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A clonal strain of *Trichomonas gallinae* is the aetiologic agent of an emerging avian epidemic disease

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ABSTRACT

Trichomonas gallinae is a protozoan parasite that is well characterised as a cause of trichomonosis in columbid and raptor species world-wide. The parasite emerged as a novel infection of British passerines in 2005, leading to epidemic mortality associated with significant declines of breeding populations of greenfinches (*Carduelis chloris*) and chaffinches (*Fringilla coelebs*). We characterised the extent of *T. gallinae* genotypic heterogeneity within the affected wild British avifauna by analysing individual isolates from 17 of the species affected. To do so, we employed improved platform-based multilocus typing tools as well as the hydrogenosomal Fe-hydrogenase gene as a single marker locus for fine-typing. We found no evidence of heterogeneity amongst the parasites infecting British passerines, indicating that a clonal strain of *T. gallinae* is the causative agent of this emerging infectious disease.

1. Introduction

Trichomonosis, characterised by necrotic ingluvitis, was first recognised as an emerging infectious disease of British finches in 2005 (Pennycott et al., 2005) and caused epidemic mortality and significant declines of greenfinch (*Carduelis chloris*) and chaffinch (*Fringilla coelebs*) populations in subsequent years (Robinson et al., 2010). The reason why *Trichomonas gallinae*, an endemic parasite of columbid populations, caused novel disease in sympatric finches is not understood, but the most likely explanations are to do with changes to environmental or genetic factors. Frequent contact rates amongst birds feeding at high densities at garden feeding stations (Kirkwood, 1998) and opportunities for novel inter-specific contacts at shared feeding sources suggest that recently increased provisioning of wild birds (Jones and Reynolds, 2008; Davies et al., 2009) might have increased opportunities for the parasite to spill-over to naïve host species. Alternatively, or in addition, novel and enabling genetic change within an endemic strain of *T. gallinae* might have occurred, expanding the range of susceptible host species and leading to the emergence of an epidemic in finches.

Previously, we used sequence data obtained from the ITS1/5.8S rRNA/ITS2 region to diagnose *T. gallinae* infection in nine greenfinches and nine chaffinches that had died as a result of necrotic ingluvitis in Britain during 2005 and 2006 (Robinson et al., 2010). Resulting sequences had 100% homology to published columbiform isolates (EU215369 from the USA, Gerhold et al., 2008; EF208019 from Mauritius, Gaspar da Silva et al., 2007; AY349182 (origin not available), Kleina et al., 2004) and passerine isolates from the USA (EU290649, Anderson et al., 2009). Also, a nested PCR, targeting a fragment of the small subunit (SSU) rRNA gene was developed for case diagnosis (Robinson et al., 2010) and the 149 base pair (bp) sequence obtained matched published isolates from columbiform species with 100% homology (EU215373, EU215374; Gerhold et al., 2008). No sequence variation within the limited number of British isolates collected was detected at either locus (Robinson et al., 2010).

In this study, we looked for evidence of genetic heterogeneity in the *T. gallinae* causing necrotic ingluvitis in British passerines, columbids and raptors, analysing DNA extracted from infected lesions or *T. gallinae* cultures isolated from 17 British bird species (comprising seven Fringillidae (finches), three Accipitridae (hawks),

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two Columbidae (pigeons), one Turdidae (blackbird), one Paridae (tit), one Passeridae (sparrow), one Prunellidae (dunnock), and one Strigidae (owl)) which had been examined as part of the Garden Bird Health *initiative* (GBH*i*) (Robinson et al., 2010) between 2004 and 2009. Samples were analysed in three ways:

First, the ITS1/5.8S rRNA/ITS2 and SSU rRNA loci were sequenced to confirm the presence of *T. gallinae* infection. Second, sequence analysis of a new single locus sequence typing gene was undertaken. The hydrogenosomal Fe-hydrogenase gene, which is a house-keeping gene common to amitochondrial protists, was chosen as this has been used successfully to evaluate evolutionary relationships amongst various organisms, including *T. vaginalis* (Voncken et al., 2002). This Fe-hydrogenase gene shows potential as a novel genotyping marker with the capacity to detect fine-scale variation amongst *T. gallinae* parasites.

Finally, since published sequence analyses had not hitherto enabled fine-scale strain differentiation amongst *T. gallinae* isolates, we utilised random amplified polymorphic DNA (RAPD) analyses for this purpose. RAPD analyses amplify an undefined and arbitrary group of loci, some of which are hypervariable, such as microsatellites, and may change from generation to generation and some of which are essentially invariant. Thus sample variation is expected in RAPD analyses, even over a very small timescale, and can provide a sensitive measure of relatedness amongst samples. RAPD analyses, however, demand stringent and consistent methodologies and high quality DNA to ensure reproducibility, both between gels and between samples. Consequently, in this study, we further optimised RAPD methodologies for the detection of *T. gallinae* parasite strain variation with the application of platform technology and new software for analysis.

Results from the sequence and RAPD analyses were appraised to determine the genotypic heterogeneity amongst *T. gallinae* causing avian disease in the UK over recent years and for any evidence of temporal, geographical or species-specific variation.

2. Materials and methods

2.1. Infected birds

Post mortem examinations were performed on wild birds of multiple species by the veterinary laboratories participating in the GBH*i* from 2004 to 2009 following a standardised protocol (Robinson et al., 2010). Necrotic ingluvitis lesions were collected from trichomonosis cases and stored at −20 °C or −80 °C. In addition, fresh necrotic ingluvitis lesions were incubated at 30 °C in Oxoid Trichomonas Media No. 2. (Oxoid, UK) with chloramphenicol (0.125 mg/ml) and were screened for the successful culture of motile trichomonads at 24, 48, 72 h and 5 days (Robinson et al., 2010). Although 35.5 °C is the optimal growth temperature for *T. gallinae* (Diamond, 1957), we used 30 °C because at this temperature the sensitivity of detection is maintained but with a shallower growth curve, thus increasing the time period during which high quality parasite DNA could be harvested. Similarly, prolonged viability of *T. vaginalis* has been demonstrated *in vitro* at lower growth temperatures (Smith, 1983). Media with motile trichomonads were routinely stored at −20 °C or −80 °C and a subset of parasites also was cryopreserved.

In addition, necrotic pharyngitis lesions were available from two wood pigeons (*Columba palumbus*) which had died with trichomonosis in 2002, before the emergence of trichomonosis in British finches, and which had been stored at −20 °C.

2.2. DNA extraction techniques for PCR and sequencing

DNA was extracted from frozen then thawed necrotic ingluvitis lesions (20–25 mg) collected from trichomonosis cases using the Biosprint 15 DNA Blood Kit (Qiagen, UK) according to the manufacturer's instructions. DNA was also extracted from parasites cultured directly from lesions and from culture of *T. gallinae* (Rivolta) Stabler (American Type Culture Collection (ATCC) Number 30230), a well characterised reference strain. Cultured parasites were spun to a pellet by centrifugation for 3 min at 14,500 rpm before DNA extraction using the same technique as for parasites in tissues.

2.3. PCR for the ITS1/5.8S rRNA/ITS2 region

PCR was used to amplify the non-coding ITS1/5.8S rRNA/ITS2 region using the TFR1 and TFR2 primers, as previously described (Gaspar da Silva et al., 2007; Robinson et al., 2010). Each PCR run contained a negative control of molecular grade water. Sequence data were compared with available National Centre for Biotechnology Information (NCBI) Genbank entries using the Basic Local Alignment Search Tool (BLAST) search function to assess evidence for variation within the British *T. gallinae* isolates and their relationship with other published Trichomonadidae isolates, including the ITS region sequence groups A–L for *T. gallinae* (Gerhold et al., 2008) (Table 1).

DNA extracts ($n = 68$) were selected from the range of British birds in which trichomonosis was diagnosed (Table 2) and from both wood pigeon cases collected in 2002. Fringillidae cases were selected from across Great Britain to explore temporo-spatial variation. The set of greenfinch and chaffinch DNA extracts included cases that had died at sites with large numbers of dead finches (>20 dead birds at one site), possibly corresponding to more-virulent strains of the parasite if strain variation was present, and from sites where only small numbers of dead finches were found (2–5 per site). DNA extracted from a trichomonad parasite cultured from the caecal contents of a Scottish pheasant (*Phasianus colchicus*) was included as an outgroup.

2.4. Nested PCR for the SSU rRNA gene

A nested PCR was used to amplify a fragment of the coding SSU rRNA gene following the protocol of Robinson et al. (2010) with trichomonad SSU rRNA primers followed by TN3 and TN4 nested primers. Each PCR run contained a negative control of molecular grade water. DNA extracts were selected from a range of British birds ($n = 22$) (Table 2), both wood pigeon cases collected in 2002 and from the *T. gallinae* reference strain. Greenfinch cases were selected to include temporo-spatial spread from 2005 to 2007.

2.5. PCR for the Fe-hydrogenase gene

PCR was used to amplify the hydrogenosomal Fe-hydrogenase gene using the primers TrichhydFOR (GTTTGGGATGGCCTCAGAAT) and TrichhydREV (AGCCGAAGATGTTGTCGAAT). These primers were designed using Primer-BLAST Primer Designing Tool software (Primer 3 – http://sourceforge.net) based on AF446077.1 (*T. gallinae* Fe-hydrogenase gene, partial cds from Voncken et al., 2002). PCR reactions were performed using standard conditions using HotStar Taq Plus DNA Polymerase (Qiagen, UK) and a thermocycle with 15 min denaturation at 94 °C, 35 cycles of 94 °C for 1 min, 53 °C for 30 s and 72 °C for 1 min, followed by a 5 min extension at 72 °C. Each set of amplifications contained a negative control of molecular grade water and a positive control of known *T. gallinae* DNA. Amplification was confirmed visually under UV by the presence of an appropriately sized band (c. 1 Kb) on an ethidium stained 1% agarose gel. PCR products were cleaned using the QIAQuick gel extraction kit (Qiagen, UK) and submitted for sequencing using the ABI 3730xl platform with the TrichhydFOR forward and

Table 1
Genbank entries used in the phylogeny based on the ITS1/5.8S rRNA/ITS2 region. A single example from each of the ITS region sequence groups A-L (Gerhold et al., 2008) is provided.

Species	Host	Origin	Genbank accession number
T. gallinae group A	e.g. House finch *Carpodacus mexicanus*	California, USA	EU290649
T. gallinae group B	e.g. Broad winged hawk *Buteo platypterus*	Florida, USA	EU215368
T. gallinae group C	e.g. Rock pigeon ATCC 30228 *Columbia livia*	Colorado, USA	EU215362
T. gallinae group D	e.g. Eurasian collared dove 829 *Streptopelia decaoacto*	Texas, USA	EU215364
T. gallinae group E	e.g. Eurasian collared dove 858 *Streptopelia decaoacto*	Texas, USA	EU215363
T. gallinae group F	e.g. Common ground-dove 1321 *Columbina passerina*	Texas, USA	EU215358
T. gallinae group G	e.g. Common ground-dove 1 *Columbina passerina*	Texas, USA	EU215359
T. gallinae group H	e.g. White winged-dove 840 *Zenaida asiatica*	Texas, USA	EU215360
T. gallinae group I	e.g. White winged-dove 1159 *Zenaida asiatica*	Texas, USA	EU215361
T. gallinae group J	e.g. Mourning dove 22 *Zenaida macroura*	Texas, USA	EU215365
T. gallinae group K	e.g. Band-tailed pigeon 1 *Patagioenas fasciata*	California, USA	EU215367
T. gallinae group L	e.g. Coopers hawk 1 *Accipiter cooperii*	Arizona, USA	EU215366
Trichomonas sp.	Mockingbird *Mimus polyglottos*	California, USA	EU290650
Tetratrichomonas gallinarum	(–) Strain MR5	–	AY349181
Histomonas meleagridis	Domesticated turkey *Meleagris gallopavo*	North Carolina, USA	HQ540396

Table 2
Numbers and identification of birds with trichomonosis from which extracted DNA was used for the ITS1/5.8S rRNA/ITS2 region and SSU rRNA gene analyses.

Family	Species	ITS1/5.8S rRNA/ITS2 region	SSU rRNA gene
Accipitridae	Common buzzard *Buteo buteo*	1	1
	Red kite *Milvus milvus*	1	0
	Sparrowhawk *Accipiter nisus*	1	2
Columbidae	Collared dove *Streptopelia decaoacto*	5	3
	Wood pigeon *Columba palumbus*	5	5
Emberizidae	Yellowhammer *Emberiza citrinella*	3	0
Fringillidae	Brambling *Fringilla montifringilla*	2	0
	Bullfinch *Pyrrhula pyrrhula*	3	0
	Chaffinch *Fringilla coelebs*	12	1
	Goldfinch *Carduelis carduelis*	4	0
	Greenfinch *Carduelis chloris*	22	8
	Siskin *Carduelis spinus*	1	0
Passeridae	House sparrow *Passer domesticus*	2	0
Paridae	Great tit *Parus major*	2	0
Prunellidae	Dunnock *Prunella modularis*	2	1
Strigidae	Tawny owl *Strix aluco*	1	1
Turdidae	Blackbird *Turdus merula*	1	0

TrichhydREV reverse primers. Integrity of the DNA sequences was assessed manually by using chromatograph inspection using Chromas 2 software (www.synthesisgene.com). The sequences from the forward TrichhydFOR primer and the reverse complement of the TrichhydREV primer PCR product were aligned in both directions for each sample using Molecular Evolutionary Genetics Analysis (MEGA) 4.1 software and ClustalW (http://www.megasoftware.net). Only common sequence was utilised. Sequences were compared with the only available Trichomonadidae gene sequences (*T. gallinae* AF446077 and *T. vaginalis* from humans XM_001310179) within NCBI Genbank using the BLAST search function.

DNA extracts from 18 British birds of nine species (Table 3) and from the reference strain were examined. The Fe-hydrogenase gene from *T. gallinae* isolates from three columbid species from the Seychelles was also amplified and sequenced to provide a comparison to the British birds. These had been isolated in 2007 from the Seychelles blue pigeon (*Alectroenas pulcherrima* $n = 4$), the Madagascar turtle-dove (*Streptopelia picturata* $n = 1$) and the zebra dove (*Geopelia striata* $n = 1$) and were available in the sample archive at the University of East Anglia (Bunbury, 2011).

2.6. Phylogeny construction

For each of the datasets obtained (ITS1/5.8S rRNA/ITS2 region, SSU rRNA, Fe hydrogenase), phylogenetic dendrograms were constructed using the Neighbour-Joining method (Saitou and Nei, 1987) and MEGA 5 software (Tamura et al., 2011). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (2000 replicates) was calculated (Felsenstein, 1985). The trees were drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) in the units of the number of base substitutions per site.

2.7. Random Amplified Polymorphic DNA (RAPD) analysis

DNA extraction was performed on *T. gallinae* cultures raised from the cryopreserved archives (see above). Cultures of motile trichomonads were screened for evidence of fungal contamination by examining wet preparations under the light microscope and stored at 4 °C. Where fungal contamination was detected, parasites were passaged repeatedly in the presence of amphotericin B (Fungizone® (Gibco, UK) according to the manufacturer's instructions until after the contamination was no longer detectable. The concentration of extracted DNA from cultured parasites was measured using a Nanodrop (Thermo Scientific, USA) and only samples with DNA concentrations greater or equal to 10 ng/μl, with good quality based on an A260:280 ratio >1.9, were selected for RAPD analysis. DNA extracts were selected for RAPD analysis from confirmed *T. gallinae* isolates cultured from a range of British birds (14 birds, 9 species) in which trichomonosis was diagnosed (Table 3) and from the *T. gallinae* reference strain.

Table 3
Numbers and identification of birds with trichomonosis from which extracted DNA was used for the Fe-hydrogenase PCR and RAPD analyses.

Case number	Species	Location	Year	Fe-hydrogenase	RAPD
62-09	**Brambling**	Norfolk, England	2009	Yes	Yes
B1514	Bullfinch	Cornwall, England	2007	Yes	No
820-07	Chaffinch	Kent, England	2007	Yes	No
796-08	Chaffinch	Derbyshire, England	2008	No	Yes
861-08	**Chaffinch**	Staffordshire, England	2008	Yes	Yes
867-08	Chaffinch	Powys, Wales	2008	Yes	No
963-05	Collared dove	Shropshire, England	2005	Yes	No
811-07	**Collared dove**	North Yorkshire, England	2007	Yes	Yes
528-07	Common buzzard	Glamorgan, Wales	2007	Yes	No
815-07	Dunnock	Warwickshire, England	2007	No	Yes
89-08	Goldfinch	Gwent, Wales	2008	No	Yes
1036-05	Great tit	Norfolk, England	2005	Yes	No
1035-07	**Great tit**	Sussex, England	2007	Yes	Yes
760-07	Greenfinch	Gloucestershire, England	2007	Yes	No
810-07	Greenfinch	North Yorkshire, England	2007	No	Yes
837-07	Greenfinch	Devon, England	2007	Yes	No
892-07	Greenfinch	Worcestershire, England	2007	No	Yes
90-08	Greenfinch	Lincolnshire, England	2008	Yes	No
99-08	Greenfinch	Norfolk, England	2008	Yes	No
858-08	Greenfinch	Bedfordshire, England	2008	Yes	No
864-08	**Greenfinch**	Bedfordshire, England	2008	Yes	Yes
R2003	Greenfinch	Somerset, England	2008	No	Yes
R2056-08	Greenfinch	Staffordshire, England	2008	No	Yes
1020-07	**Sparrowhawk**	Devon, England	2007	Yes	Yes
484-07	Wood pigeon	Cambridgeshire, England	2007	Yes	No
1337-07	Wood pigeon	Shropshire, England	2007	No	Yes
1	Seychelles blue pigeon	Mahé, Seychelles	2007	Yes	No
4	Seychelles blue pigeon	Mahé, Seychelles	2007	Yes	No
5	Seychelles blue pigeon	Mahé, Seychelles	2007	Yes	No
7	Seychelles blue pigeon	Mahé, Seychelles	2007	Yes	No
61	Madagascar turtle-dove	Mahé, Seychelles	2007	Yes	No
12	Zebra dove	Mahé, Seychelles	2007	Yes	No

∗ Samples marked in **bold** have results available for both Fe-hydrogenase PCR and RAPD analyses.

The protocol for RAPD analysis published by Gaspar da Silva et al. (2007), adapted from Felleisen (1998), was modified by using 5′[6_FAM] fluorescently labelled OPD primers (Corley-Smith et al., 1997) (OPD3, OPD5, OPD7, and OPD8), supplied by Sigma Aldrich, UK. PCR reactions were used with 5 μL of 10X PCR buffer (Qiagen, UK), 3 μL of 25 mM $MgCl_2$ (Qiagen, UK), 0.5 μL of 5 U/μL HotStar Taq Plus DNA Polymerase (Qiagen, UK), 25 ng genomic template DNA, 0.4 μL of 100 mM dNTP mix (Bioline, UK), 3 μL of 10 μM OPD primer and molecular grade water to complete the 50 μL per reaction. After an initial 15 min denaturation at 94 °C, 40 cycles of 94 °C for 1 min, 36 °C for 1 min and 72 °C for 2 min were carried out, followed by a 15 min extension at 72 °C using a thermal cycler (Geneamp PCR System 9700). Each set of amplification reactions contained a negative control of water and a positive control of purified *T. gallinae* DNA obtained from parasites cultured from an affected greenfinch.

The RAPD analyses were repeated in triplicate. The amplified PCR products were visualised under UV light after ethidium bromide staining of a 2% agarose gel and the product sizes were determined using Ready-Load 100 bp DNA ladder (Invitrogen, UK). Band profiles from each replicate, for each OPD primer, were compared visually to verify that the results were highly reproducible.

Two replicates of each PCR product were submitted to the John Innes Genome Laboratory, Norwich, where they were diluted 1 in 30 with molecular grade water. 1 μl of the diluted PCR product was added to 8.9 μl of HiDi Formamide (Applied Biosystems 4311320) and 0.1 μl of ROX Ladder (Web Scientific MRK1000) for a total of 10 μl per sample. Samples were loaded directly onto an Applied Biosystems 3730xl DNA Analyzer without heat denaturation which enabled automated reading of RAPD products, thereby optimising the protocol. Samples were run with Dye set D on a 50 cm array with Pop7 using a Run Voltage of 8.0 V and Run Time of 6000 s with all other settings set to default values. The fsa file outputs from the 3730xl DNA Analyzer were imported into GeneMarker software, version 1.90 beta (SoftGenetics LLC, USA) and calibrated against the ROX Ladder 1000× to determine the fragment size for each peak representing the genotype of the sample. A panel was constructed for each OPD primer set. The panel consisted of all the alleles for all the genotypes from all the samples for that primer set. The sample data files were analysed using the panel, recording the presence or absence of each peak, or allele of the genotype, and expressed in binary matrix format. The size of bands assessed was restricted within the range of the ROX 1000 Ladder. The band profiles from both replicates were manually examined and verified independently for each OPD primer, to remove inconsistencies or artifacts, and compared to confirm high reproducibility of results; only bands that were present in both replicates were included in the RAPD analyses. Clustering analyses were performed to generate a dendrogram for each of the four OPD primer sets in isolation, with similarity between isolates expressed as a proportion. The GeneMarker merge project tool was then used to perform a meta-analysis of data combined from all four, individual OPD primer sets. Where sample availability and quality permitted, DNA isolates were used from the same birds for both the Fe-hydrogenase PCR and RAPD analyses (Table 3) to enable direct comparison.

3. Results

3.1. PCR for the ITS1/5.8S rRNA/ITS2 region

An identical nucleotide sequence (214 nucleotides, Genbank accession number GQ150752) was obtained for *T. gallinae* from all the British bird trichomonosis cases following amplification of the ITS1/5.8S rRNA/ITS2 region regardless of host species, geographical region or year (Fig. 1). Sequence data from columbiform

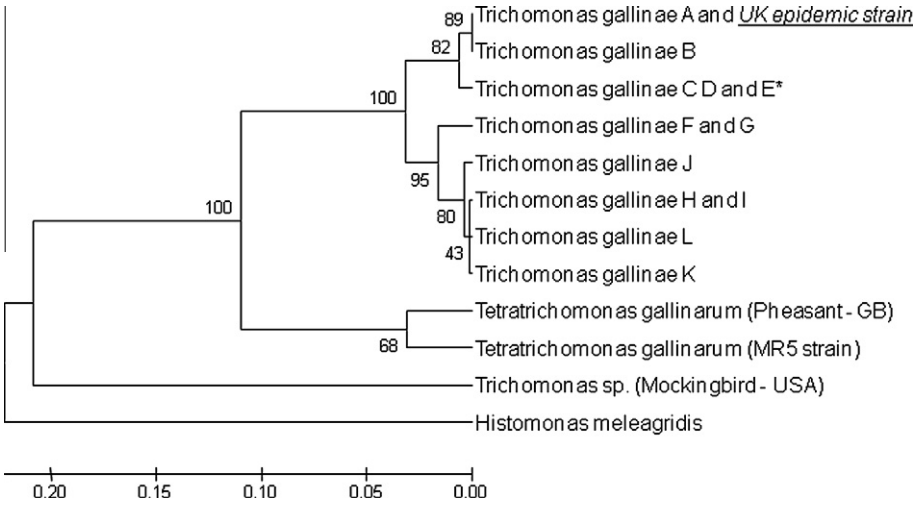

Fig. 1. Phylogeny of avian trichomonad isolates based on the ITS1/5.8S rRNA/ITS2 region. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura–Nei model. The bootstrap consensus tree inferred from 2000 replicates is taken to represent the evolutionary history of the taxa analysed. Branches corresponding to partitions reproduced in less than 40% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (2000 replicates) are shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically as follows. When the number of common sites was <100 or less than one quarter of the total number of sites, the maximum parsimony method was used; otherwise BIONJ method with MCL distance matrix was used. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 12 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 195 positions in the final dataset. Evolutionary analyses were conducted in MEGA5. Asterisk denotes that *T. gallinae* group E includes ATCC 30230.

T. gallinae isolates collected before, and following, the emergence of finch trichomonosis were identical to those for *T. gallinae* from the other British birds. The reference strain *T. gallinae* differed from the British isolates with one substitution and one deletion, but still grouped with the British isolates with a high bootstrap value (Fig 1).

3.2. Nested PCR for the SSU rRNA gene

An identical nucleotide sequence (149 nucleotides Genbank accession number GQ214405) was obtained for *T. gallinae* from all the British bird trichomonosis cases that were examined using nested PCR. Sequence data from columbiform isolates collected before, and following, the emergence of finch trichomonosis were identical to those for *T. gallinae* from the other British birds, as was the sequence of the reference strain of *T. gallinae* (data not shown).

3.3. PCR for the Fe-hydrogenase gene

High quality nucleotide sequence of variable length (930–999 nucleotides) was obtained from all of the British bird trichomonosis cases and from the isolates from the Seychelles columbids. No sequence variation was detected amongst the British bird isolates. Multiple attempts to amplify the Fe-hydrogenase gene from DNA extracted from the 2002 pigeons with trichomonosis were unsuccessful. A Neighbour-Joining phylogenetic tree was constructed from a 903 nucleotide sequence available from all samples (Fig. 2, Table 3). All clusters were supported by high bootstrap values. Marked divergence in the sequence data was found with 18 point nucleotide substitutions between the British isolates collected during the course of this study and the *T. gallinae* sequences from historically archived strains (the North American reference strain and AF446077, the only *T. gallinae* isolate available within Genbank) (Table 4; Fig. 2). Sequence diversity also was observed in *T. gallinae* from different columbid species on the Seychelles, which showed divergence not only from the British and North American strains, but also from each other, indicating that a number of strains of *T. gallinae* are circulating in the Seychelles' avifauna (Fig. 2).

3.4. Random Amplified Polymorphic DNA analyses

Visual comparison of the band profiles on the agarose gels for each of the four OPD primer sets indicated high reproducibility of results. Modification of the RAPD protocol, utilising 5′[6_FAM]-labelled primers with automated reading of gel profiles was successful; again high reproducibility was achieved between the two sets. Data were incorporated from 24–62 bands of size 92–999 bp for the four OPD primer sets. An indication of divergence between isolates is provided by considering the proportion of bands shared, described as the percentage similarity.

Some variation was observed amongst the results of the four OPD primers. Three of the OPD primer sets supported minimal variation amongst the British passeriform and columbiform isolates, with percentage similarity amongst isolates of >95% for OPD3 (Fig. 3a) and OPD5 (data not shown), and >85% similarity for OPD8 (data not shown). For OPD7, nine of the British passeriform and columbiform isolates had >90% similarity, however, the remaining three isolates (a greenfinch found dead in 2008 (84% similarity), a brambling from 2009 (75% similarity) and a dunnock from 2007 (58% similarity)) gave more divergent results (data not shown).

The *T. gallinae* reference strain showed the greatest amount of difference from the other strains tested, and this was consistent for each of the OPD primers sets. The isolate collected from an adult female sparrowhawk from England in 2007 also showed divergence from the other British isolates: this isolate clustered with the *T. gallinae* reference strain with each of the OPD3 and OPD7 primer sets, but clustered with the British bird isolates with each of the OPD5 and OPD8 primer sets (data not shown).

A meta-analysis incorporating data from each of the OPD primer sets produced a summary dendrogram which supports a single grouping for all British bird isolates, apart from the 2007 sparrowhawk isolate, with 77% overall similarity amongst the RAPD band patterns, and with both the 2007 sparrowhawk isolate and the reference strain as outliers (Fig. 3b).

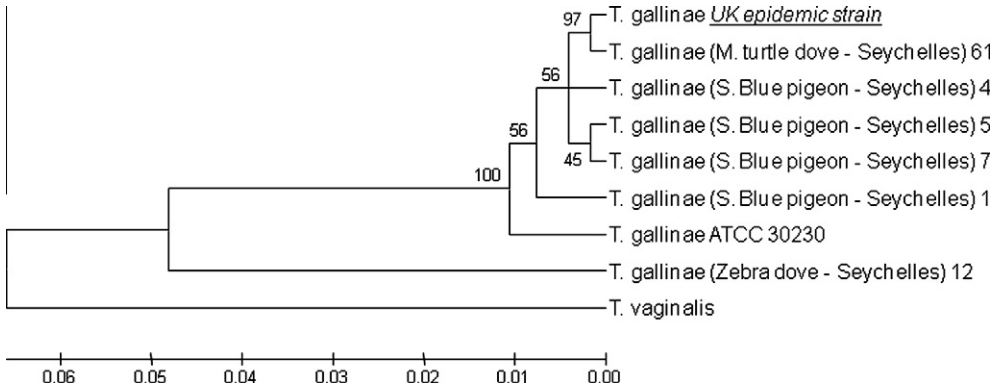

Fig. 2. Phylogeny of *Trichomonas gallinae* isolates based on the Fe-hydrogenase gene. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura–Nei model. The bootstrap consensus tree inferred from 2000 replicates is taken to represent the evolutionary history of the taxa analysed. Branches corresponding to partitions reproduced in less than 40% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (2000 replicates) are shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically as follows. When the number of common sites was <100 or less than one quarter of the total number of sites, the maximum parsimony method was used; otherwise BIONJ method with MCL distance matrix was used. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved nine nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 803 positions in the final dataset.

Table 4
Genetic differences (number and percentage of base pair polymorphisms) between the sequence data for the hydrogenosomal Fe-hydrogenase gene (903 bp sequence) from *T. gallinae* collected from British bird species (used as baseline), the Seychelles columbid species and the Genbank Trichomonadidae entries.

Sample	Genbank identifier	Number of substitutions	Number of insertions	Number of deletions
T. gallinae British bird species	JF681136	0	0	0
T. gallinae	AF446077	18 (2%)	0	0
T. gallinae ATCC 30230		18 (2%)	0	0
T. gallinae (S. blue pigeon – Seychelles) 1	JF681137	16 (1.8%)	0	0
T. gallinae (S. blue pigeon – Seychelles) 4	JF681138	8 (0.9%)	0	0
T. gallinae (S. blue pigeon – Seychelles) 5	JF681139	10 (1.1%)	0	0
T. gallinae (S. blue pigeon – Seychelles) 7	JF681140	9 (1.0%)	0	0
T. gallinae (M. turtle-dove – Seychelles) 61	JF681141	5 (0.6%)	0	0
T. gallinae (Zebra dove – Seychelles) 12	JF681142	77 (8.5%)	1 (0.1%)	0
T. vaginalis	TVAG_037570	103 (11%)	0	0

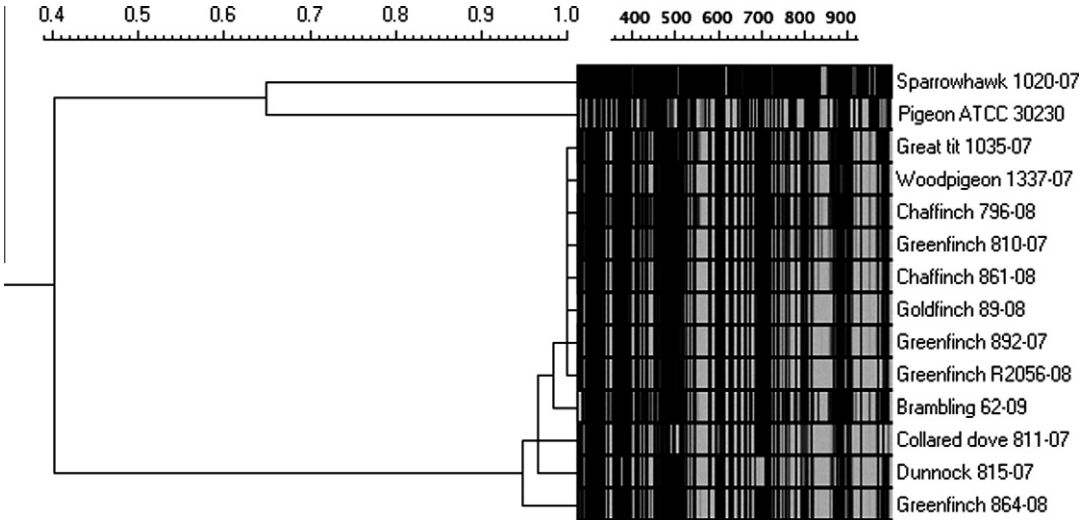

Fig. 3. Random Amplified Polymorphic DNA analyses from the *T. gallinae* isolates from British birds. (a) RAPD dendrogram for the OPD3 primer set. Left scale denotes similarity between isolates expressed as a proportion. Right scale denoted band size (bp). (b) RAPD dendrogram for meta-analysis incorporating the four OPD primer sets in combination. Scale denotes similarity between isolates expressed as a proportion.

4. Discussion

We used a variety of complementary molecular methods to investigate the genetic heterogeneity of *T. gallinae* in British avifauna following the emergence of finch trichomonosis. Analysis of *T. gallinae* from over 50 diseased passerines showed no evidence for multiple strains being present within this newly infected population, indicating the emergence of a clonal strain as the aetiologic agent of the epidemic. This is in contrast to the heterogeneity displayed at the ITS region amongst endemic infection of

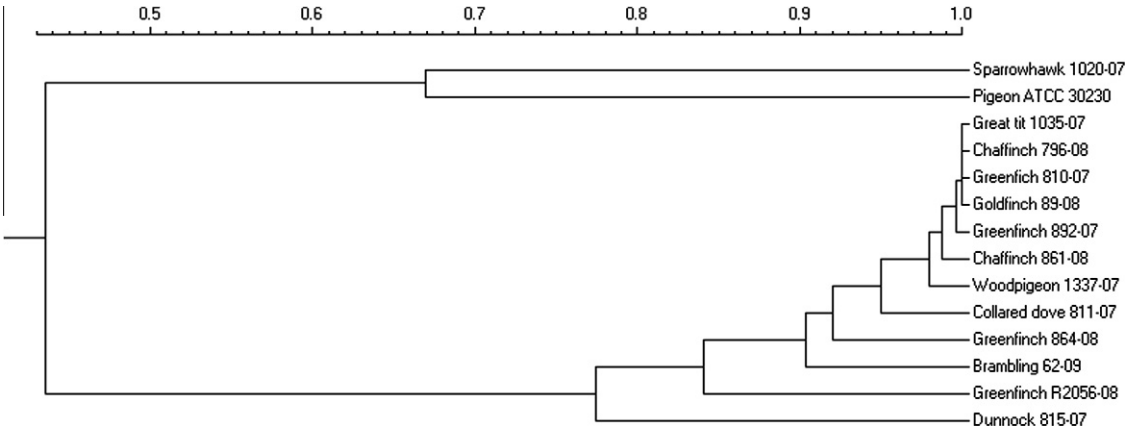

Fig. 3 (*continued*)

columbids and raptors in the USA (Gerhold et al., 2008), Spain (Sansano-Maestre et al., 2009) and Austria (Grabensteiner et al., 2010), or the heterogeneity found following spillover to introduced columbids in Mauritius (Gaspar da Silva et al., 2007). Gerhold et al. (2008) found variation to support at least two clades within the *T. gallinae* species complex, one of which they describe as a canonical *T. gallinae* group (including *T. gallinae* region sequence group A), the other as more closely related to *T. vaginalis*. All the British bird isolates were identical in sequence to the *T. gallinae* ITS region sequence group A. It is interesting to note that a house finch (*Carpodacus mexicanus*) isolate from the USA reported by Gerhold et al. (2008) is also within ITS region sequence group A.

We found no change in the sequence data over time (2005–2009), by geographical region or by species amongst the British isolates. *T. gallinae* isolates from finches collected from sites with high levels of mortality (>20 dead birds) had the same sequence data as all other isolates, consequently, we found no evidence for genotypic strain variation that might relate to virulence in British passerines.

The Fe-hydrogenase gene sequence data showed considerable variation between the Seychelles and British *T. gallinae* strains. Considerable sequence variation of this gene was also found within the small number of Seychelles *T. gallinae* isolates. When compared to this, the lack of sequence variation amongst the large number of British *T. gallinae* tested (Table 4) indicates that the British finch trichomonosis epidemic was caused by a clonal parasite strain.

Minimal variation was found in the multilocus genotypes obtained by RAPD on DNA extracted from a more limited set of cultured isolates from British birds. These findings also support a clonal epidemic strain of *T. gallinae* responsible for the finch trichomonosis epidemic. The modified RAPD technique employed in this study showed clear divergence of the British bird parasite strain from the *T. gallinae* reference strain. Several protocol modifications were applied to *T. gallinae* for the first time in this study to improve accuracy and reduce subjective and gel-gel variation. In addition, use of the novel meta-analysis tool (GeneMarker) enabled the power of the RAPD analyses to be increased, by combining findings for all four OPD primer sets.

As with sequence analysis of the Fe-hydrogenase gene, the RAPD analyses indicate that the *T. gallinae* reference strain is divergent from the British bird isolates. In contrast to the sequence data, however, the RAPD analysis indicated that the single sparrowhawk isolate was distinct from the other British bird isolates (Fig. 3). This raptor isolate is the only indication we have that multiple parasite strains were circulating in British columbiforms prior to the emergence of finch trichomonosis. Due to the demands of DNA quality and purity required for RAPD analysis, only a single sample of *T. gallinae* isolated from a raptor and no columbiform samples predating emergence of the finch trichomonosis epidemic were available. It may be that *T. gallinae* strain diversity exists within the British columbiform population that was not identified within the small number of pigeons and doves examined in this study. Further prospective studies of *T. gallinae* from a range of columbiform species, from clinical trichomonosis and asymptomatic trichomoniasis cases, are required to investigate parasite strain diversity and to inform our understanding of spillover dynamics to new species.

Whilst all the OPD primers indicated a clonal strain of *T. gallinae* in the British passeriform and columbiform birds, fine-scale variation amongst these parasite strains was detected, although no temporal, spatial or host species trends were apparent. RAPD analyses are often able to detect strain variation not apparent in single locus sequence data because the primers bind to a variety of genomic targets which can include rapidly evolving, non-coding sequences such as microsatellites. Thus the complete absence of variation within British birds at the Fe-hydrogenase locus, in spite of its relatively fine discriminatory ability, and the variation detected by RAPD analyses is not unexpected and is readily reconciled. Moreover, the extent of variation amongst samples in the combined RAPD analysis was limited, indicating the widespread presence of a single, clonal *T. gallinae* strain in the British passerine population subsequent to the emergence of finch trichomonosis.

RAPD analysis has several disadvantages when compared with phylogenetic analyses of sequence data. RAPD analyses require high quality DNA extracted from pure parasite cultures and this limits the number of isolates that can be studied using this technique. Also, all aspects of the methodology must be standardised as far as possible to reduce variation (e.g. sample collection, Taq enzyme source and batch, PCR machine, primer to template concentration ratio) which constrains the use of this approach (Soll, 2000). In comparison, PCR and sequencing can be conducted on parasite DNA extracted from infected tissues which are unsuitable for parasite culture: consequently, a much larger sample size was available for this technique. The results of the current study indicate that sequence analysis of the Fe hydrogenase gene provides a superior genotyping marker to discriminate amongst *T. gallinae* strains when compared with sequence analysis of the ITS1/5.8S rRNA/ITS2 region or the SSU rRNA gene, both of which detect minimal variation.

Whilst RAPD analyses can be usefully employed to investigate relatedness of strains where discriminatory sequence loci are not available, the novel Fe-hydrogenase sequence analyses appear preferable to RAPD studies since the results are more robust and DNA sample requirements are less stringent. The Fe-hydrogenase

gene offers a useful marker to investigate intra- and inter-specific strain variation within the Trichomonadidae in the future. Alternatively, microsatellite studies, which have recently been employed to robustly identify genetic heterogeneity amongst *T. vaginalis* isolates (Conrad et al., 2011), could be used to further investigate the epidemiology of *T. gallinae*.

Anderson et al. (2009) investigated the molecular epidemiology of *T. gallinae* infecting northern Californian passerine species using ribosomal DNA sequence and found no variation with sympatric free-ranging columbiform species or raptors. These authors highlighted communal feeding and water sources, such as bird feeders and bird baths in suburban habitats, as a potential route for the inter-species spread of the parasite and suggested that localised spill-over from infected columbiforms to house finches is most likely to have occurred at these sites. Congregation at game bird feeders was also hypothesized to be an important risk factor for the epidemic spread of trichomonosis in Spanish wood pigeons (Höfle et al., 2004).

The clonal nature of the British passerine epidemic of trichomonosis suggests that the infection of these novel hosts has been recent, which is consistent with our field observations (Robinson et al., 2010). Although the identification and extent of genetic diversity within the source *T. gallinae* population is unknown, the clonal nature of the passerine epidemic strain suggests that it has arisen recently from a bottleneck (Sprat and Maiden, 1999), such as would be represented by a single spillover event. The feeding of garden birds is an increasingly popular pastime with the British public, and this provides novel opportunities for species mixing and unusually high or prolonged congregations of birds at feeding stations (Kirkwood, 1998; Jones and Reynolds 2008; Davies et al., 2009). The BTO Garden Bird Feeding Survey (Chamberlain et al., 2005), which examines the number of birds using human-provided food and water in gardens over winter (Oct–Mar, inclusive), shows that the average weekly peak count per garden of wood pigeons and collared doves rose from 0.09 and 0.91 individuals respectively in the 1970s, to 1.20 and 2.37 individuals during the last decade (T. Harrison, *pers comm.*). This upward trend in the use of gardens by columbids is attributed to the intensification of arable cultivation, which promotes increased overwinter survival and spill-over from farmland to garden habitats (Gibbons et al., 1993). In turn, this might have led to increased mixing of Fringillidae and Columbidae species in garden habitats, providing increased opportunities for cross-species parasite spread. As with Anderson et al. (2009), therefore, we hypothesise that spillover occurred at food and water sources shared with columbids at supplementary (garden) feeding stations.

Both prospective and retrospective analyses of *T. gallinae* strains from a range of host species and geographical regions are required to increase our understanding of the epidemiology of trichomonosis in wild bird populations. Full genome sequence analysis from a British finch *T. gallinae* isolate is underway and once the sequence data are available, microsatellite studies of British *T. gallinae* isolates will be undertaken.

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