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Molecular characterization of *Trichomonas gallinae* isolates recovered from the Canadian Maritime provinces' wild avifauna reveals the presence of the genotype responsible for the European finch trichomonosis epidemic and additional strains.

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1 **Molecular characterization of *Trichomonas gallinae* isolates recovered from the**
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4 **strains.**

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21 **Running title:** Molecular characterization of *T. gallinae* in Canadian wild avifauna

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24 **Summary** (150-200 words)

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26 Finch trichomonosis, caused by *Trichomonas gallinae*, emerged in the Canadian
27 Maritime provinces in 2007 and has since caused ongoing mortality in regional purple
28 finch (*Carpodacus purpureus*) and American goldfinch (*Carduelis tristis*) populations.
29 *Trichomonas gallinae* was isolated from (1) finches and rock pigeons (*Columbia livia*)
30 submitted for post mortem or live-captured at bird feeding sites experiencing
31 trichomonosis mortality; (2) bird seed at these same sites; and (3) rock pigeons live-
32 captured at known roosts or humanely killed. Isolates were characterized using internal
33 transcribed spacer (ITS) region and iron hydrogenase (Fe-hyd) gene sequences. Two
34 distinct ITS types were found. Type A was identical to the UK finch epidemic strain and
35 was isolated from finches and a rock pigeon with trichomonosis; apparently healthy rock
36 pigeons and finches; and bird seed at an outbreak site. Type B was obtained from
37 apparently healthy rock pigeons. Fe-hyd sequencing revealed six distinct subtypes. The
38 predominant subtype in both finches and the rock pigeon with trichomonosis was
39 identical to the UK finch epidemic strain A1. Single nucleotide polymorphisms in Fe-hyd
40 sequences suggest there is fine-scale variation amongst isolates and that finch
41 trichomonosis emergence in this region may not have been caused by a single spill-
42 over event.

43 **Keywords:** *Trichomonas gallinae*, trichomonosis, genotype, ITS, Fe-hydrogenase,
44 subtype, finch, pigeon

45 **Key Findings** (3-5 bullets of < 90 characters each, including spaces)

- 46 - Two *T. gallinae* ITS sequence types found in the Canadian Maritime provinces'
47 avifauna
- 48 - *T. gallinae* ITS sequence type in Canadian finches identical to UK finch epidemic
49 strain
- 50 - Bird seed from an outbreak yielded *T. gallinae* with the UK finch epidemic strain
51 ITS sequence
- 52 - Fe-hyd gene sequencing revealed fine-scale variation with six *T. gallinae*
53 subtypes
- 54 - Fe-hyd subtype of the UK finch epidemic strain was predominant in Canadian
55 finches

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63 Introduction

64 *Trichomonas gallinae* is a protozoan parasite which commonly infects the upper
65 digestive tract of columbids (*i.e.*, pigeons and doves) and birds of prey (*i.e.*, eagles,
66 hawks and owls) and less frequently can also infect a variety of other avian taxa
67 including passerines (such as finches and sparrows) (Forrester and Foster; 2009; Amin
68 *et al.* 2014). In 2005, trichomonosis was first recognized as an emerging infectious
69 disease of wild finches in Great Britain (GB) (Pennycott *et al.* 2005; Lawson *et al.* 2006).
70 The species affected in the summer/autumn seasonal epidemic were primarily
71 greenfinch (*Chloris [Carduelis] chloris*) and chaffinch (*Fringilla coelebs*). Although pre-
72 existing sporadic reports of disease in free-ranging finches do exist, the 2005-2006 (and
73 on-going) outbreak is the first reported instance of large-scale epidemic mortality due to
74 trichomonosis in any passerine species (Lawson *et al.* 2012). In the years following the
75 initial outbreak in the western and central counties of England and Wales, finch
76 trichomonosis spread to eastern England (2007) and then to southern Fennoscandia
77 (2008) and Germany (2009); epidemiological and historical banding return data
78 supported chaffinch migration as the most likely mechanism of the observed pattern of
79 disease spread (Neimanis *et al.* 2010; Robinson *et al.* 2010; Lawson *et al.* 2011a,
80 Peters *et al.* 2009). The disease range of finch trichomonosis has continued to extend
81 further eastward within continental Europe and had reached Austria and Slovenia by
82 2012 (Ganas *et al.* 2014). Concurrently, finch trichomonosis spread westward from
83 Britain with finch mortality incidents reported in Northern Ireland from 2006 and in the
84 Republic of Ireland from 2007 (Lawson *et al.* 2012).

85 In the late summer/early autumn of 2007, trichomonosis was first recognized in
86 the purple finch (*Carpodacus purpureus*) populations of Nova Scotia, Canada (Forzán
87 *et al.* 2010). In the following summer and autumn, the Canadian Wildlife Health
88 Cooperative (CWHC), Atlantic region, confirmed additional Canadian mortalities from
89 trichomonosis in the purple finch populations of Nova Scotia and Prince Edward Island
90 (PEI) and in American goldfinch (*Carduelis tristis*) populations of New Brunswick
91 (Forzán *et al.* 2010). In 2009, the CWHC confirmed finch trichomonosis incidents in all
92 three Canadian Maritime provinces during the same seasons, and diagnosed the
93 disease in a new species, the pine siskin (*Carduelis pinus*) (CWHC *unpublished data*).
94 The diagnosis of trichomonosis in the Canadian Maritime provinces in the summer and
95 autumn of three consecutive years and the infection of multiple finch species not known
96 to be previously affected by the disease, suggests that finch trichomonosis is an
97 emerging disease in this region. Prior to the emergence of trichomonosis in the
98 passerine bird populations of the Canadian Maritime provinces, this disease was not
99 diagnosed in any of the region's wild avian species since the CWHC, Atlantic Region,
100 began collecting diagnostic wildlife health data in 1992. While it is assumed that *T.*
101 *gallinae* is present in the columbid populations of the Canadian Maritime provinces due
102 to the parasite's ubiquitous distribution in wild pigeon and dove populations worldwide
103 (Amin *et al.* 2014), to our knowledge reports of *T. gallinae* in the region's columbid
104 populations have not been documented. Lastly, it is noteworthy that the Canadian
105 Maritime provinces represent the eastern limit of North America with closest
106 geographical proximity to the UK and that finch trichomonosis emerged in the years
107 immediately subsequent to the onset of epidemic mortality in British finches.

108 The aims of the present study were to firstly investigate the sequence diversity of
109 *T. gallinae* recovered from finches and columbids from the Canadian Maritime
110 provinces. Secondly, to compare the Canadian *T. gallinae* sequences with those
111 published from other countries, including isolates from GB. Finally, to provide a
112 description of the temporal, geographical and species-specific variation amongst the
113 isolates examined from the Canadian Maritime provinces. Genotyping of isolates was
114 determined by polymerase chain reaction (PCR) and sequencing of the ITS region
115 (5.8S rDNA and flanking internal transcribed spacer regions, ITS1 and ITS2) and the
116 hydrogenosomal Fe-hydrogenase (Fe-hyd) gene to evaluate finer scale evolutionary
117 relationships amongst these organisms (Lawson *et al.* 2011b; Chi *et al.* 2013).

118 **Materials and Methods**

119 *Columbid and passerine capture methods*

120 When suspected finch trichomonosis incidents in the Canadian Maritime
121 provinces were reported by members of the public, the CWHC, Atlantic region,
122 facilitated the immediate submission of recently dead passerines by encouraging
123 property owners to submit specimens for a detailed post-mortem examination (PME).
124 When PME confirmed trichomonosis (on the basis of gross and microscopic lesions or
125 microscopic lesions alone consistent with trichomonosis with or without a positive
126 culture of *Trichomonas* sp. from upper alimentary tract lesions), the site was visited to
127 live-capture all species of birds present and sample them for *Trichomonas* sp. by culture
128 (see culture technique below). In addition, food and water sources provided at these

129 sites were individually sampled for *Trichomonas* sp. by culture (see culture technique
130 below).

131 To investigate the heterogeneity of *T. gallinae* in sympatric columbid populations
132 in PEI, known locations of high columbid population densities were selected for
133 extensive trapping, without pre-existing knowledge of the presence of *T. gallinae* or
134 trichomonosis within these populations.

135 All birds were captured under a Canadian Wildlife Federation license (permit
136 #SC2707) and Canadian Council on Animal Care guidelines (UPEI protocol #10-020,
137 6003687) by standard methods including mist net, whoosh net, and walk-in box trap.
138 Passerines were captured for this study by using a mist net (Bleitz Wildlife Foundation
139 California, 50D-2 ply mesh, 1½" mesh, 7' X 42', Stock # 26N-50/2) for two days per
140 location in May-September of 2009 and 2011. Columbid species required extensive
141 time for acclimatization to the box trap, and as a result, columbids were ground trapped
142 at each site on multiple days sometimes occurring over a period of several weeks in
143 May-December of 2009 and 2010. A ground box trap (Safeguard single compartment
144 pigeon trap, 28"L x 24"W x 8"H, with eight entry doors, and a capacity to hold up to 30
145 birds) was baited with bird seed for a minimum of 12 days prior to commencing
146 trapping. It is important to note that during the allotted baiting period, all regular
147 supplementary feed sources were removed from the property to ensure the birds fed in
148 the baited area. Mourning dove, another target species, were difficult to capture with the
149 ground box trap so their capture was also facilitated by use of a whoosh-net (Hawkseye
150 Nets Virginia Beach, VA, USA - 2 1/8" mesh, 23' whoosh net). Similar to the box-trap

151 protocol, the area over which the whoosh-net was fired was baited with bird seed for a
152 minimum of 5 days prior to attempted capture.

153 Birds captured by all methods were sexed and aged by plumage (hatch year or
154 adult) when possible, weighed, banded and examined for clinical signs consistent with
155 trichomonosis such as the typical oropharyngeal lesions, fluffed up feathers, saliva on
156 the face, food at the commissures of the beak or matted in the feathers of the head or
157 chest and/or reluctance or inability to fly. If *T. gallinae* was isolated from a bird with no
158 clinical evidence of trichomonosis it was designated as “apparently healthy”. If *T.*
159 *gallinae* was isolated from a bird with clinical signs consistent with trichomonosis, the
160 infection was defined as a clinical case of trichomonosis. Opportunistic sampling was
161 also undertaken for *Trichomonas* sp. by culture of wild passerines and columbids
162 admitted to the Atlantic Veterinary College Teaching Hospital and of rock pigeons that
163 were humanely killed during removal from cattle barns in the winter months on PEI.

164 *Trichomonad culture*

165 Prior to swabbing live birds, the end of a sterile calcium-alginate cotton-tipped
166 swab with an aluminum shaft (Puritan™ Medical, Fisher Scientific, Canada, catalogue
167 number 22-029-501) was bent into a gentle curve representing ~ 120 ° angle to match
168 the natural anatomical curvature of the oral cavity as it opens into the esophagus. The
169 distance between the start of the curve and cotton tip was equivalent to the distance
170 between the oral cavity and crop, and the positioning of this curve depended on the
171 species of bird. Anatomically, bending the swab at the 120° angle facilitated the
172 movement of the swab from the oral cavity to the crop. After bending, the swab was

173 moistened with sterile saline and gently inserted into the oral cavity of the bird by
174 pushing the tip against a commissure of the beak. The swab was slowly and gently
175 advanced into the esophagus to the level of the crop while allowing the bird to swallow.
176 Due to the thinness of the esophageal and ingluvial walls in passerine birds this
177 procedure was done with extreme caution and only by experienced individuals to avoid
178 iatrogenic damage. Once in the crop, the swab was gently rotated, moved up and down
179 and removed. Care was taken to swab any visible oropharyngeal trichomonosis lesions.
180 The crop and lesions of dead birds were swabbed once the upper digestive tract was
181 opened for PME. After collection, all swabs were used to immediately inoculate an
182 InPouch TF™ test medium kit (BioMed Diagnostics, White City, OR, USA) on-site prior
183 to transport back to the laboratory for incubation at 37°C and daily monitoring for 10
184 days. If the site was not in the province of PEI, the samples were placed in a Hova-
185 Bator egg incubator (circulated air model no. 2362N, 20.3 watt, 115 volt AC,
186 G.Q.F.MFG. Co. Inc. Savannah, GA) set at 37°C for transport to the laboratory at the
187 University of Prince Edward Island.

188 Bird seed and water sources at sites experiencing trichomonosis mortality were
189 independently swabbed. The swabs were used to immediately inoculate an InPouch
190 TF™ test medium kit on-site prior to transport back to the laboratory for incubation at
191 37°C and daily monitoring for 10 days. If the site was not in the province of PEI, the
192 samples were placed in a Hova-Bator egg incubator for transport to the laboratory at the
193 University of Prince Edward Island as described above.

194 *Parasite culture and cryopreservation*

195 Parasite cultures were monitored daily using a double chamber hemacytometer,
196 counts of motile trichomonads were performed on both grids and if results did not
197 correlate within 10%, the process was repeated and the average of the four counts was
198 taken instead of the two. Once parasites reached mid-log phase, they were
199 cryopreserved by adding 100µl of 100% glycerol to 1ml of the parasite culture. This total
200 volume was subdivided into four separate 500µl aliquots and stored in liquid nitrogen.
201 An additional 1ml aliquot of the original parasite culture was collected to be used for
202 DNA extraction.

203 *PCR for ITS region and Fe-hyd gene regions*

204 *Trichomonas* sp. DNA was obtained from culture isolates using a QIAamp DNA
205 Mini Kit (QIAGEN, Toronto, ON, Canada) as per the manufacturer's instructions for cell
206 cultures. DNA extracts of 42 isolates (Table 1) were examined using PCR protocols
207 specific for the ITS1/5.8S rRNA/ITS2 region (subsequently referred to as the ITS region)
208 and Fe-hyd gene. DNA amplification of the ITS region (~ 300 bp) was performed using
209 trichomonad-specific primers TFR1 (5'-TGCTTCAGTTCAGCGGGTCTTCC-3') and
210 TFR2 (5'-CGGTAGGTGAACCTGCCGTTGG-3') (Felleisen 1997) while amplification of
211 the Fe-hyd gene (~ 900 bp) used the primers TrichhydFOR (5'-
212 GTTTGGGATGGCCTCAGAAT-3') and TrichhydREV (5'-
213 AGCCGAAGATGTTGTGCGAAT-3') (Lawson *et al.* 2011b; Chi *et al.* 2013). Each PCR
214 reaction mixture contained 12.5µl Amplitaq Gold Master Mix (Applied Biosystems, Life
215 Technologies, Burlington, ON, Canada), 4.5µl nuclease free water, 2.5µl forward primer
216 (10µM), 2.5µl reverse primer (10µM) and 3µl of undiluted target DNA and was

217 performed in duplicate. For each reaction, negative controls substituted target DNA with
218 3µl of nuclease-free water and positive controls used 3µl of *T. gallinae* DNA (purple
219 finch isolate from Forzán *et al.* 2010; parasite species confirmed by sequencing the ITS
220 region) and *T. gallinae* DNA from a British greenfinch (species confirmed by sequencing
221 the Fe-hyd gene) respectively. PCR parameters for the ITS region amplification were
222 94°C for 2 minutes, followed by 40 cycles of 94°C for 30 seconds, 67°C for 30 seconds,
223 72°C for 2 minutes and a final extension at 72°C for 15 minutes. PCR parameters for
224 Fe-hyd gene amplification were 94°C for 15 minutes, followed by 35 cycles of 94°C for 1
225 minute, 66°C for 30 seconds, 72°C for 1 minutes and a final extension at 72°C for 5
226 minutes. PCR amplicons were then examined via 1% agarose gel electrophoresis with
227 ethidium bromide.

228 *DNA sequencing and phylogeny reconstruction*

229 PCR products were sequenced in both directions at the McGill University and
230 Genome Québec Innovation Centre, Montréal, Québec, Canada. Sequences were
231 aligned with published trichomonad sequences from GenBank using BioEdit (Hall
232 1999). Phylogenies were constructed separately for the ITS region and Fe-hyd gene by
233 neighbour-joining (NJ), maximum parsimony (MP) and maximum likelihood (ML)
234 methods using MEGA version 6.0 (Tamura *et al.* 2013). Statistical support for NJ, ML,
235 and MP tree topologies were bootstrap-sampled 1,000 times and support values (%) of
236 NJ, MP and ML analysis were superimposed on the NJ consensus trees.

237 For phylogeny reconstruction using the ITS region, NJ tree evolutionary
238 distances were computed using the Jukes-Cantor method (Jukes and Cantor 1969) and

239 were reported in the units of the number of base substitutions per site. The MP tree was
240 obtained using the Subtree-Pruning-Regrafting algorithm (Nei and Kumar 2000) with
241 search level 1 in which the initial trees were obtained with the random addition of
242 sequences (10 replicates). The ML tree was constructed using Jukes-Cantor
243 substitution model (Jukes and Cantor 1969) as determined by the lowest Bayesian
244 Information Criterion (BIC) score and highest Akaike Information Criterion, corrected
245 (AICc) value (Tamura *et al.* 2013). Initial tree(s) for the heuristic search were obtained
246 by applying the NJ method to a pairwise distance matrix estimated using Maximum
247 Composite Likelihood (MCL). For ITS region trees, there were a total of 37 nucleotide
248 sequences using 209 positions in the final dataset. All positions containing gaps and
249 missing data were eliminated. Bootstrap values (1000 replicates) for each NJ, MP and
250 ML trees were computed following Felsenstein (1985).

251 For phylogeny reconstruction based on the Fe-hyd gene, the NJ, MP and ML
252 used the same parameters as for the ITS region sequences including bootstrap
253 replicates (1000). For Fe-hyd trees, there were 15 nucleotide sequences with a total of
254 803 positions in the final dataset. All positions containing gaps and missing data were
255 eliminated.

256 **Results**

257 *Parasite recovery from columbids, finches and environmental samples*

258 Forty-two trichomonad isolates were collected between 2009 and 2011 from rock
259 pigeons ($n = 12$), finch species ($n = 29$) and bird seed ($n = 1$) from the Canadian
260 Maritime provinces (Table 1 and Figure 1). Thirty-seven live mourning doves were

261 captured, swabbed and cultured for this study, and none of these individuals were
262 positive for *T. gallinae*. Additionally, no water samples were positive for *T. gallinae*. In
263 the individuals that died of trichomonosis, no gross or microscopic lesions consistent
264 with another disease being the primary problem (e.g., avipoxvirus infection or
265 salmonellosis) were identified at post mortem or with histopathology.

266 *ITS region sequence and phylogeny*

267 ITS region sequences of 300 nucleotides were derived for the 42
268 trichomonad isolates recovered from finches, rock pigeons and from a bird seed sample
269 in the Canadian Maritime provinces. Two distinct ITS region types were recognized that
270 share 98.5% similarity, (1) Sequence Type A (GenBank: KF214772) was identified in 39
271 *T. gallinae* isolates collected from American goldfinches ($n = 7$; 5 apparently healthy
272 individuals and 2 with trichomonosis), purple finches ($n = 22$; 8 apparently healthy
273 individuals and 14 with trichomonosis), rock pigeons ($n = 9$; 8 apparently healthy
274 individuals and 1 with trichomonosis) and an aggregate of moist bird seed removed
275 from several birdfeeders and deposited in a compost bin at a site confirmed to be
276 experiencing finch trichomonosis ($n = 1$) and (2) Sequence Type B (GenBank:
277 KF214773) was identified in *T. gallinae* isolates from 3 apparently healthy rock pigeons
278 (Table 1).

279 The ITS region phylogeny confirms that the *T. gallinae* isolates formed a
280 monophyletic assemblage within the trichomonads with two well-supported groups,
281 Type A & B (Figure 2). Type A contains 39 PEI isolates from finches and rock pigeons
282 as well as the bird seed sample (GenBank: KF214772) and also representative isolates

283 including the UK finch epidemic strain (GenBank: GQ150752) and other isolates from
284 finches, columbids and raptors from Brazil, Europe, Mauritius, Australia and the USA
285 (Figure 2). Type B contains an additional three isolates derived from PEI rock pigeons
286 (GenBank: KF214773) with no evidence of trichomonosis, along with representative
287 isolates derived from columbids, raptors and a canary from diverse geographic regions
288 including the USA, Europe and Australia (Figure 2).

289 *Fe-hyd gene sequence and phylogeny*

290 The Fe-hyd nucleotide sequences (901 nucleotides) were obtained from all finch
291 and rock pigeon isolates from the Canadian Maritime provinces (n=41). Multiple
292 attempts to amplify the Fe-hyd gene from DNA extracted from the bird seed sample
293 (isolate 42) were unsuccessful. Six different Fe-hyd sequence subtypes were
294 discovered that share between 98.1-99.8% similarities. The six Fe-hyd subtypes
295 identified in the present study are indicated in Table 1. The first subtype (GenBank:
296 KJ184167) included American goldfinch isolates 1-6, purple finch isolates 8, 11-17 and
297 19-29 and rock pigeon isolates 32, 34-35 and 37-38 that were identical to the clonal UK
298 finch epidemic strain (GenBank: JF681136, Lawson *et al.* 2011b). The second subtype
299 (GenBank: KJ184168) included purple finch isolates 9 and 10, while the third subtype
300 (GenBank: KJ184169) included American goldfinch isolate 7 and purple finch isolate 18
301 respectively; each subtype differed by one unique single nucleotide polymorphism
302 (SNP) from the UK finch epidemic strain A1. Similarly, the fourth subtype (GenBank:
303 KJ184170) from rock pigeon isolate 39 was identical to an isolate from a Madagascar
304 turtle dove (*Streptopelia picturata*) from the Seychelles (GenBank: JF681141), while the

305 fifth subtype (GenBank: KJ184171) from rock pigeon isolate 40 differed by one SNP.
306 The sixth subtype (GenBank: KJ184172) included rock pigeon isolates 33, 36 and 41
307 that were identical to an isolate from a wood pigeon (*Columba palumbus*) from the UK
308 (GenBank: KC529662).

309 The Fe-hyd phylogeny shows two distinct clusters of sequences. Isolates 1-6, 8,
310 11-17, 19-32, 34-35 and 37-38 (GenBank: KJ184167), isolates 7 and 18 from American
311 goldfinch and purple finch (GenBank: KJ184169) and the isolate from purple finches 9
312 and 10 (GenBank: KJ184168) all grouped with the UK finch epidemic strain A1
313 (GenBank: JF681136). The second cluster contains the two PEI rock pigeons isolates
314 39 and 40 (GenBank: KJ184170 and KJ184171 respectively) in a well-supported (98%
315 by all three phylogeny methods) cluster with an isolate from a Madagascar turtle dove
316 from the Seychelles (A2) (Figure 3).

317 The three other PEI rock pigeon isolates 33, 36 and 41 (GenBank: KJ184172)
318 grouped with a *T. gallinae* isolate from a wood pigeon from the UK (C4). These
319 sequences along with the remaining *T. gallinae* Fe-hyd gene sequences show a less
320 cohesive branching structure (Figure 3.).

321 Discussion

322 This study utilised ITS region and the Fe-hyd gene sequencing to investigate the
323 genetic diversity of *T. gallinae* in finch and columbid populations of the Canadian
324 Maritime provinces following the emergence of finch trichomonosis in this region.

325 The ITS region analysis revealed that two *T. gallinae* sequence types are present
326 in the wild avifauna of the Canadian Maritime provinces. In phylogenies based on ITS
327 region sequence data, *T. gallinae* splits into two very distinct groups as noted by
328 previous authors (Gerhold *et al.* 2008; Sansano-Maestre *et al.* 2009; Grabensteiner *et*
329 *al.* 2010, Lawson *et al.* 20011b).

330 All finch isolates in this study, whether they originated from apparently healthy
331 birds or birds with trichomonosis, were identical to the *T. gallinae* Type A that has been
332 previously identified in European finches and is widespread in North American
333 columbids (Gerhold *et al.* 2008; Lawson *et al.* 2011b; Girard *et al.* 2014). Importantly,
334 this same type was identified in nine rock pigeons (eight apparently healthy individuals
335 and one with trichomonosis) (Table 1). Thus, the ITS region sequence typing alone
336 cannot discriminate whether the origin of trichomonosis in finches in the Canadian
337 Maritime provinces is a translocation of the European finch strain or is simply the result
338 of contact with infected sympatric columbids. However, because both American
339 goldfinch and purple finch populations in the Canadian Maritimes are considered local
340 resident populations with limited distance North-South migrations (mainly associated
341 with weather conditions and food availability) and rock pigeons are non-migratory year-
342 round residents, a plausible scenario for transmission between these species at local
343 bird feeding stations is reasonable without requiring movement of the disease from
344 Europe to the Canadian Maritime provinces.

345 A common factor in the emergence of trichomonosis in finches in all geographical
346 locations is that the mortality is identified where large numbers of birds congregate at
347 private birdfeeding and watering stations (Forzán *et al.* 2010; Neimanis *et al.* 2010;

348 Robinson *et al.* 2010). Therefore, it has been suggested that indirect transmission
349 associated with contaminated bird seed, water bowls, or bird baths plays a role in the
350 epidemiology of this disease (Boal *et al.* 1999; Neimanis *et al.* 2010; Robinson *et al.*
351 2010; Gerhold *et al.* 2013). In the present study, *T. gallinae* was not detected in water
352 collected from sites where trichomonosis mortalities were occurring. This was surprising
353 given that Bunbury *et al.* (2007) were successful in recovering *T. gallinae* from puddles
354 and Gerhold *et al.* (2013) found that *T. gallinae* was able to survive for up to 20 minutes
355 in both distilled and chlorinated water when organic matter (detritus, leaves and soil)
356 was present. One caveat to our water sampling success was that property owners
357 undergoing bird mortalities in their backyards became more diligent in cleaning feeders
358 and waterers. Thereby reducing the likelihood of recovering parasites from water
359 samples collected in our study. In support of this fact, the only successful isolation of *T.*
360 *gallinae* from bird seed was from a composite sample disposed of in a compost bin at a
361 property experiencing trichomonosis mortality. This isolation supports the experimental
362 evidence that showed *T. gallinae* can survive in moist grain for 120 hours (Kocan 1969).
363 Furthermore, ITS typing confirmed that the bird seed isolate was Type A, identical to *T.*
364 *gallinae* isolates recovered from sick birds on the same property.

365 Interestingly, we also identified three rock pigeons infected with Type B *T.*
366 *gallinae*, a type that has been reported in columbids from the USA, eastern Spain and
367 Austria as well as in raptors from eastern Spain (Gerhold *et al.* 2008; Sansano-Maestre
368 *et al.* 2009). In a prevalence study of *T. gallinae*, Sansano-Maestre *et al.* (2009)
369 examined pigeons and raptors with gross lesions consistent with trichomonosis and
370 apparently healthy birds with no identifiable lesions and found that Type A *T. gallinae*

371 were recovered more frequently from birds with gross lesions of trichomonosis, whereas
372 Type B *T. gallinae* were recovered from individuals with no lesions, suggesting a
373 relationship between Type A and increased virulence. Sansano-Maestre *et al.* (2009)
374 also speculated that Type B parasites may be adapted to pigeon hosts as this Type was
375 much more prevalent in pigeons than in raptors. Similar to Sansano-Maestre *et al.*
376 (2009) study, we found that all Type B isolates were recovered from apparently healthy
377 rock pigeons, and all finch species and rock pigeons with evidence of clinical
378 trichomonosis were infected with Type A. However, it is important to note that while all
379 isolates recovered from either finches or pigeons with clinical evidence of trichomonosis
380 were Type A, Type A isolates were also recovered from apparently healthy birds. Also,
381 while rock pigeon isolates were not all Type B, all Type B isolates in our study were
382 recovered exclusively from rock pigeons, all of which were apparently healthy
383 individuals. Thus our results are consistent with the hypothesis put forward by Sansano-
384 Maestre *et al.* that there may be a relationship between Type A and increased virulence.

385 Through examination of multiple gene regions (ITS region, Fe-hyd gene and
386 small sub-unit rDNA), as well as random amplified polymorphic DNA analyses, Lawson
387 *et al.* (2011b) examined over 50 isolates obtained from finch trichomonosis cases and
388 found no evidence for multiple strains, concluding that a clonal strain of Type A was
389 responsible for the emergence of epidemic trichomonosis in GB. Lawson *et al.*, (2011b)
390 further speculated that due to the clonal nature of the passerine epidemic strain, it most
391 likely recently arose from a bottleneck, such as a single spill-over event (*i.e.*, host-
392 switching) from columbids to sympatric finches. In the present study, ITS region
393 sequence analysis revealed that all Type A isolates from the Canadian Maritime

394 provinces were identical to the UK finch epidemic strain. Furthermore, our examination
395 of the Fe-hyd gene also revealed that several finch and rock pigeon isolates were
396 identical to the UK finch epidemic strain (Lawson *et al.* 2011b). However, it is equally
397 important that Fe-hyd sequence analysis also revealed single nucleotide polymorphisms
398 amongst some of the Canadian Type A isolates. Based on Fe-hyd nucleotide sequence
399 analysis, four Canadian Type A isolates, including American goldfinch, purple finch and
400 rock pigeon isolates, and one Canadian Type B isolate, only from a rock pigeon, were
401 found to be different from both the clonal UK epidemic strain and the Canadian Maritime
402 provinces' isolates similar to the clonal UK epidemic strain mentioned above (see
403 Figures 2 and 3). This suggests divergence not only from the British finch and
404 Seychelles columbid strains they were compared to, but also from each other, indicating
405 that a number of strains of *T. gallinae* are present in the wild avifauna of the Canadian
406 Maritime provinces. Analysis of the Fe-hyd gene sequences from the Canadian
407 Maritime provinces bird isolates showed that there is fine-scale variation amongst
408 isolates akin to that observed in UK columbid populations. This observation suggests
409 that the emergence of finch trichomonosis in this region may have been caused by
410 multiple spill-over events, either from sympatric columbids, another bird species as yet
411 unknown to be infected with the parasite or from virulent *T. gallinae* developing
412 independently within the Canadian Maritime provinces' finch populations. In support of
413 this view a recent paper has reported the presence of the UK finch epidemic subtype A1
414 in North American columbids (Girard *et al.* 2014) similar to the findings in this study.

415 Indeed, when historic *T. gallinae* DNA samples were subtyped, the A1 subtype
416 had also been isolated from Mauritian columbids sampled in 2004 (*unpublished data*)

417 suggesting distribution of this subtype may actually be longstanding and global. Other
418 reports of finch trichomonosis in North America have since emerged in west and east-
419 central United States of America (Gerhold 2009) and western Canada (Canadian
420 Cooperative Wildlife Health Centre *unpublished data*) in 2009. During the winter and
421 spring of 2009, the Southeastern Cooperative Wildlife Disease Study (SCWDS)
422 conducted PME's on passerines of multiple species, including American goldfinch,
423 house finch (*Carpodacus mexicanus*), northern cardinal (*Cardinalis cardinalis*), pine
424 siskin and purple finch, submitted from mortality incidents from the eastern United
425 States and found that whilst the majority had salmonellosis, at least 12 birds were
426 suffering from trichomonosis or had concurrent infection with both of these pathogens
427 which result in upper alimentary tract lesions (Hernandez *et al.* 2013; Gerhold 2009).

428 As with GB, there is evidence of some finch trichomonosis incidents in North
429 America prior to the emergence of finch trichomonosis in the Canadian Maritime
430 Provinces in 2007. On the western coast of the USA, Anderson *et al.* (2002) screened
431 birds for trichomonad parasites on admission to a northern California wildlife
432 rehabilitation facility over a period of four years (2001-2005) and found evidence of a
433 low prevalence of the infection in the house finch (1.7%) with a high case fatality rate
434 (95.5%); these authors hypothesised that the infection may be endemic in this (and
435 other) passerine species in the region. Moreover, an outbreak affecting house finches,
436 house sparrows and American goldfinches, contemporaneous with American mourning
437 dove mortality (*Zenaida macroura*), occurred in the Midwest (Kentucky, Ohio and
438 Indiana) in the autumn of 2002. A combination of trichomonosis and West Nile virus
439 (WNV) infection was diagnosed as the cause of mortality (estimated total of 200 birds)

440 although the relative importance of these agents was not described (NWHC 2002). In
441 the summer of 2006, a mixed species mortality incident of circa 200 birds involving
442 house finches, American goldfinches and a gray catbird (*Dumetella carolinensis*) was
443 reported to the SCWDS. Eighteen birds were submitted for PME with trichomonosis
444 confirmed in ten cases and WNV infection detected in one bird (Gerhold 2009).

445 Various potential routes exist through which the UK finch epidemic strain of *T.*
446 *gallinae* could have been introduced to the Canadian Maritime Provinces. Bird migration
447 is believed to be the primary route of spread of the disease within Europe. Large
448 numbers of the finch and columbid species in which trichomonosis has been most
449 frequently diagnosed in GB in recent years have been banded (1960-2012 inclusive)
450 (greenfinch $n=2,107,976$, chaffinch $n=1,287,396$, goldfinch (*Carduelis carduelis*) $n=$
451 $466,108$, siskin (*Carduelis spinus*) $n=503,097$ and collared dove $n=37,780$, wood pigeon
452 $n=45,823$): however, no banded birds of these species have been recovered in North
453 America over that period suggesting international exchange is negligible (Robinson and
454 Clark 2013). Indeed, there are remarkably few exchanges of any British wild bird
455 species recorded with North America, with the most frequent being for seabirds and
456 waders, including the kittiwake (*Rissa tridactyla*) $n=73$, Manx shearwater (*Puffinus*
457 *puffinus*) $n=25$, knot (*Calidris canutus*) $n=19$, turnstone (*Arenaria interpres*) $n=14$, and
458 fulmar (*Fulmarus glacialis*) $n=13$; all other species with <10 individual birds recorded as
459 North American band recoveries are seabirds, shorebirds or waterfowl species in which
460 *T. gallinae* infection has not been recorded (Robinson and Clark, 2013). Collectively,
461 therefore bird migration from Europe is an unlikely route of introduction. Since *T.*
462 *gallinae* is not capable of long-term environmental persistence, movement with fomites

463 is also an implausible method of parasite translocation. Anthropogenic movement of
464 captive birds, whether deliberate (e.g. cage and aviary birds, game birds, zoological
465 collections) or accidental (e.g. wild bird stowaways or stray racing pigeons) could have
466 occurred; however, there is no available evidence to support or refute this hypothesis
467 further. Collectively, therefore, whilst the emergence of finch trichomonosis in the
468 Canadian Maritime Provinces occurred shortly after the emergence of the disease in GB
469 in time, there is no clear candidate for a plausible route of introduction of the finch
470 epidemic strain of *T. gallinae* from the UK.

471 Instead, there is evidence that favours the hypothesis that finch trichomonosis
472 emerged locally in the Canadian Maritime Provinces, through spillover from sympatric
473 birds; this route is most consistent with the SNPs in Fe-hyd subtypes found amongst the
474 finch and columbid isolates from PEI. The occurrence of endemic finch trichomonosis in
475 western USA (Anderson *et al.* 2009), and other isolated finch mortality incidents due to
476 the disease, indicates that parasite strains with the potential to cause disease in
477 passerines have been present in North America for some time.

478 Future studies should examine *T. gallinae* isolates using multiple gene regions,
479 or full genome sequencing, in order to provide more detailed information about their
480 genetics which could lead to a better understanding of the epidemiology of avian
481 trichomonosis and the mechanisms of disease emergence.

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- 606

607 Figure 1. Geographical distribution of the sites in the Canadian Maritime provinces
608 where *Trichomonas gallinae* isolates were collected. Superscripts correspond to the
609 birds from which the isolate was recovered: ^F = finch; ^P = pigeon; and ^{BS} = bird seed.
610 Refer to Table 1 for additional details for each isolate.

611

612 Figure 2. Neighbour-joining 60% bootstrap-consensus tree based on *Trichomonas*
613 *gallinae* ITS region sequences. Values at nodes represent the bootstrap percentages
614 from 1,000 replicates for neighbour-joining, maximum parsimony and maximum
615 likelihood respectively. There were a total of 209 positions in the final dataset as all
616 positions containing gaps and missing data were eliminated. GenBank accession
617 numbers are given along with host names or isolate designations and country for each
618 trichomonad. Isolates in bold are from birds sampled in the present study. For additional
619 isolate details see Table 1. * indicates UK Finch epidemic strain.

620

621 Figure 3. Neighbour-joining 60% bootstrap-consensus tree based on *Trichomonas*
622 *gallinae* Fe-hydrogenase gene sequences. Values at nodes represent the bootstrap
623 percentages from 1,000 replicates for neighbour-joining, maximum parsimony and
624 maximum likelihood respectively. There were a total of 803 positions in the final dataset.
625 GenBank accession numbers are given beside host names or isolate designations and
626 country for each trichomonad. Isolates in bold are from birds sampled and designated
627 into the six Fe-hyd subtypes identified in the present study. For additional isolate details
628 see Table 1. * indicates UK Finch epidemic strain.

Table 1. Case data and *Trichomonas gallinae* isolates used for the ITS region and Fe-hydrogenase (Fe-hyd) gene PCR analyses. The last two digits of the year of collection are indicated as the first two digits of the case number. Bird state (alive or dead) indicates whether the sample was collected in-field from live-sampling or at necropsy. ITS typing and Fe-hyd subtyping results from sequence data are recorded.

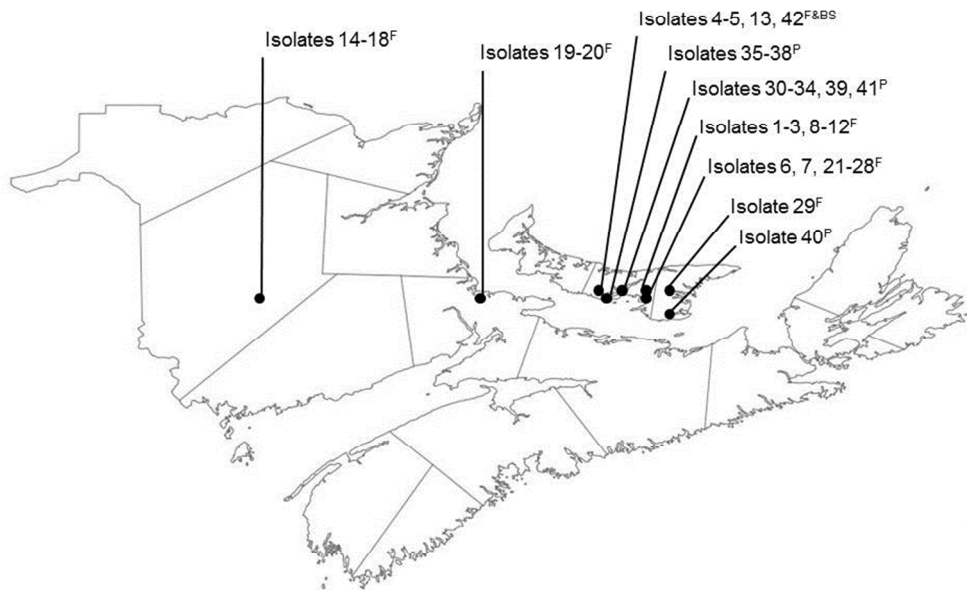
Isolate ID	Case no.	Species	Bird state	Trichomonosis Status	Site location	ITS type	Fe-hyd subtype
1	09-01	American goldfinch	Alive	Apparently Healthy	Vernon River, PEI	A	1
2	09-02	American goldfinch	Alive	Apparently Healthy	Vernon River, PEI	A	1
3	09-07	American goldfinch	Alive	Apparently Healthy	Vernon River, PEI	A	1
4	09-14	American goldfinch	Alive	Apparently Healthy	New Haven, PEI	A	1
5	09-28	American goldfinch	Alive	Apparently Healthy	New Haven, PEI	A	1
6	11-116	American goldfinch	Alive [†]	Trichomonosis	Orwell, PEI	A	1
7	11-122	American goldfinch	Alive	Trichomonosis	Orwell, PEI	A	3
8	09-04	Purple finch	Alive ^{††}	Trichomonosis	Vernon River, PEI	A	2
9	09-08	Purple finch	Alive	Trichomonosis	Vernon River, PEI	A	2
10	09-12	Purple finch	Alive	Apparently Healthy	Vernon River, PEI	A	2
11	09-13	Purple finch	Alive	Apparently Healthy	Vernon River, PEI	A	1

12	09-15	Purple finch	Alive	Trichomonosis	Vernon River, PEI	A	1
13	09-24	Purple finch	Alive	Apparently Healthy	New Haven, PEI	A	1
14	11-29	Purple finch	Dead	Trichomonosis	Durham Bridge, NB	A	1
15	11-31	Purple finch	Alive ^{††}	Apparently Healthy	Durham Bridge, NB	A	1
16	11-32	Purple finch	Alive	Apparently Healthy	Durham Bridge, NB	A	1
17	11-46	Purple finch	Alive	Apparently Healthy	Durham Bridge, NB	A	1
18	11-50	Purple finch	Alive ^{††}	Apparently Healthy	Durham Bridge, NB	A	3
19	11-100	Purple finch	Dead	Trichomonosis	Pointe-du-Chêne, NB	A	1
20	11-136	Purple finch	Alive	Apparently Healthy	Pointe-du-Chêne, NB	A	1
21	11-113	Purple finch	Alive	Trichomonosis	Orwell, PEI	A	1
22	11-114	Purple finch	Alive [†]	Trichomonosis	Orwell, PEI	A	1
23	11-115	Purple finch	Alive	Trichomonosis	Orwell, PEI	A	1
24	11-117	Purple finch	Alive	Trichomonosis	Orwell, PEI	A	1
25	11-119	Purple finch	Alive	Trichomonosis	Orwell, PEI	A	1
26	11-120	Purple finch	Alive	Trichomonosis	Orwell, PEI	A	1
27	11-121	Purple finch	Alive	Trichomonosis	Orwell, PEI	A	1
28	11-124	Purple finch	Alive	Trichomonosis	Orwell, PEI	A	1
29	11-146	Purple finch	Dead	Trichomonosis	Montague, PEI	A	1
30	10-08	Rock pigeon	Alive	Apparently	Charlottetown,	A	1

				Healthy	PEI		
31	10-09	Rock pigeon	Alive	Apparently Healthy	Charlottetown, PEI	A	1
32	10-12	Rock pigeon	Alive	Apparently Healthy	Charlottetown, PEI	A	1
33	10-14	Rock pigeon	Alive	Apparently Healthy	Charlottetown, PEI	B	6
34	10-16	Rock pigeon	Alive	Apparently Healthy	Charlottetown, PEI	A	1
35	11-06	Rock pigeon	Alive	Apparently Healthy	New Dominion, PEI	A	1
36	11-07	Rock pigeon	Alive	Apparently Healthy	New Dominion, PEI	B	6
37	11-08	Rock pigeon	Alive	Apparently Healthy	New Dominion, PEI	A	1
38	11-09	Rock pigeon	Alive	Apparently Healthy	New Dominion, PEI	A	1
39	11-12	Rock pigeon	Dead	Trichomonosis	Charlottetown, PEI	A	4
40	11-13	Rock pigeon	Dead	Apparently Healthy	Murray River, PEI	A	5
41	11-151	Rock pigeon	Dead	Apparently Healthy	Charlottetown, PEI	B	6
42	09-BF	Bird seed	N/A	N/A	New Haven, PEI	A	NE ^{††}

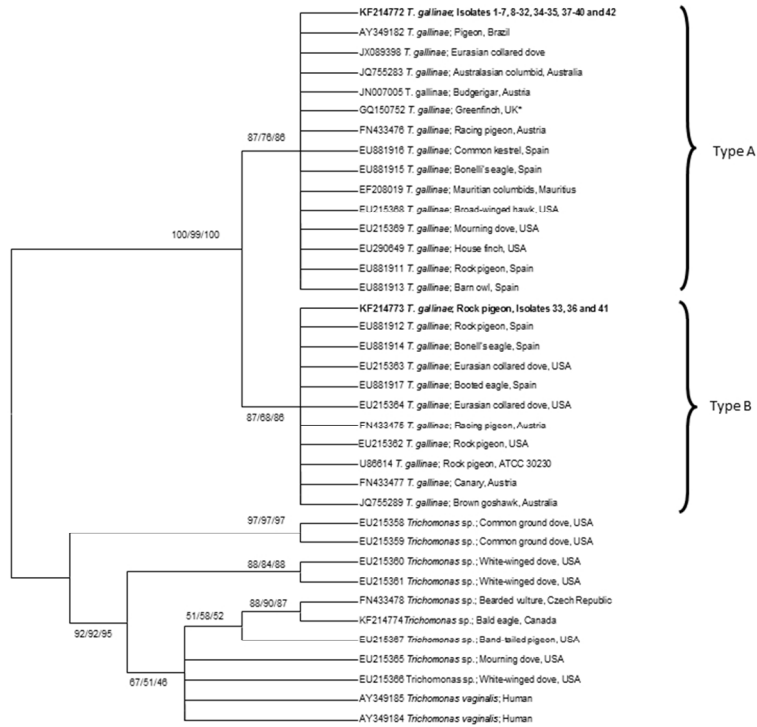
[†] died immediately after swabbing (approx. 45 minutes), confirmed trichomonosis as cause of death via post-mortem examination. ^{††} died after swabbing (days to weeks), confirmed trichomonosis as cause of death via post-mortem examination

^{††}NE – Not evaluated as the PCR was unsuccessful.



160x120mm (152 x 152 DPI)

Review



160x120mm (152 x 152 DPI)



160x120mm (152 x 152 DPI)