 international Biological Effects Quality Assurance in Monitoring Programmes (BEQUALM) project.  
136 The diagnosis of these lesion types in the dab and flounder liver follows the guidelines set out by Feist  
137 et al. (2004)<sup>14</sup>. Upon landing, dab of 20 to 30 cm total length from each site in each year were  
138 immediately removed from the catch and placed into flow-through tanks containing aerated seawater<sup>14</sup>.  
139 The sex, size (total length) and presence of grossly visible signs of disease were recorded for each fish  
140 using the methodology specified by the International Council for the Exploration of the Sea (ICES)<sup>38</sup>.  
141 Following grossly visible disease assessment, fish were euthanised and, upon opening of the body  
142 cavity, the liver was assessed for the presence of visible tumours according to the guidelines set out by  
143 Feist et al. (2004)<sup>14</sup>. Liver samples were removed and fixed for 24 h in 10% neutral buffered formalin  
144 (NBF) before transfer to 70% industrial methylated spirit (IMS) for subsequent histological assessment.  
145 Livers were processed for formalin fixed paraffin embedded histology in a vacuum infiltration  
146 processor using standard histological protocols and embedded in paraffin wax. Using a rotary  
147 microtome, sections of 3-4 µm were taken and subsequently stained with haematoxylin and eosin  
148 (H&E). Slides were examined for microscopic tumours (hepatocellular adenoma and HCC) and pre-  
149 tumours (vacuolated foci of cellular alteration (FCA), eosinophilic FCA, basophilic FCA), according to  
150 BEQUALM and ICES criteria<sup>14</sup> using a Nikon Eclipse E800 microscope.

151

152 *Total RNA isolation, cDNA synthesis and Rb cDNA isolation from individual fish*

153 For each fish an additional sample of liver (approximately 20 mg) was removed from near the sample  
154 used in histology analysis, for parallel molecular analyses, specifically isolation of the *Rb* cDNA. Total  
155 RNAs were extracted using the High Pure RNA Tissue kit (Roche Diagnostics Ltd, West Sussex, U.K.)  
156 according to the supplier's instructions. RNA quality (integrity of 18S and 28S ribosomal bands) was  
157 evaluated by electrophoresis on a 1% agarose-formaldehyde gel. First strand cDNAs were synthesized  
158 from 1 µg of total RNA using the SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen Ltd, Paisley,  
159 U. K.) and according to the supplier's instructions.

160 Three overlapping parts of the coding sequence of the *Rb* cDNA: RbA1, RbA2 and RbB,  
161 containing the region of functional importance were amplified. Primer pairs used to amplify the region  
162 between 620 and 1942 bp of the *Rb* cDNA (Accession number: **AY973250**) are described in Table S3  
163 (contained in Supplemental Information). One µL of the reverse transcribed product was used as a  
164 template for subsequent polymerase chain reaction (PCR) in a 25 µL final volume using 2.5 units of the  
165 Expand High Fidelity<sup>PLUS</sup> enzyme (Roche Diagnostics Ltd, West Sussex, U.K.), primers at a final  
166 concentration of 1 µM and following the supplier's protocol. PCR reactions were performed using the  
167 following programme: one cycle at 94°C for 2 min and 40 amplification cycles at 94°C for 30 s, 60°C  
168 (RbA1) or 65°C (RbA2 and RbB) for 30 s, and 72°C for 1 min. 10 µL of each PCR product were then  
169 forward and reverse sequenced commercially (Macrogen, Amsterdam, Netherlands). Both strands for  
170 each overlapping fragment were assembled using the sequence-editing software CodonCode Aligner  
171 version 4.0. Sequences were aligned using ClustalW 1.81.

172

### 173 *Statistical analysis*

174 Statistical analyses were performed using R 3.0 (R Development Core Team 2013). The distribution  
175 of different tumour stages and genetic profiles among sites, and the relation between the genetic

176 profiles and tumour stages were first analysed by correspondence analyses, using the “dudi.coa”  
177 function (ade4 package). The distribution of chemicals among sites was assessed by a principal  
178 component analysis (PCA), using the “dudi.pca” function (ade4 package). The effect of the site,  
179 genotype, sex and age of fish on the presence (pre-tumour and tumour) - absence (normal) of tumour  
180 was also tested using generalized linear models (GLIM). All of these factors were included in the  
181 model. Statistical analyses were performed using GLIM (Poisson family, link log), with the anova.glm  
182 function in R. The best-fit model was selected using Akaike information criterion (AIC). Full  
183 explanation of the models used to derive Figures 1-4 are given in Supplemental Information as  
184 Supplemental Methods, SM1.

185

## 186 **Results**

### 187 *Fish biometric distribution relative to locality*

188 The size and weight ranges for the fish used in this study are provided in Table S2 in the Supplementary  
189 Information section. In terms of the biometric data for the 165 fish sampled in this study there were  
190 significant differences in the composition of the individuals at specific sampling locations as follows.  
191 Fish sampled at North Dogger/Central Dogger were significantly larger/smaller than other sampling  
192 sites (Table S2). Fish sampled at Dogger sites were also significantly older than fish sampled at  
193 Newhaven (Table S2). However, no significant differences between fish sampled at all the sampling  
194 sites were evident for Fulton Condition Index, liver weight or hepatosomatic index (HSI) (Table S2).  
195 PCA statistical analysis of all the factors subsequently indicated a significant effect of site, genotype and  
196 age of fish on the presence-absence of liver tumours (GLIM, site:  $p = 0.006$ ; genotype:  $p = 0.028$ ; age:  $p$   
197  $= 0.0007$ ; sex:  $p = 0.057$ ). We shall thus present the results in the order of site/locality, phenotype,  
198 genotype, age and sex.

199

200 *Distribution of metals, PCBs and PBDEs relative to locality*

201 The concentrations of contaminants in dab liver differed significantly by site (Table S4a-c) and this  
202 dataset has been used to produce a PCA plot to characterise the distribution of individual chemicals in  
203 relation to site (Figure 1). For instance, the liver of fish sampled from Newhaven was characterised by  
204 relatively low levels of PCB contamination (Figure 1; Table S4c), whereas that of fish sampled from  
205 North Dogger was characterised by high concentrations of Cd ( $406 \pm 122 \mu\text{g/kg}$  liver tissue)(Figure 1;  
206 Table S4a). Associations between different chemical contaminants are presented in Table S5. Principal  
207 component analysis showed the following highlights: the liver of fish from Rye Bay was characterised  
208 by contamination with the greatest number, and highest concentrations, of PCB congeners (particularly  
209 CB101, 105, 110, 138, 153 and 187)(Table S4c); fish from Newhaven less so (though PCBs still  
210 formed the dominant profile)(Table S4c); those from Central, West and North East Dogger being  
211 weakly associated to metals, PBDEs and PCB contamination; and those from North Dogger being most  
212 associated to metals (with the highest association for Cd) (Figure 1; Table S4a-c).

213

214 *Sampling site-specific distribution of tumour phenotypes*

215 The occurrence of normal, pre-tumour (including all FCA types), and tumour liver phenotype form a  
216 gradient progressing from the Newhaven to North Dogger sites (Figure 2; Figure S1). Correspondence  
217 analysis revealed a gradient as follows: normal livers were mostly found in fish sampled at the  
218 Newhaven site (81%) and then at Rye Bay (67%) and North East Dogger (66%) to a lesser extent  
219 (Figure 2; Figure S1). This latter site also contained fish displaying pre-tumours (24%), whilst this  
220 pathology also dominated in fish from the West Dogger (31%) and Central Dogger sites (36%)(Figure  
221 2; Figure S1). In terms of prevalence, tumours were most prevalent in the livers of fish from the North

222 Dogger site (20%) (Figure 2; Figure S1). North Dogger was thus characterised by high Cd levels (406  
223  $\pm$  122  $\mu$ g/kg liver tissue) and high liver tumour prevalence (20%).

224  
225 *Different Rb genetic profiles are found between sites and tumour phenotypes*

226 *Rb* genetic profiles were characterised in fish samples from six sites within a North Sea and English  
227 Channel dab population. Four nucleotides were found to be changed in the *Rb* coding sequence at 996  
228 bp (G to A), 1088 bp (T to C), 1514 bp (G to T) and 1592 bp (G to T) leading to 17 different genetic  
229 profiles annotated from A to Q (Table 1). All of these changes occurred within the *Rb* sequence  
230 encoding the functionally important and conserved A and B domains.

231 Differing *Rb* profiles were associated with fish captured at different North Sea and English  
232 Channel locations (Figure S2). Correspondence analysis (Figure 3) revealed three groupings: one  
233 associates fish from Newhaven, Rye Bay, Central Dogger, North East Dogger with profiles A, B, C, E,  
234 H, P and Q; a second associates fish from West Dogger with profiles D, F, G and I; and the third  
235 associates fish from North Dogger with profiles L, M, N and O (Figure 3, Table 1).

236 Additionally, several *Rb* profiles were identified in livers of fish displaying normal, pre-tumour  
237 and tumour phenotypes. Correspondence analysis (Figure 4) showed that five *Rb* profiles; A, D, I, Q  
238 and P were associated with normal liver phenotype, ten profiles; B, C, E, F, H, J, K, M, N and O are  
239 associated with liver pre-tumour stages, and profiles G and L are associated with a liver tumour  
240 phenotype (Figure 4). The differences in these *Rb* profiles hinge around only four nucleotide positions  
241 of the *Rb* sequence (Table 1). On close examination of the *Rb* gene status at samples from West  
242 Dogger, genotypes seen in pre-tumour fish (profiles C and D, Table 1) are also seen in normal fish  
243 from that site, giving an indication that normal fish from that site on a pathogenesis trajectory to liver  
244 tumour (Figures 2- 4, Table 1).

245

246 *Age and sex*

247 The age of fish has a significant effect on the liver phenotype (normal and tumour) (GLIM1,  $p = 0.0007$ ,  
248 see Supplementary Information, SM1, for full statistics). Fish from Dogger Bank are significantly older  
249 than fish from Newhaven ( $p < 0.05$ , Supplementary Information, Table S2). However, the age of fish  
250 from a given site displaying normal and tumour phenotypes is similar (GLIM2,  $p = 0.0756$ , see  
251 Supplementary Information, SM1, for full statistics). The sex of fish has no effect on the phenotype  
252 observed (normal and tumour) using the number of fish sampled in this study (GLIM1,  $p = 0.06$  see  
253 Supplementary Information, SM1, for full statistics).

254 In summary, we link the presence of liver tumours in dab to specific contaminant classes and  
255 *Rb* gene status in liver tissue next to that used in histology, providing a potential mechanism for future  
256 characterisation and prediction of disease prevalence in such populations.

257

## 258 **Discussion**

259 For the first time, this study provides a link between genetic deterioration, visible disease progression  
260 and specific environmental contaminant profiles in discrete populations of marine fish. Specifically, we  
261 are the first to link genetic profiles (using the *Rb* gene) to histopathological diagnosis of normal, pre-  
262 tumour and tumour, in liver tissue of the same individual fish from different sampling sites. These  
263 sampling sites have also been characterised in terms of predominant contaminant classes present in the  
264 fish liver tissue, thus providing an indication of the potential causality in generation of differing *Rb*  
265 genetic profiles. Such profiles also indicate that normal fish from the Dogger Bank also possess *Rb*  
266 profiles associated with pre-tumour disease (Figure 2, Table 1) suggesting that such fish are possibly  
267 heading towards liver tumours.

268

269 *Characteristic Rb profiles are associated with disease phenotype*

270 In terms of *Rb* genetic profiles, four nucleotide positions were altered, corresponding to a region of  
271 functional importance of the *Rb* gene, leading to 17 genetic profiles (Table 1). *Rb* profiles were not  
272 randomly distributed, with specific profiles associated with both sampling site (Figure 3) and liver  
273 phenotype (Figure 4). Of the *Rb* gene alterations characterised (Table 1), several were similar to those  
274 found in tumours sampled from a different dab population in the Irish Sea from a previous study<sup>15</sup>. The  
275 exception is one change occurring at 996 bp, corresponding to a G/G to G/A change, which has not  
276 been identified previously.

277         Regarding the precise molecular-level biological mechanisms of cause (pollutant-induced  
278 mutational activation/inactivation of key genes) and effect (pre-tumour and tumour liver phenotypes),  
279 understanding the implications of these *Rb* allele zygosity patterns (contained in Table 1) are key. For  
280 instance, focussing on *Rb* profile L (Table 1), which associates with both tumour phenotype (Figure 4)  
281 and North Dogger sampling site (Figure 3), this entails heterozygosity at two of the four nucleotide  
282 positions and a homozygous alteration at another (1592 bp). For the transitional, pre-tumour phenotype,  
283 the *Rb* profiles E, F, J, K, and O all display homozygous T allele at position 1592 bp. Such alterations  
284 in an established tumour suppressor gene may reflect driving steps in the multi-stage progression  
285 towards the tumour endpoint (as evidenced in rodent studies by Wang et al. (2012)<sup>39</sup>) and as such  
286 require further biochemical characterisation.

287         Of important note is the lack of any homozygous A/A detected at position 996 bp of the *Rb*  
288 sequence (Table 1) in any of the 165 fish analysed. The latter nucleotide alteration would theoretically  
289 lead to a change of amino acid involving a lysine (K) instead of glutamic acid (E). The glutamic acid  
290 (with polar acid properties) to lysine (with polar basic properties) alteration also occurs within the

291 functionally conserved Domain A of the protein that is responsible for a key LxCxE motif and  
292 transcription factor binding <sup>40</sup>. This theoretical change is identified as lethal phenotype Rb<sup>-/-</sup> in mice  
293 embryos <sup>41</sup>. The existence of such phenotype in dab may have already had, or could have future,  
294 repercussions at the population level and is of interest from the perspective of population sustainability  
295 of the dab.

296 Related to the lethality and phenotype discussion is age, an important cofactor involved in the  
297 epidemiology of tumour development. The analyses show that the age of fish is a potentially  
298 confounding factor. In general, fish are older at Dogger Bank than at Newhaven (Table S2). In this  
299 study, no significant differences between the age of fish displaying a normal or a tumour phenotype at  
300 each site were observed. However, the limited number of fish and associated age classes make it  
301 difficult to demonstrate clear links with tumour formation in our study. Since tumourigenesis is  
302 typically a multi-stage event involving several gene activation/inactivation events, one would expect  
303 older fish to display a higher prevalence of pre-tumour and tumour phenotypes. Taking into account  
304 previously published work, dab with HCA (a pre-tumour phenotype) were found in older age classes  
305 sampled from North Dogger Bank, yet no cases of HCC (actual tumour phenotype) were observed in  
306 fish of age >5 yr at this site <sup>7</sup>. Thus adding weight to the notion of an Rb<sup>-/-</sup> lethal phenotype.

307 Sex is also considered a confounding factor in the epidemiology of flatfish tumour development  
308 <sup>40</sup>. In our study, using a relatively small sample size of 165 fish ( $n = 11-37$  at each sampling site), using  
309 the statistical approach described, no influence of sex was detected for any of the variables investigated  
310 but this is undermined by low numbers of males at certain sites (Table S2). N, W and C Dogger, in  
311 particular, has bigger and older fish, and the majority are females, which may in turn be due to relatively  
312 low numbers of animals sampled during current study. In previous work, focusing on age primarily as a  
313 confounding factor, yet importantly using very large dataset, evidence suggested that (despite some



314 significant differences between the mean age of fish sampled from specific sites) the mean age of all  
315 male (5.3 yr) and all female (4.8 yr) fish sampled during the programme was similar, and relevantly,  
316 data demonstrated a very similar prevalence of specific diseases in male and female dab<sup>7</sup>.

317

318 *Characteristic Rb profiles are associated with sampling site*

319 Focussing on sampling sites, of particular interest are the results from North Dogger where fish livers  
320 exhibit the highest prevalence (20%) of advanced stage tumour (Figure 2; Figure S1), possess specific  
321 *Rb* genetic profiles (Figure 3), and display a high concentration of Cd ( $406 \pm 122$   $\mu\text{g}/\text{kg}$  liver  
322 tissue)(Figure 1; Table S4a). While site-specific disease profiles have been reported between sampling  
323 years<sup>6</sup>, these results highlight North Dogger Bank as a site of concern for prevalence of carcinogenesis  
324 and involvement of Cd. Cd is a heavy metal with no essential role in organisms, classified as a human  
325 carcinogen by the International Agency for Research on Cancer, and induces cancer in several  
326 organs/tissue of animals by multiple direct and indirect mechanisms<sup>43-45</sup>. The liver is a target organ of  
327 Cd toxicity in animals including fish<sup>42</sup>. Cd is a weak genotoxic chemical that inhibits DNA damage  
328 repair pathways<sup>46</sup> and apoptosis induced by toxicants<sup>47</sup>. Cd co-exposure thus enhances the  
329 carcinogenic potential, or may act as a promoter, of other genotoxic chemicals, such as PAHs  
330 previously identified in the molecular aetiology of liver carcinogenesis in Atlantic killifish (*Fundulus*  
331 *heteroclitus*)<sup>48</sup>, to cause cancer. This is particularly relevant for dab populations that are chronically  
332 exposed to a mixture of environmental contaminants such as the case at Dogger Bank. While the PAH  
333 levels are not characterised in this study, the levels of hydroxypyrene and EROD activity ( $124 \pm 52$  ng/g  
334 and  $83 \pm 58$  pmol/min/mg protein respectively at North Dogger, Table S6) indicate that PAHs are  
335 present but at levels significantly lower than the reference sites (for instance  $124 \pm 52$  ng/g,  $124 \pm 52$

336 pmol/min/mg protein for Newhaven)(Table S6). Further work involving controlled laboratory exposure  
337 is required to confirm the exposure-effect relationship.

338

### 339 *Wider implications of Rb involvement in fish tumour pathologies*

340 In terms of wider implications and utility of this work, there are two to consider: development of an  
341 early warning system and ‘mutator phenotype’. Genetic modifications can occur earlier than  
342 microscopic histopathological changes in the tumourigenesis process. Here we have linked for the first  
343 time, *Rb* profiles in samples dissected from tissue located beside liver tissue, in the same individual  
344 fish, displaying a particular liver phenotype (Figure 4). Profile data also indicates that normal fish from  
345 Dogger sampling locations also possess *Rb* profiles associated with pre-tumour disease, providing an  
346 indication that such fish are heading towards development of a liver tumour. Relating *Rb* profiles to  
347 specific early neoplastic pre-tumour phenotype (different FCAs) may be used to predict future tumour  
348 prevalence likelihoods and is subject of a current study. A limitation of the study to highlight, however,  
349 is that the molecular analysis was conducted using liver tissue next to, yet not the exact same, liver  
350 tissue sample used for histopathology assessment. Inherent in such an approach is the scope for false  
351 negatives/positives, and that tissues of the same liver may show heterogeneity of cell type. More  
352 recently, a laser capture microdissection technique to address this limitation has been optimised in dab  
353 <sup>49</sup>. Nonetheless, this work associates *Rb* profile status with liver pathology. In addition, a second  
354 mechanism of possible RB interaction, via regulators of chromatin structure including  
355 methyltransferases, may be involved <sup>20-21, 50</sup>. Taken together our results and those from the literature  
356 highlight possible involvement of *Rb* in both genetic and epigenetic mechanisms in the aetiology of dab  
357 liver tumourigenesis.

358 Mutations in critical cell cycle control genes such as *Rb* represent a cellular defect that may  
359 catalyse the accumulation of further mutations, characteristic of a ‘mutator phenotype’ accelerating the  
360 disease process <sup>51-52</sup>. The genetic instability found in our study reflects the accumulation of DNA  
361 damage which is a key event driving the tumourigenesis process. In the absence of normal *Rb* gene,  
362 genomic instability and chromosomal aberrations are allowed to accumulate leading to tumour  
363 initiation, progression and metastasis <sup>53</sup>. The prevalence of cancer in most fish populations is extremely  
364 low with background levels similar to those seen in terrestrial wild animal populations and humans <sup>7</sup>.  
365 The high prevalence of HCA and other liver tumour types in dab and other marine flatfish populations  
366 from coastal environments <sup>3,7,42,54</sup> may be accounted for by the mutator phenotype theory. Herein we  
367 also show that the flatfish model provides an opportunity to study the mechanistic molecular etiology,  
368 including the relative contributing factors from the environment and the genotype, in the multi-step  
369 initiation and progression of vertebrate liver cancer.

370 This work represents a novel approach attempting to link genetic causes (by contaminant-  
371 induced damage in a conserved gene) to population-level biological endpoints (high prevalence of liver  
372 tumours). We assessed genetic changes in a key cancer gene, *Rb*, and made a histopathological  
373 diagnosis of normal, pre-tumour and tumour in the livers of 165 fish collected at four sites at Dogger  
374 Bank and two sites in the east English Channel. Four genetic changes were found within the *Rb*  
375 sequence at functionally important sites. Characteristic *Rb* genetic profiles were found in samples  
376 beside the tissue exhibiting different tumour phenotypes, giving insight into the mechanistic molecular-  
377 level cause of the observed liver pathologies, as well as a possible early warning tool for regulatory  
378 authorities. Characteristic *Rb* profiles were also found for sampling sites with differing contaminant  
379 burdens. This study highlights the involvement of *Rb* and specific contaminants (particularly cadmium)  
380 in the molecular aetiology of dab liver tumourigenesis.

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389

390 **Supporting Information Available**

391 Tumour phenotype prevalence data and distribution of *Rb* genetic alleles at each sampling location are  
392 supplied as additional Figures. The sampling site coordinates, biometric data, analytical chemistry data  
393 plus correlation associations among chemical contaminants, and biomarkers of PAH exposure  
394 (hydroxyrene levels and EROD activities) are also supplied as additional Tables. The primers used for  
395 the isolation for the *Rb* cDNA are also available as an additional Table. This information is available  
396 free of charge via the Internet at <http://pubs.acs.org/>.

397

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541  
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543 **Figure and Table Legends**

544 **Figure 1.** Principal component analysis showing the association between concentrations of chemicals  
545 in liver of fish and sampling site ( $n = 30$  pools of 5 fish). Axis1 represents 60% of variance. Axis2  
546 represents 17% of variance.

547 **Figure 2.** Correspondence analysis showing the distribution of phenotypes (normal, pre-tumour,  
548 tumour) across North Sea/English Channel sampling sites ( $n = 165$ ). Axis1 represents 95% of variance.  
549 Axis2 represents 5% of variance.

550 **Figure 3.** Correspondence analysis showing the distribution of *Rb* genotypes across North Sea/English  
551 Channel sampling sites ( $n = 165$ ). Axis1 represents 38% of variance. Axis2 represents 29% of variance.

552 **Figure 4.** Correspondence analysis showing the association between *Rb* genotypes and liver  
553 histopathological phenotypes ( $n = 165$  fish). Axis1 represents 60% of variance. Axis2 represents 40%  
554 of variance.

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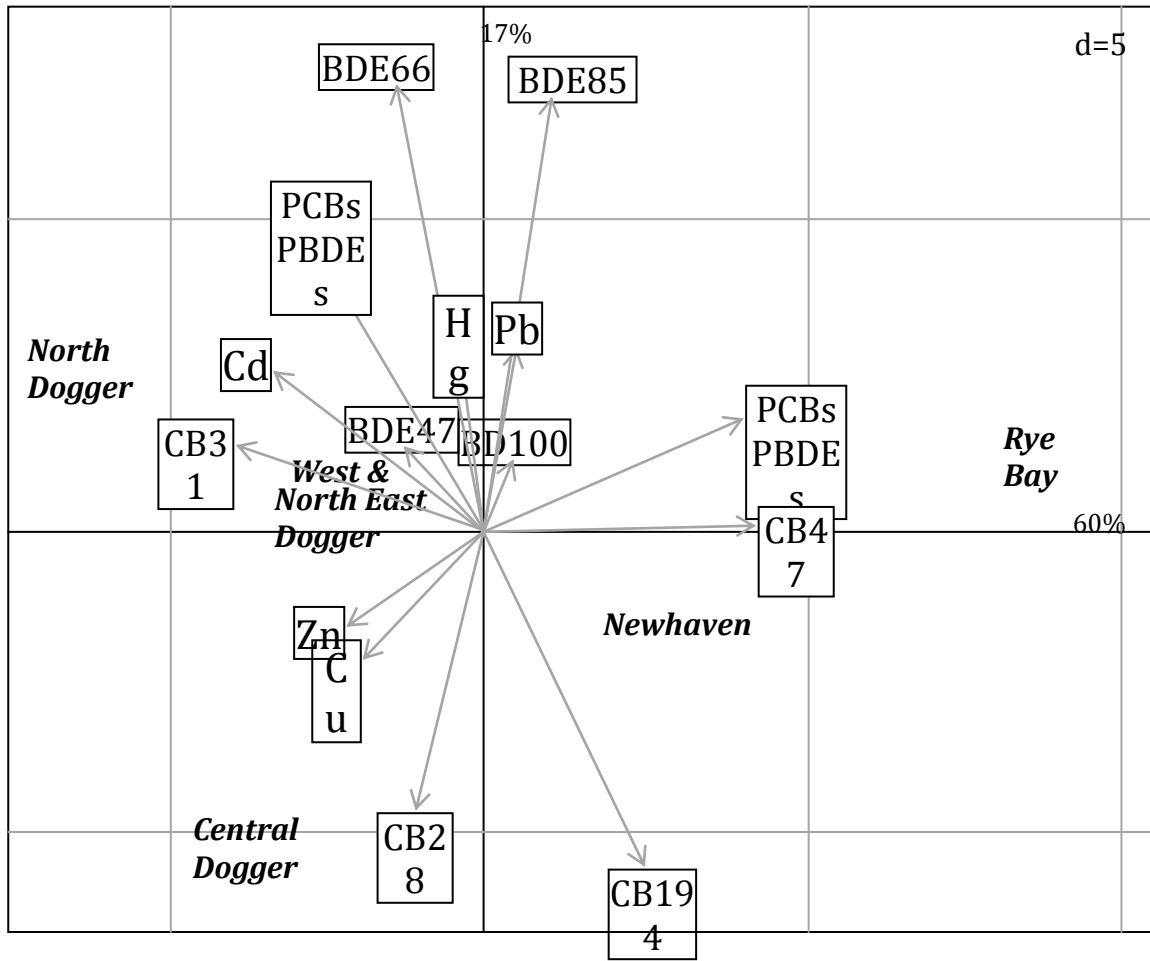
556 **Table 1.** Spectrum of *Rb* genetic profiles identified in a North Sea/English Channel dab population  
557 from differing localities ( $n = 165$  individual fish).

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561 **Figure 1**



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569 **Figure 2**

					5.2 %		d=0.2
tumour				<i>North East Dogger</i>			
			<i>West Dogger</i>		normal		<i>Newhaven</i>
	<i>North Dogger</i>						94.8 %
		pretumour		<i>Rye Bay</i>			
		<i>Central Dogger</i>					

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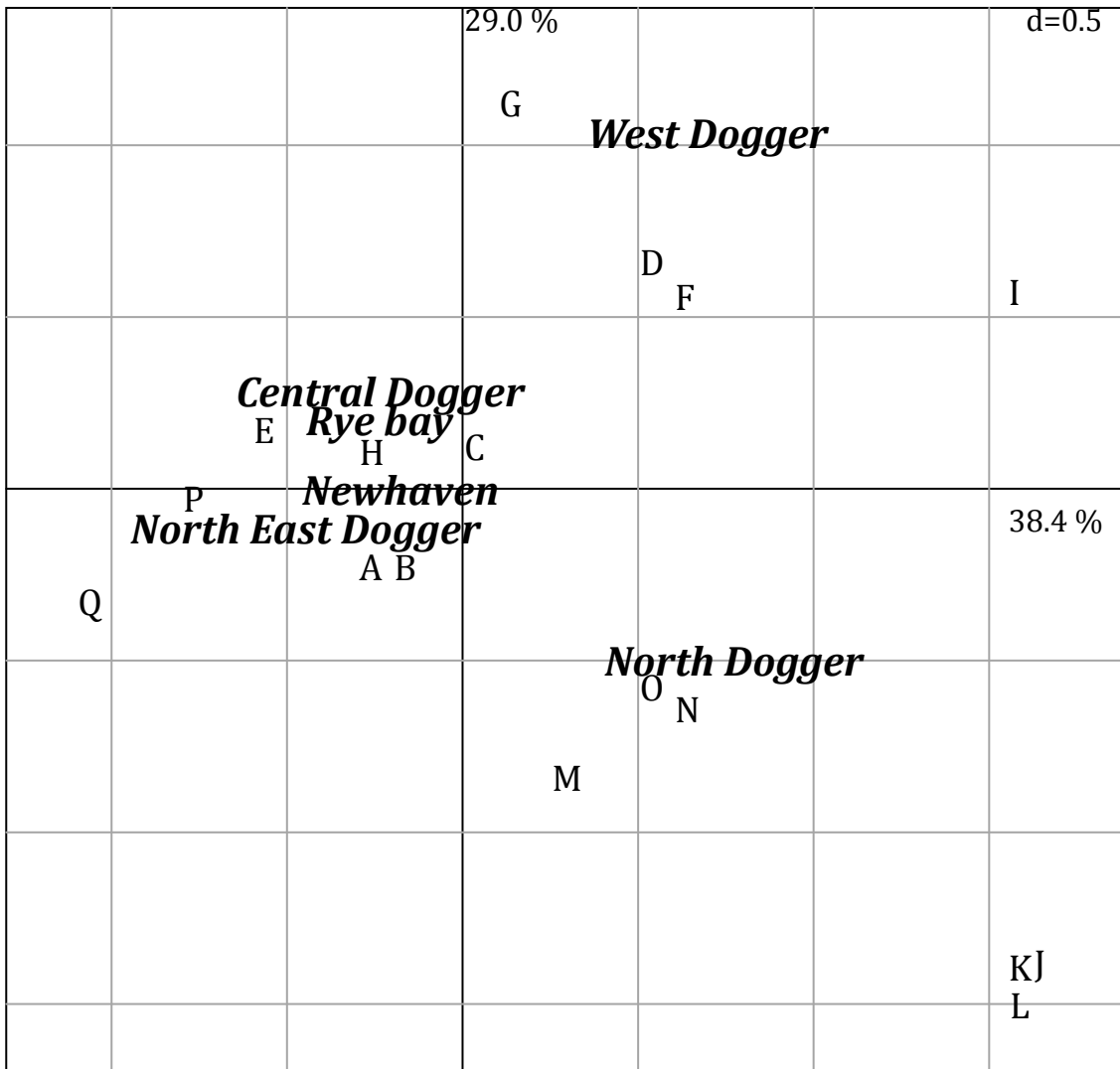
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577 **Figure 3**



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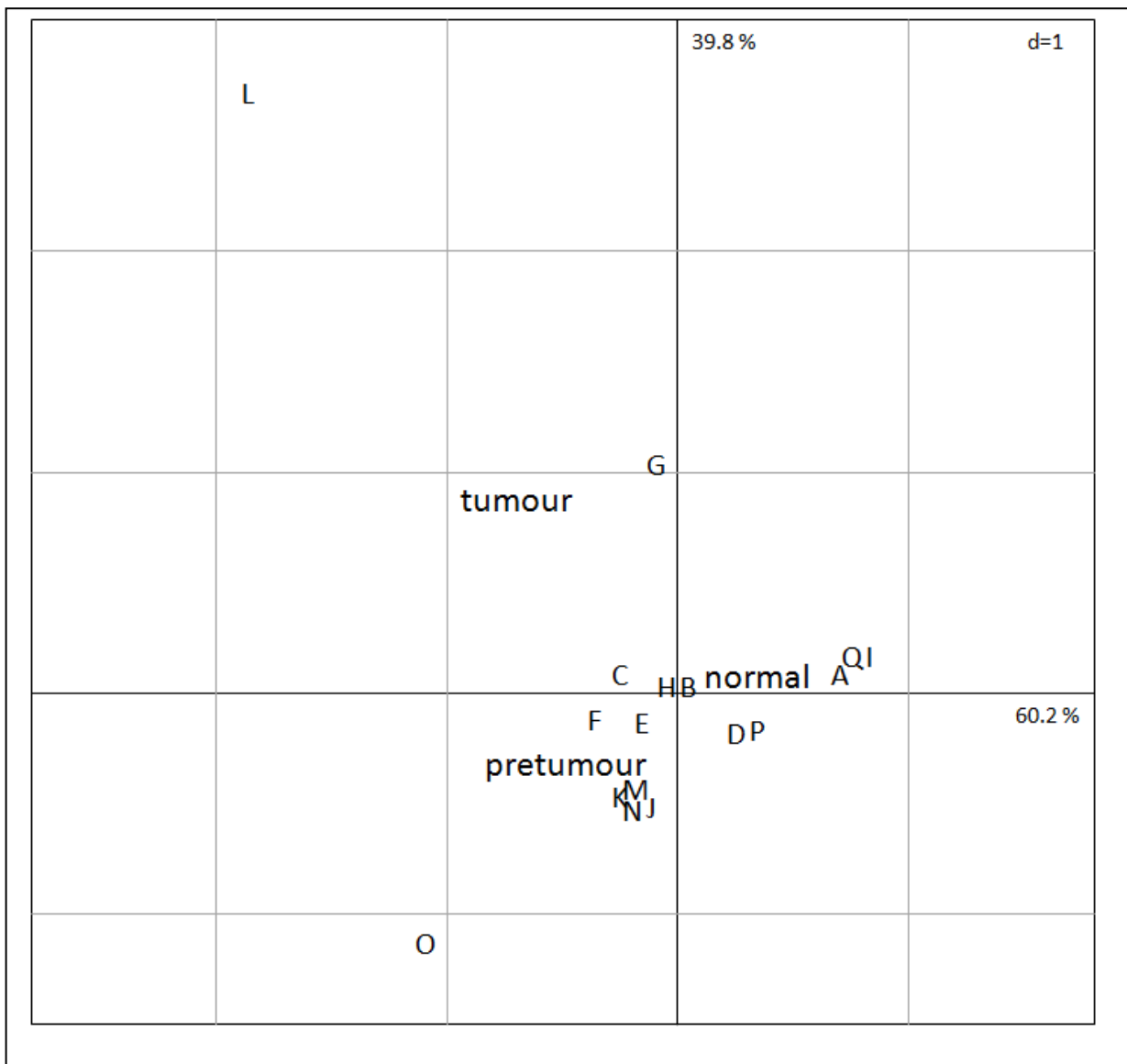
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585 **Figure 4**

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604 **Table 1**

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Profile name	<i>Rb</i> cDNA genetic changes			
	996 bp	1088 bp	1514 bp	1592 bp
A	G	T	G	G
B	G	T/C	G/T	G/T
C	G/A	T/C	G/T	G/T
D	G/A	T	G	G
E	G	C	T	T
F	G/A	C	T	T
G	G/A	T/C	G	G
H	G	T/C	G	G
I	G/A	T	G/T	G/T
J	G/A	C	G/T	T
K	G	C	G/T	T
L	G/A	C	G/T	G/T
M	G	C	G	G
N	G	T/C	G	G/T
O	G	T/C	T	T
P	G	C	G/T	G/T
Q	G	T	G	G/T

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