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1 **Mitochondrial DNA haplotype analysis of liver fluke in bison from Bialowieza**
2 **Primeval Forest indicates domestic cattle as the likely source of infection.**

3

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17

18 Abstract

19 We have determined the mitochondrial genotype of liver fluke present in Bison (*Bison*
20 *bonasus*) from the herd maintained in the Bialowieza National Park in order to
21 determine the origin of the infection. Our results demonstrated that the infrapopulations
22 present in the bison were genetically diverse and were likely to have been derived from
23 the population present in local cattle. From a consideration of the genetic structure of
24 the liver fluke infrapopulations we conclude that the provision of hay at feeding stations

25 may be implicated in the transmission of this parasite to the bison. This information
26 may be of relevance to the successful management of the herd.

27 *Key words:* Bison; *Fasciola hepatica*; genetic diversity; Bialowieza.

28

29 **1. Introduction**

30

31 The Bialowieza Primeval Forest (52°45'53"N, 23°51'39"E) covers an area of
32 approximately 1500 km² and straddles the border between Poland and Belarus. It is
33 characterised by stands of trees that are considered to be representative of those once
34 present over much of prehistoric northern Europe (Falinski, 2003) and supports a wide
35 variety of native European fauna, although management as a game reserve in previous
36 centuries has led to the extinction of some predators (such as the bear) and the
37 introduction of some non-native species (Sidorovich et al., 1996). Its most famous
38 “charismatic megafauna” is the European Bison, *Bison bonasus* and this species plays a
39 significant role in maintaining the ecology of the forest (Jaroszewicz et al. 2009). There
40 are two populations of bison, one in the Belarus part of the forest and the other in the
41 Bialowieza National Park (BNP), a 100 km² area of commercially unexploited forest on
42 the Polish side of the border. The bison in the BNP are separated from farmland and
43 domestic animals and their numbers are controlled to some extent by provision of
44 winter fodder and culling. The native bison herd was exterminated in 1918 and re-
45 established using twelve animals (some of which were descendants of the Bialowieza
46 bison) from zoological gardens in the 1950s. As a result of this process, the herd has
47 very limited genetic diversity, with mitochondrial DNA studies indicating only three
48 haplotypes (Wójcik et al., 2009) in 195 individuals. The herd’s nuclear genome is

49 similarly restricted, with only four alleles being found at the Major Histocompatibility
50 Complex (MHC) DRB3 locus in contrast to that in the American bison, *Bison bison*,
51 which, although also having suffered population bottle-necks, has 15 alleles (Radwan et
52 al., 2007; Traul et al., 2005). The four alleles present in the Bialowieza herd, however,
53 are highly divergent. The potential effects of loss of genetic variability and in particular
54 restricted MHC variability on the survival of species have been recently reviewed
55 (Radwan et al., 2010a).

56 Since the early 1980s, male bison in the Bialowieza herd have been suffering
57 from balanoposthitis, a chronic disease of the external genital organs associated with
58 infection with *Arcanobacterium* spp. (Lehnen et al., 2006) and it has been postulated
59 that this infection, which may endanger the herd, is a consequence of the lack of MHC
60 diversity (Udina and Shaikhaev, 1998). The Bialowieza herd is also susceptible to
61 infection by helminth parasites, with up to 44% of animals culled in 2001 carrying the
62 liver fluke, *Fasciola hepatica* (Kizeiwicz, 2008). In recent years, this incidence has
63 risen to 100% of adults, some of which show very heavy infections (> 600 flukes)
64 (Demiaszkiewicz et al., 2008). Infection with this parasite has been shown to modulate
65 the host's immune system towards a T helper 2 cytokine profile (Brady et al., 1999). It
66 has recently been proposed that this immunomodulatory effect may be due to secreted
67 helminth molecules that mimic the action of mammalian cathelicidins and may act to
68 reduce the inflammatory component of the immune response to bacterial infections
69 (Robinson et al. 2011). This raises the possibility that the balanoposthitis infection in
70 the Bialowieza herd may become a chronic infection due to the presence of a
71 concomitant liver fluke infection. In these circumstances, it is desirable to investigate
72 the origin of the liver flukes present in these animals in order to devise strategies which

73 may reduce the frequency of infection. We have determined the mitochondrial
74 haplotypes of flukes from bison and cattle from the Bialowieza region and compared
75 these with those seen in flukes from the wider northern European cattle population.

76

77 **2. Materials and Methods**

78

79 Flukes were obtained from bison culled in 2007 (Demiaszkiewicz et al. 2008)
80 and transported in absolute alcohol. Approximately 25mm³ of fluke tissue was placed
81 into 500µl of 10% w/v Chelex® (Fluka) solution incorporating 10µl proteinase K
82 (Sigma) at a concentration of 20mg/ml. This was heated at 55°C for one hour, followed
83 by gentle vortexing and a further incubation at 95°C for 30 minutes. The mixture was
84 gently vortexed and spun down at 10,000g for 10 seconds. The 250 µl of supernatant
85 was taken, diluted 1:10 in deionised water and stored at -20°C. Details of the primers
86 and procedures used for mitochondrial DNA analysis, data assembly and analysis of
87 population structure have been given elsewhere (Walker et al. 2011a; Teofanova et al.
88 2011). The sequences of each unique haplotype from the Polish flukes samples were
89 submitted to GenBank and have been assigned Accession numbers HM 487168 to HM
90 487199. The Bison fluke dataset was supplemented in analysis by sequences from five
91 flukes from cattle from eastern Poland and 444 flukes from a Dutch fluke dataset.
92 Details of the origin of these flukes and their Accession numbers are given elsewhere
93 (Teofanova et al. 2011, Walker et al. 2011a). Median-Joining networks were calculated
94 using “Network 4.5” (Flexus Technology Ltd) software which incorporates the
95 algorithm developed Bandelt and colleagues (Bandelt et al., 1999).

96

97 **3. Results**

98

99 A total of twenty-six sequences suitable for analysis were obtained from samples
100 of the flukes present in the infrapopulations from six bison (number of flukes analysed
101 per infrapopulation, 4,4,4,4,5,5). Following alignment and analysis, it was shown that
102 the twenty-six flukes carried twelve distinct mitochondrial haplotypes. Within the
103 population, the haplotypes followed a leptokurtic distribution, with the most common
104 haplotype occurring eleven times, the two next most frequent five times and twice,
105 respectively, and the remaining haplotypes being present in single flukes. There were
106 twenty-nine polymorphic sites seen in the 1160 nucleotides present in the analysed
107 sequences and the average number of nucleotide differences between pairs of samples
108 (P_i) was 0.00605. All mitochondrial sequences were consistent with the flukes being *F.*
109 *hepatica* rather than *F. gigantica* or other fasciolids. Infrapopulations from individual
110 bison generally contained several (< 4) haplotypes.

111

112 In order to investigate the genetic relationship between the flukes present in the
113 bison and those present in local domestic cattle, the dataset was supplemented with
114 flukes from Polish cattle and a Median Joining Network plotted (Figure 1a). This
115 shows that the flukes from the bison were derived from two well-defined clades with
116 almost all of the individual flukes being associated with the taxa forming the nucleus of
117 the clade or separated from these taxa by only one or two nucleotide changes. The
118 flukes from the local cattle were associated with only with one of these clades. In view
119 of the possibility that the small number of Polish flukes from cattle present in the
120 dataset could be distorting the analysis, we repeated it with the incorporation of a larger

121 dataset (N=444) containing flukes from elsewhere in northern Europe. To simplify the
122 display, a “star contraction” of 3 was applied to the data before calculating the network:
123 this condenses minor nodes (differing by less than three nucleotide changes) within a
124 clade. Figure 1b shows that, under these conditions, the Polish flukes from both cattle
125 and bison are associated with the two major clades seen with flukes from northern
126 Europe.

127

128 **4. Discussion**

129

130 The high prevalence of liver fluke infection in the bison herd in BNP
131 (Demiaszkiewicz et al., 2008) is indicative of the general immunological “weakness” of
132 this population. The results presented in this study prove that the liver flukes are
133 *Fasciola hepatica* and that, although the establishment of the BNP has allowed the
134 preservation of many ancient flora and fauna, these parasites do not appear to be
135 distinctive and are drawn from the same population as that which is common in present-
136 day cattle and sheep in northern Europe (Walker et al. 2011a). The BNP is separated
137 from adjacent farmland by a fence which should have prevented the ingress of infected
138 cattle or sheep and the subsequent transmission of *F. hepatica* to local snail populations.
139 There are a number of possibilities with regard to the route by which the population of
140 *F. hepatica* described in this study may have become part of the Bialowieza forest eco-
141 system. Wild herbivorous animals may have acted as a vector; deer are plentiful in the
142 forest and have been reported to be infected with liver fluke in the past although a recent
143 study of forty-one deer found that only three showed evidence of fasciolosis
144 (Demiaszkiewicz, in preparation). Hares are known to act as vectors for liver fluke both

145 in the Bialowieza region and elsewhere (Shimalov, 2001; Rondelaud et al., 2001).
146 However, infra-populations (the number of parasites in a single host) are typically small
147 in hares and rarely exceed three or four flukes (Shimalov, 2001; Walker et al. 2011b).
148 The diversity of haplotypes seen in the bison population is such that it would have
149 required multiple introductions by infected hares. This same argument would also be
150 valid with regard to the possibility that there had been an introduction of a population of
151 infected lymnaeid snail intermediate hosts. Although Polish lymnaeid snail populations
152 may have a high prevalence of infection (Kozak and Wędrychowicz, 2010) individual
153 snails are rarely infected by more than one or two miracidia (Kaplan et al., 1997) and
154 the asexual reproduction occurring at this stage of the life cycle acts to reduce genetic
155 diversity. The similarity of the mitochondrial haplotypes found in liver flukes from the
156 bison herd and those from cattle implicate cattle – directly or indirectly - in the
157 introduction of *F. hepatica* into the bison herd. Up to approximately fifty years ago
158 domestic cattle were grazed in the clearings of the Bialowieza National Park, however
159 heavy infection with liver fluke has only become evident in the last decade (Kizeiwicz,
160 2008), posing the question as to what was acting as the definitive mammalian host for
161 the parasite during the intervening years. Recruitment to the bison herd has been shown
162 to be related to climatic conditions, with the abundance of oak seeds (masting) in the
163 preceding year and the depth of snow in winter being major factors (Myserud et al.,
164 2007). To ameliorate the effects of snowy weather, hay is provided at a number of
165 feeding sites, which leads to the bulk of the herd gathering at these sites during the
166 winter months. It is possible that the fluke may have been inadvertently introduced into
167 the forest as metacercariae on contaminated hay. Liver fluke metacercariae are known
168 to be able to remain infective on foliage for up to eight months at above -10°C (Boray

169 and Enigk, 1964). Hay sourced from local farms (Kowalczyk et al. 2011) would, if
170 contaminated with *F. hepatica* metacercariae, transmit a population of flukes derived
171 from the local cattle population; this would explain why the distribution of haplotypes
172 and diversity seen in the bison flukes resembles that of the cattle flukes.

173

174 Although the high prevalence of the fasciolosis in the bison herd means that the
175 infection is probably self-maintaining, the likely origin of the flukes in local farm stock
176 means that they will be amenable to anthelmintic drugs should it become necessary to
177 treat individual bison. The long-term benefits of providing winter feeding have been
178 questioned (Wolk and Krasińska, 2004, Kowalczyka et al., 2011), as the congregation
179 of animals round the feeding stations may be conducive to the spread of infectious and
180 parasitic diseases (Radwan et al., 2010b). The findings in this study and that of others
181 (Jaroszewicz et al., 2009) indicate that further consideration should be given to the
182 question of supplementary winter feeding and the source of such feed should be
183 controlled, not only with regard to seeds from non-native species (as it is at present) but
184 also for possible contamination with parasite propagules.

185

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187

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190

191 **References**

192

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- 276

276 Figure Legend

277

278 Figure 1a. Median Joining Network for Polish flukes. White nodes – flukes from
279 Bison; black nodes - flukes from local cattle; red median vector node – virtual
280 (hypothetical) node. The size of each node is proportional to the number of
281 individual flukes bearing that haplotype. The distances between nodes are
282 proportional to genetic distance, as indicated by the positions of nucleotide
283 changes, which are shown by red numerals.

284

285 Figure 1b. Median Joining Network for Polish and other northern European Flukes.

286 Nodes containing haplotypes found in the Polish flukes from bison and cattle are
287 shown in grey, white nodes – other northern European cattle flukes.

288

Figure 1a

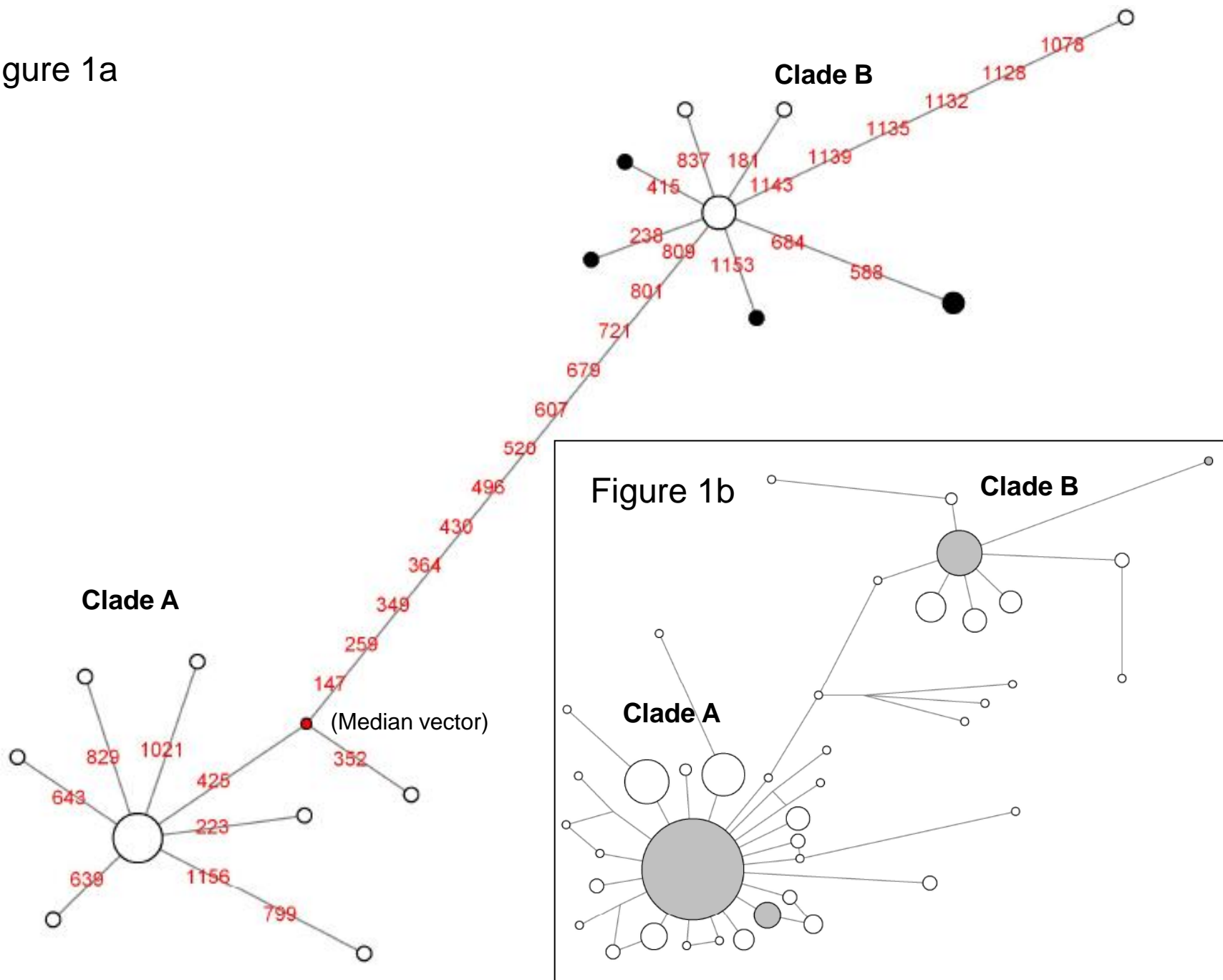


Figure Legend

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Figure 1b. Median Joining Network for Polish and other northern European Flukes. Nodes containing haplotypes found in the Polish flukes from bison and cattle are shown in grey, white nodes – other northern European cattle flukes.

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