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

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Summary (150-200 words)

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

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

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

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

Introduction

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

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

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

Materials and Methods

Columbid and passerine capture methods

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

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

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

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

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

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

Trichomonad culture

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

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

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

Parasite culture and cryopreservation

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

PCR for ITS region and Fe-hyd gene regions

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

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

DNA sequencing and phylogeny reconstruction

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

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

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

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

Results

Parasite recovery from columbids, finches and environmental samples

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

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

ITS region sequence and phylogeny

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

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

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

Fe-hyd gene sequence and phylogeny

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

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

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

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

Discussion

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

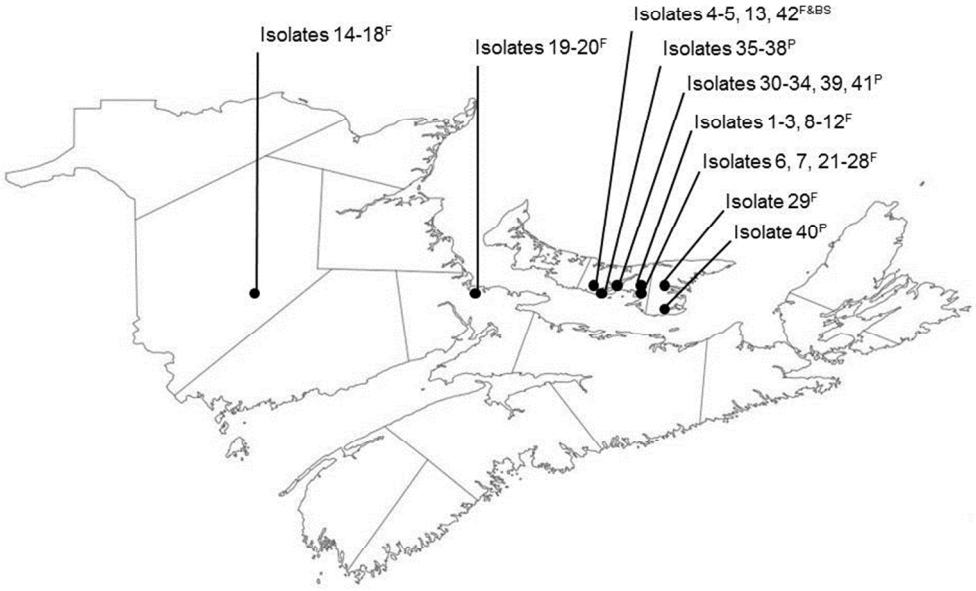
Isolate ID	Case no.	Species	Bird state	Trichomonosis Status	Site location	ITS type	Fe-hyd subtype
1	09-01	American goldfinch	Alive	Apparently Healthy	Vernon River, PEI	A	1
2	09-02	American goldfinch	Alive	Apparently Healthy	Vernon River, PEI	A	1
3	09-07	American goldfinch	Alive	Apparently Healthy	Vernon 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.



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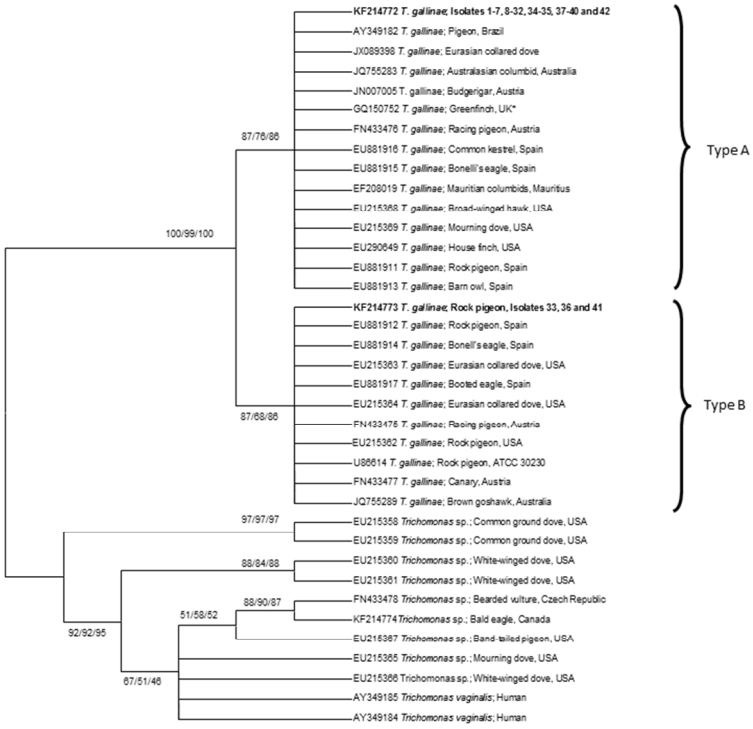

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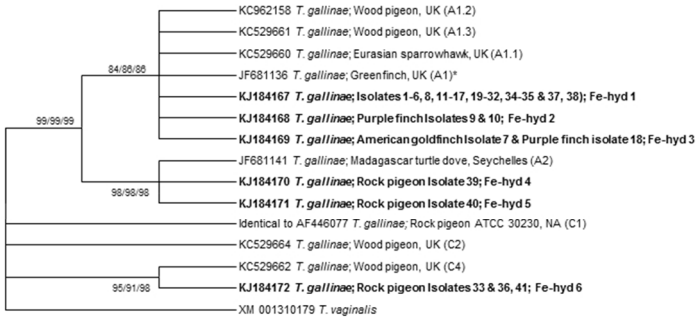

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