Multi-locus sequence typing confirms the clonality of *Trichomonas gallinae* isolates circulating in European finches

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SUMMARY

In recent years, *Trichomonas gallinae* emerged as the causative agent of an infectious disease of passerine birds in Europe leading to epidemic mortality of especially greenfinches *Chloris chloris* and chaffinches *Fringilla coelebs*. After the appearance of finch trichomonosis in the UK and Fennoscandia, the disease spread to Central Europe. Finch trichomonosis first reached Austria and Slovenia in 2012. In the present study the genetic heterogeneity of *T. gallinae* isolates from incidents in Austria and Slovenia were investigated and compared with British isolates. For this purpose comparative sequence analyses of the four genomic loci ITS1-5.8S-ITS2, 18S rRNA, *rpb1* and Fe-hydrogenase were performed. The results corroborate that one clonal *T. gallinae* strain caused the emerging infectious disease within passerine birds and that the disease is continuing to spread in Europe. The same clonal strain was also found in a columbid bird from Austria. Additionally, the present study demonstrates clearly the importance of multi-locus sequence typing for discrimination of circulating *T. gallinae* strains.

Key words: *Trichomonas gallinae*, finch trichomonosis, emerging infectious disease, passerine birds, European finches, sequence analysis, ITS1-5.8S-ITS2, 18S rRNA, Fe-hydrogenase, *rpb1*.

INTRODUCTION

The flagellated protozoan, *Trichomonas gallinae*, is the causative agent of avian trichomonosis (Stabler, 1954; Locke and James, 1962). The parasite is globally distributed and infects the upper digestive tract of a wide range of bird species, including columbiformes, accipitriformes, strigiformes, psittaciformes and passeriformes (Forrester and Foster, 2008). Such infections of the upper digestive tract may lead to pathological changes ranging from mild inflammation of the mucosa to large caseous lesions that can obstruct the lumen of the oesophagus (Kocan and Herman, 1971). Therefore, diseased wild birds frequently die due to starvation. However, avian *T. gallinae* infection can result in a broad spectrum of clinical presentations ranging from aclinical parasite carriage to lethal infection.

Typically, this disease is known as a cause of morbidity and mortality in columbiform species and birds of prey (Forrester and Foster, 2008). In recent years, trichomonosis was described as an emerging infectious disease of wild finches in Europe. The first appearance of the disease was recognized in 2005 in the UK with a late summer/autumn seasonal epidemic mortality of passerine hosts especially greenfinches *Chloris chloris* and chaffinches *Fringilla coelebs* (Pennycott *et al*. 2005; Lawson *et al*. 2006). In addition, trichomonosis was also diagnosed in a small number of other passerine birds including bullfinch *Pyrrhula pyrrhula*, goldfinch *Carduelis carduelis*, brambling *Fringilla montifringilla*, siskin *Carduelis spinus*, house sparrow *Passer domesticus*, yellowhammer *Emberiza citronella*, dunnock *Prunella modularis* and great tit *Parus major* (Robinson *et al*. 2010). From April to September 2006, over 1000 finch trichomonosis incidents involving single or multiple individuals were recorded in the UK with the greatest incidence in western and central England and Wales (Robinson *et al*. 2010). In 2007 there was a pronounced eastward shift in the spatial distribution of the disease within England which was followed by the spread of finch trichomonosis to southern Fennoscandia in the following year (Lawson *et al*. 2011*b*). During the second half of 2008, appearance of greenfinch and chaffinch mortality was reported in southern Fennoscandia with the index incident documented in Sweden, followed by Norway and Finland (Neimanis *et al*. 2010). Large-scale greenfinch population declines have occurred as a result of this emerging infectious disease in the UK (Lawson *et al*. 2012) and Finland (Lehikoinen *et al*. 2013).

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Furthermore, since April 2009 an increased incidence of greenfinch mortality due to finch trichomonosis was recognized in northern Germany (Peters *et al*. 2009). Lawson *et al*. (2011*b*) hypothesized that finch trichomonosis spread from the UK to Fennoscandia with seasonal migratory chaffinches. Ring return data show that chaffinches migrate directly from the UK to Fennoscandia across the North Sea in the spring (Norman, 2002). For their return migration route in autumn, chaffinches pass through Denmark, Germany, the Netherlands and Belgium, before they cross the English Channel (Norman, 2002). Therefore, the location and timing of the emergence of finch trichomonosis in northern Germany in 2009 is consistent with the hypothesized route of spread of the parasite mediated by chaffinch migration. In May 2010, finch trichomonosis was first diagnosed in France (Gourlay *et al*. 2011) and in summer 2012, the disease was first confirmed as a cause of finch mortality in Slovenia (Zadravec *et al*. 2012). This further spread of the disease could also be associated with finch movement.

To diagnose *T. gallinae* as the causative agent of infection, and to determine the phylogenetic relationship amongst isolates, sequence analyses are commonly performed. Most investigations of sequence data are based on the ITS1-5.8S-ITS2 region and the 18S rRNA gene, which are used to discriminate *T. gallinae* from other trichomonads and to resolve certain lineages (Felleisen, 1997; Kleina *et al*. 2004; Gerhold *et al*. 2008; Sansano-Maestre *et al*. 2009; Grabensteiner *et al*. 2010). Within phylogenetic analyses several genes encoding proteins such as glyceraldehyde-3-phosphate dehydrogenase, malate dehydrogenase, enolase, α- and β-tubulin have also been used to resolve the evolutionary relationships between different *Trichomonadida* (Viscogliosi and Müller, 1998; Wu *et al*. 1999; Gerbod *et al*. 2004; Keeling, 2004; Gerhold *et al*. 2008). However, these genetic markers were insufficient to reliably resolve inter- and intraspecific relationships, because they underwent recombination, horizontal gene transfer or duplication (Viscogliosi and Müller, 1998; Wu *et al*. 1999; Gerbod *et al*. 2004; Keeling, 2004; Stechmann *et al*. 2006; Rogers *et al*. 2007). For this reason, two single copy genes, *rpb1* and Fe-hydrogenase, were established as novel genotyping markers (Malik *et al*. 2011; Lawson *et al*. 2011*a*). The *rpb1* gene encodes for the largest subunit of the RNA Polymerase II, which carries out the transcription of all protein-coding genes in eukaryotes (Archambault and Friesen, 1993; Cramer *et al*. 2000). The hydrogenosomal Fe-hydrogenase gene belongs to the housekeeping genes of amitochondrial protozoa. The enzyme catalyses the activation of hydrogen and functions to either couple hydrogen oxidation to energy-yielding processes or reduces protons as a mechanism to recycle reduced electron carriers that accumulate during fermentation (Vignais and Billoud, 2007). Phylogenetic analyses based on both single copy genes were recently applied and the *rpb1* gene was used successfully to resolve species and isolate-level parabasilid relationships, including two *T. gallinae* isolates (Malik *et al*. 2011). Sequence data of the Fe-hydrogenase gene were analysed from multiple *T. gallinae* isolates derived from British passeriformes, columbiformes, accipitriformes and strigiformes, demonstrating the potential for detecting fine-scale variation amongst *T. gallinae* strains (Chi *et al*. 2013).

Sequence analyses of *T. gallinae* isolates from finch trichomonosis cases based on the ITS1-5.8S-ITS2 and the 18S rRNA regions showed no variation between the British and the Fennoscandian parasite strains (Lawson *et al*. 2011*b*). Moreover, the comparison of sequence data obtained for the Fe-hydrogenase gene and the random amplified polymorphic DNA data confirmed the lack of sequence variation amongst a large number of British *T. gallinae* isolates from different passerine host species (Lawson *et al*. 2011*a*). These results indicated that the British finch trichomonosis epidemic was caused by a clonal strain of the parasite (Lawson *et al*. 2011*a*) and they supported the spread of the disease to southern Fennoscandia from the UK (Lawson *et al*. 2011*b*). Recently, Chi *et al*. (2013) demonstrated that the finch epidemic strain of *T. gallinae* was preponderant amongst the British columbiformes and birds of prey which died between the years 2007 and 2012 and were examined. The application of the Fe-hydrogenase locus enabled identification of the wide strain diversity of the parasite in these wild bird groups.

In the present paper, we report for the first time the occurrence of finch trichomonosis in Austria, which emerged in summer 2012, accompanied by mortality predominant in greenfinches. The primary aims of the present study were to (i) investigate the genetic heterogeneity within *T. gallinae* isolates from incidents in Austria and Slovenia, where the disease occurred recently, and (ii) to establish whether these *T. gallinae* isolates were identical to those from Britain where finch trichomonosis first emerged. In order to address these questions, comparative analyses of *T. gallinae* isolates were performed using four different genetic markers which comprised the ITS1-5.8S-ITS2 region, the 18S rRNA gene, the *rpb1* gene and the Fe-hydrogenase gene.

MATERIALS AND METHODS

Source of protozoa

A total of 29 trichomonad isolates obtained from wild birds of six different species from 26 sites, and 15 counties were investigated in the present study. A detailed list of the isolates is displayed in Table 1. All 17 protozoan isolates originating from Austria were

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Table 1. List of trichomonads investigated in the present study. Clonal cultures of protozoan isolates are labelled with C

Host species	Isolate	Location (Site No.)	Year	GenBank accession numbers			
				ITS1-5.8S-ITS2 region	18S rRNA gene	rpb1 gene	Fe-hydrogenase gene
Greenfinch	8800-3-C1	Austria, Burgenland (1)	2012	HG008050	HG008106	HG008113	HG008114
Greenfinch	8800-3-C3	Austria, Burgenland (1)	2012	HG008050	HG008106	HG008113	HG008114
Greenfinch	8800-3-C6	Austria, Burgenland (1)	2012	HG008050	HG008106	HG008113	HG008114
Greenfinch	8800-3-C7	Austria, Burgenland (1)	2012	HG008050	HG008106	HG008113	HG008114
Greenfinch	8800-3-C8	Austria, Burgenland (1)	2012	HG008050	HG008106	HG008113	HG008114
Greenfinch	8800-3-C11	Austria, Burgenland (1)	2012	HG008050	HG008106	HG008113	HG008114
Greenfinch	8800-3-C14	Austria, Burgenland (1)	2012	HG008050	HG008106	HG008113	HG008114
Greenfinch	8800-3-C16	Austria, Burgenland (1)	2012	HG008050	HG008106	HG008113	HG008114
Greenfinch	8800-3-C17	Austria, Burgenland (1)	2012	HG008050	HG008106	HG008113	HG008114
Greenfinch	8800-3-C18	Austria, Burgenland (1)	2012	HG008050	HG008106	HG008113	HG008114
Greenfinch	8800-3-C19	Austria, Burgenland (1)	2012	HG008050	HG008106	HG008113	HG008114
Greenfinch	8800-3-C20	Austria, Burgenland (1)	2012	HG008050	HG008106	HG008113	HG008114
Greenfinch	11490-C1	Austria, Upper Austria (2)	2012	HG008050	HG008106	HG008113	HG008114
Greenfinch	11490-C2	Austria, Upper Austria (2)	2012	HG008050	HG008106	HG008113	HG008114
Greenfinch	11490-C3	Austria, Upper Austria (2)	2012	HG008050	HG008106	HG008113	HG008114
Greenfinch	11490-C4	Austria, Upper Austria (2)	2012	HG008050	HG008106	HG008113	HG008114
Greenfinch	11490-C5	Austria, Upper Austria (2)	2012	HG008050	HG008106	HG008113	HG008114
Greenfinch	11490-C6	Austria, Upper Austria (2)	2012	HG008050	HG008106	HG008113	HG008114
Greenfinch	11490-C9	Austria, Upper Austria (2)	2012	HG008050	HG008106	HG008113	HG008114
Greenfinch	11490-C10	Austria, Upper Austria (2)	2012	HG008050	HG008106	HG008113	HG008114
Greenfinch	11490-C11	Austria, Upper Austria (2)	2012	HG008050	HG008106	HG008113	HG008114
Greenfinch	11490-C12	Austria, Upper Austria (2)	2012	HG008050	HG008106	HG008113	HG008114
Greenfinch	11794-1-C1	Austria, Lower Austria (3)	2012	HG008050	HG008106	HG008113	HG008114
Greenfinch	11794-1-C2	Austria, Lower Austria (3)	2012	HG008050	HG008106	HG008113	HG008114
Greenfinch	11794-1-C3	Austria, Lower Austria (3)	2012	HG008050	HG008106	HG008113	HG008114
Greenfinch	11794-1-C6	Austria, Lower Austria (3)	2012	HG008050	HG008106	HG008113	HG008114
Greenfinch	11794-1-C7	Austria, Lower Austria (3)	2012	HG008050	HG008106	HG008113	HG008114
Greenfinch	11794-1-C8	Austria, Lower Austria (3)	2012	HG008050	HG008106	HG008113	HG008114
Greenfinch	11794-1-C10	Austria, Lower Austria (3)	2012	HG008050	HG008106	HG008113	HG008114
Greenfinch	11794-1-C11	Austria, Lower Austria (3)	2012	HG008050	HG008106	HG008113	HG008114
Greenfinch	11794-1-C12	Austria, Lower Austria (3)	2012	HG008050	HG008106	HG008113	HG008114
Greenfinch	12794-1	Austria, Upper Austria (4)	2012	HG008050	HG008106	HG008113	HG008114
Greenfinch	12983-1	Austria, Styria (5)	2012	HG008050	HG008106	HG008113	HG008114
Greenfinch	12983-2	Austria, Styria (5)	2012	HG008050	HG008106	HG008113	HG008114
Greenfinch	12997	Austria, Styria (6)	2012	HG008050	HG008106	HG008113	HG008114
Greenfinch	015405-C2	Austria, Vienna (7)	2012	HG008050	HG008106	HG008113	HG008114
Greenfinch	015405-C3	Austria, Vienna (7)	2012	HG008050	HG008106	HG008113	HG008114
Greenfinch	015405-C4	Austria, Vienna (7)	2012	HG008050	HG008106	HG008113	HG008114
Greenfinch	015405-C5	Austria, Vienna (7)	2012	HG008050	HG008106	HG008113	HG008114

Table 1. (*Cont.*)

Host species	Isolate	Location (Site No.)	Year	GenBank accession numbers			
				ITS1-5.8S-ITS2 region	18S rRNA gene	rpb1 gene	Fe-hydrogenase gene
Greenfinch	015405-C7	Austria, Vienna (7)	2012	HG008050	HG008106	HG008113	HG008114
Greenfinch	015405-C8	Austria, Vienna (7)	2012	HG008050	HG008106	HG008113	HG008114
Greenfinch	015405-C9	Austria, Vienna (7)	2012	HG008050	HG008106	HG008113	HG008114
Greenfinch	015405-C10	Austria, Vienna (7)	2012	HG008050	HG008106	HG008113	HG008114
Hawfinch	13329-C1	Austria, Lower Austria (8)	2012	HG008050	HG008106	HG008113	HG008114
Hawfinch	13329-C2	Austria, Lower Austria (8)	2012	HG008050	HG008106	HG008113	HG008114
Hawfinch	13329-C4	Austria, Lower Austria (8)	2012	HG008050	HG008106	HG008113	HG008114
Hawfinch	13329-C6	Austria, Lower Austria (8)	2012	HG008050	HG008106	HG008113	HG008114
Hawfinch	13329-C8	Austria, Lower Austria (8)	2012	HG008050	HG008106	HG008113	HG008114
Hawfinch	13329-C9	Austria, Lower Austria (8)	2012	HG008050	HG008106	HG008113	HG008114
Hawfinch	13329-C10	Austria, Lower Austria (8)	2012	HG008050	HG008106	HG008113	HG008114
Greenfinch	14313	Austria, Vienna (9)	2012	HG008050	HG008106	HG008113	HG008114
Greenfinch	14660-1-C1	Austria, Lower Austria (10)	2012	HG008050	HG008106	HG008113	HG008114
Greenfinch	14660-1-C2	Austria, Lower Austria (10)	2012	HG008050	HG008106	HG008113	HG008114
Greenfinch	14660-1-C3	Austria, Lower Austria (10)	2012	HG008050	HG008106	HG008113	HG008114
Greenfinch	14660-1-C5	Austria, Lower Austria (10)	2012	HG008050	HG008106	HG008113	HG008114
Greenfinch	14660-2-C1	Austria, Lower Austria (10)	2012	HG008050	HG008106	HG008113	HG008114
Greenfinch	14660-2-C2	Austria, Lower Austria (10)	2012	HG008050	HG008106	HG008113	HG008114
Greenfinch	14660-2-C5	Austria, Lower Austria (10)	2012	HG008050	HG008106	HG008113	HG008114
Greenfinch	14660-2-C6	Austria, Lower Austria (10)	2012	HG008050	HG008106	HG008113	HG008114
Greenfinch	019383	Austria, Lower Austria (11)	2012	HG008050	HG008106	HG008113	HG008114
Greenfinch	021326	Austria, Vienna (12)	2012	HG008050	HG008106	HG008113	HG008114
Feral pigeon	021337	Austria, Vienna (13)	2012	HG008050	HG008106	HG008113	HG008114
Hawfinch	000091	Austria, Vienna (14)	2013	HG008050	HG008106	HG008113	HG008114
Yellowhammer	1914-2	Austria, Burgenland (15)	2013	HG008050	HG008106	HG008113	HG008114
Greenfinch	TGAL-A	Slovenia, Central Slovenia (16)	2012	HG008050	HG008106	HG008113	HG008114
Greenfinch	TGAL- B	Slovenia, Upper Carniola (17)	2012	HG008050	HG008106	HG008113	HG008114
Brambling	TGAL- PIN	Slovenia, Central Slovenia (18)	2012	HG008050	HG008106	HG008113	HG008114
Chaffinch	TGAL-SCIN	Slovenia, Central Slovenia (18)	2013	HG008050	HG008106	HG008113	HG008114
Greenfinch	760-07	England, Gloucestershire (19)	2007	GQ150752[a]	GQ214405[a]	HG008113	JF681136[b]
Greenfinch	837-07	England, Devon (20)	2007	GQ150752[a]	GQ214405[a]	HG008113	JF681136[b]
Greenfinch	90-08	England, Lincolnshire (21)	2008	GQ150752[a]	GQ214405[a]	HG008113	JF681136[b]
Greenfinch	99-08	England, Norfolk (22)	2008	GQ150752[a]	GQ214405[a]	HG008113	JF681136[b]
Chaffinch	861-08	England, Staffordshire (23)	2008	GQ150752[a]	GQ214405[a]	HG008113	JF681136[b]
Greenfinch	864-08	England, Bedfordshire (24)	2008	GQ150752[a]	GQ214405[a]	HG008113	JF681136[b]
Greenfinch	R2003	England, Somerset (25)	2008	GQ150752[a]	GQ214405[a]	HG008113	HG008114
Greenfinch	R2056	England, Staffordshire (26)	2008	GQ150752[a]	GQ214405[a]	HG008113	HG008114

[a] Published (Robinson *et al*. 2010).
[b] Published (Lawson *et al*. 2011*a*).

cultured post-mortem in the years 2012 and 2013 from the oropharynx of 13 greenfinches, two hawfinches *Coccothraustes coccothraustes*, one feral pigeon *Columba livia* and one yellowhammer. Post-mortem examination detected crop and/or oesophageal lesions that were typical of finch trichomonosis in these birds.

The four trichomonad isolates from Slovenia obtained from two greenfinch carcasses with upper alimentary tract lesions that were examined post-mortem and two live wild birds caught in mist nets, one brambling and one chaffinch, were received as *in vitro* cultures of the parasite. Live bird sampling in Slovenia was performed with permission from the Slovenian Environment Agency, Ministry of Agriculture and Environment, Republic of Slovenia (permit number 35601-125/2009-8).

The eight isolates from the UK obtained from seven greenfinches and one chaffinch were received as extracted DNA solutions. The British isolates were selected from 2007 and 2008, around the period of hypothesized spread from eastern England to Fennoscandia.

The clonal isolate *T. gallinae*/Budgerigar/Austria/5895-C1/06 (Grabensteiner *et al*. 2010) was also included in the study.

In vitro *cultivation*

Cultures of the Austrian *T. gallinae* isolates were established from oesophagus and/or crop lesions collected at post-mortem examination from birds with confirmed trichomonosis. The material was placed in 9 mL of Medium 199 containing Earle's salts, L-glutamine, 25 mM HEPES and L-amino acids (Gibco™, Invitrogen, Vienna, Austria). In addition, 15% heat-inactivated FBS (Gibco™, Invitrogen, Vienna, Austria) and 0·22% rice starch (Sigma–Aldrich, Vienna, Austria) were added. Following incubation at 40 °C, growth of trichomonads was monitored daily by light microscopy. Parasites were sub-cultured every 2–3 days of incubation by transferring 1 mL of culture into a new sterile 50 mL tube (Sarstedt, Wiener Neudorf, Austria) containing 9 mL of fresh medium before aliquots were collected for further analyses. For seven protozoan isolates (Table 1) clonal cultures were established by micromanipulation and *in vitro* propagation as previously described (Hess *et al*. 2006). If clonal cultures were available, they were used for further investigations. All of the progenitor and clonal cultures were cryo-preserved at −150 °C by adding 5% dimethylsulphoxide.

In vitro cultures of the Slovenian *T. gallinae* isolates were obtained primarily from swab samples taken from the crop and oesophagus of fresh passerine carcasses of two greenfinches and live apparently healthy birds. In total 18 live birds, 11 chaffinches, one greenfinch, one brambling, two great tits, two blue tits *Cyanistes caeruleus* and one robin *Erithacus rubecula* were caught in mist nests in November and December 2012 in Vrhnika, Slovenia and sampled for detection of *T. gallinae*. Growth of the parasite in culture medium was only detected in the samples taken from the two greenfinch carcasses and two live birds, one brambling and one chaffinch. The brambling was in good body condition and apparently healthy. Contrary to this, the chaffinch was in poor body condition, displayed severe neck distension consistent with necrotic ingluvitis and/or food impaction and died shortly after capture, which was congruent with finch trichomonosis. All isolates were cultivated in Diamond's media. The medium contained the following: 80 mg of K_2HPO_4 and 80 mg of KH_2PO_4 (Kemika, Zagreb, Croatia), 2 g of trypticase peptone and 1 g of yeast extract (BD, Heidelberg, Germany), 0·5 g of maltose (Merck, Ljubljana, Slovenia), 100 mg of L-cysteine hydrochloride (Sigma-Aldrich, Maribor, Slovenia), 20 mg of L-ascorbic acid (Kemika, Zagreb, Croatia) and 50 mg agar (Biolife, Ljubljana, Slovenia) per 90 mL of distilled water. After sterilization for 10 min at 121 °C, 10 mL heat-inactivated lamb serum (home-made), 100 000 units of crystalline penicillin C (Sigma-Aldrich, Maribor, Slovenia) and 100 mg of streptomycin sulphate (Pliva, Zagreb, Slovenia) were added to the medium. The presence of motile parasites was determined by light microscopy. After receiving the cultures at the clinic in Vienna, Austria, the protozoan cells were transferred to Medium 199 containing FBS and rice starch and were incubated at 40 °C. Following a few passages of the parasites to fresh medium, aliquots were collected for the further investigations.

For the establishment of protozoan cultures from British cases oesophageal lesions obtained from post-mortem examinations were incubated at 30 °C in Trichomonas Media No. 2 (Oxoid, UK) and screened for motile trichomonads at 48, 72 h and 5 days using light microscopy (Robinson *et al*. 2010).

PCR amplification, sequencing and sequence analyses

For DNA extraction, 1 mL of progenitor or clonal cultures from Austrian and Slovenian isolates was used. The samples were centrifuged at 500 ***g*** for 5 min and after removing the supernatant the pellets were frozen at −20 °C. DNA was extracted using the DNeasy® Blood and Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. For the British isolates extraction of DNA from trichomonad cultures was performed using the Biosprint 15 DNA Blood Kit (Qiagen, UK) for purification of DNA from tissue (Robinson *et al*. 2010). Specific primer pairs were used for PCRs to amplify the following four different genomic regions

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Table 2. List of primers used for amplification

Primer	DNA region targeted	Sequence	Annealing temperature	Reference
TFR1	ITS1-5.8S-ITS2 region	5′-TGC TTC AGT TCA GCG GGT CTT CC-3′	55 °C	(Felleisen, 1997)
TFR2	ITS1-5.8S-ITS2 region	5′-CGG TAG GTG AAC CTG CCG TTG G-3′	55 °C	(Felleisen, 1997)
Hm long f	18S rRNA gene	5′-AGG AAG CAC ACT ATG GTC ATA G-3′	55 °C	(Hess *et al*. 2006)
Hm long r	18S rRNA gene	5′-CGT TAC CTT GTT ACG ACT TCT CCT T-3′	55 °C	(Hess *et al*. 2006)
TG Fe-hyd for	Fe-hydrogenase gene	5′-GTT TGG GAT GGC CTC AGA AT-3′	58 °C	(Lawson *et al*. 2011*b*)
TG Fe-hyd rev	Fe-hydrogenase gene	5′-AGC CGA AGA TGT TGT CGA AT-3′	58 °C	(Lawson *et al*. 2011*b*)
TG rpb1 for	*rpb1* gene	5′-GAC AAA GAG AGC CCA GAT GTC-3′	58 °C	this study
TG rpb1 rev	*rpb1* gene	5′-ACG ACA TGT GTG AGA GAA CCT-3′	58 °C	this study

of the parasite: the ITS1-5.8S-ITS2 region, the 18S rRNA gene, the *rpb1* gene and the Fe-hydrogenase gene (Table 2). Amplifications were carried out in 25 μL reaction mixtures employing the HotStarTaq Master Mix Kit (Qiagen, Hilden, Germany). A reaction mixture consisted of 12·5 μL of HotStarTaq Master Mix, 8 μL of distilled water, 1 μL of forward primer, 1 μL of reverse primer (all primers were used at concentrations of 10 pmol μL^{-1}) and 2·5 μL of DNA template. After the initial denaturation step at 95 °C for 15 min, the reaction mixtures were subjected to 40 cycles of heat denaturation at 94 °C for 30 s, primer annealing at 55 or 58 °C for 1 min (Table 2) and DNA elongation at 72 °C for 2 min, followed by the final elongation step at 72 °C for 10 min.

The PCR products were visualized using agarose gel electrophoresis. PCR products of the expected sizes (approximately 350 bp for the ITS1-5.8S-ITS2 region, 1·5 kb for the 18S rRNA gene, 1·5 kb for the *rpb1* gene and 1 kb for the Fe-hydrogenase gene) were excised from the agarose gel and purified using the QIAquick gel extraction kit® (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Sequencing service was provided by LGC Genomics (Berlin, Germany). All PCR amplification products were sequenced in both directions. Sequence alignments were carried out by Accelrys Gene version 2.5 (Accelrys, San Diego, CA) software. In order to find identical GenBank database entries the BLAST search algorithm was performed with nucleotide sequences of the newly sequenced loci.

RESULTS

A total of 21 trichomonad isolates from Austria and Slovenia were obtained from five different passerine and one columbiform species (Table 1). For seven of the 21 isolates clonal cultures were established. The sequence data obtained for the ITS1-5.8S-ITS2 region, the 18S rRNA region, the *rpb1* gene and the Fe-hydrogenase gene were used for comparative analyses. Identical nucleotide sequences of the four analysed genomic regions were identified for trichomonad isolates, including the clonal cultures, from all of the Austrian and Slovenian *T. gallinae* infections regardless of host species and geographical region. The lengths of the identical consensus sequences were 271 bp for the ITS1-5.8S-ITS2 region (HG008050), 1378 bp for the 18S rRNA gene (HG008106), 1356 bp for the *rpb1* gene (HG008113) and 910 bp for the Fe-hydrogenase gene (HG008114). No multiple parasite strain concurrence within an individual host was observed in the seven trichomonosis cases from which clonal cultures were established.

Investigation of the genetic relationship between the trichomonad isolates from Austria and Slovenia with the ones collected from British finches was performed. For this purpose partial sequences of the *rpb1* gene of eight selected *T. gallinae* isolates from British finches were examined in this study. In addition, the Fe-hydrogenase gene was amplified and sequenced from two of these British isolates for which the data were not yet available. Comparison of the consensus sequences for the ITS1-5.8S-ITS2 region (HG008050) and the 18S rRNA gene (HG008106) with the sequences GQ150752, GQ150753 and GQ214405 (Robinson *et al*. 2010) obtained from the British trichomonad isolates showed no sequence variation. No sequence variation was detected amongst the Austrian, Slovenian and British parasite isolates based on the Fe-hydrogenase gene (JF681136 (Lawson *et al*. 2011*b*) and HG008114). Comparison of the newly determined sequences from this study based on the *rpb1* gene (HG008113) revealed 100% sequence identity for all examined *T. gallinae* isolates from Austria, Slovenia and the UK.

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Table 3. Percentage of sequence identity between the trichomonad isolates from European finches and *Trichomonas gallinae*/Budgerigar/Austria/5895-C1/06 and *Trichomonas gallinae*/Cooper's hawk/Arizona USA/COHA4

Isolate	Percentage of sequence identity to trichomonad isolates from European finches			
	ITS1-5.8S-ITS2 region	18S rRNA gene	*rpb1* gene	Fe-hydrogenase gene
Trichomonas gallinae/Budgerigar/Austria/5895-C1/06	100%[a]	100%[a]	100%[a]	99·5%[a]
Trichomonas gallinae/Cooper's Hawk/Arizona USA/COHA4	100%[b]	99·9%[b]	100%[c]	nd

nd stands for not determined.
[a] This study.
[b] Published (Gerhold *et al*. 2008).
[c] Published (Malik *et al*. 2011).

Performing BLAST search algorithm on the ITS1-5.8S-ITS2 region (HG008050) and the 18S rRNA gene (HG008106) sequences obtained from the finch-trichomonad isolates resulted in many GenBank entries with 100% sequence identity. However, identical sequences for both loci were detected in several clonal *T. gallinae* isolates from budgerigar *Melopsittacus undulatus* and chicken *Gallus gallus* (FN433476 and FN433484; Grabensteiner *et al*. 2010). In order to compare these isolates and the finch-trichomonad strain the loci *rpb1* and Fe-hydrogenase were analysed for one of those, *T. gallinae*/Budgerigar/Austria/5895-C1/06. No sequence variation was found in the *rpb1* gene between the finch-trichomonad isolates and the isolate 5895-C1 (Table 3). However, the comparison of the sequence for the Fe-hydrogenase gene of the isolate 5895-C1 (HG008115) showed only 99·5% identity to the consensus finch-trichomonad sequence (HG008114). Interestingly, the Fe-hydrogenase sequence for 5895-C1 demonstrated 100% identity to the sequence JF681141 obtained for a *T. gallinae* isolate from a Madagascar turtle-dove *Streptopelia picturata* from Mahé, Seychelles (Lawson *et al*. 2011*a*) and to the isolate obtained from a Scottish budgerigar B306472 (Chi *et al*. 2013). Another interesting isolate, Cooper's hawk 4 (*Accipiter cooperii*) from Arizona, USA, demonstrated identical sequences for two typing loci. The ITS1-5.8S-ITS2 region (EU215369; Gerhold *et al*. 2008) and the *rpb1* gene (HM016230; Malik *et al*. 2011) demonstrated 100% identity to the sequences determined for finch-trichomonad isolates (HG008050 and HG008114, respectively) (Table 3). However, when the finch-trichomonad sequence of the 18S rRNA gene (HG008106) was compared with the GenBank entry for this isolate (EU215372; Gerhold *et al*. 2008), two single nucleotide polymorphisms (SNPs) were identified (Table 3). No Fe-hydrogenase sequence data are available for this isolate.

DISCUSSION

Here we report for the first time the occurrence of finch trichomonosis in Austria which emerged in summer 2012. Subsequent to this further geographical spread of the disease, the genetic heterogeneity amongst *T. gallinae* isolates collected from finch trichomonosis incidents in Austria and Slovenia and their genetic relationship with British isolates was investigated. The analyses were based on the following four different genomic regions: the ITS1-5.8S-ITS2, 18S rRNA gene, the *rpb1* gene and the Fe-hydrogenase gene. Comparison of sequence data obtained for the four genomic loci displayed no sequence variation between all tested Austrian and Slovenian isolates. Moreover, 100% identity was found between the newly determined sequences and the ones obtained from finch trichomonosis cases in the UK. The Austrian and Slovenian isolates also showed 100% sequence identity to the Fennoscandian finch-trichomonad isolates at the ITS1-5.8S-ITS2 region and the 18S rRNA gene (Robinson *et al*. 2010; Lawson *et al*. 2011*a*, *b*). This result corroborates the hypothesis of Lawson *et al*. (2011*a*) that one clonal strain of *T. gallinae* is the causative agent of the emerging infectious disease within passerine birds in Europe. It also underlines the continuing dissemination of this parasite strain within Europe.

Analyses of the newly obtained sequences indicated that several earlier reported *T. gallinae* isolates might be the same strain as that which caused the finch trichomonosis epidemic, since sequences of two loci, the ITS1-5.8S-ITS2 region and the 18S rRNA gene, showed 100% identity. Most of these isolates were from budgerigars and another was isolated from a chicken (Grabensteiner *et al*. 2010). To clarify this observation, two additional loci, the *rpb1* and the Fe-hydrogenase genes, were analysed for one of these isolates, *T. gallinae*/Budgerigar/Austria/5895-C1/06. The sequences for the *rpb1* gene were fully identical, whereas a variation of five SNPs was found in the

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sequences for the Fe-hydrogenase gene. Although clinical signs of infection with the finch-trichomonad strain (Lawson *et al*. 2011*b*) and experimental data from *in vitro* studies with the trichomonad isolate 5895-C1 (Amin *et al*. 2012*a*, *b*) indicate high pathogenicity for both isolates, it is clear from the Fe-hydrogenase sequence data that these parasite strains are related but not the same. Similarly, the comparative analyses of sequences between the finch-trichomonad isolates and the available sequence data for the isolate Cooper's hawk 4 (Gerhold *et al*. 2008; Malik *et al*. 2011) demonstrated that the only difference between these two isolates was detected in the 18S rRNA gene. Collectively, these findings indicate the necessity of multi-locus sequence analysis when characterizing *T. gallinae* isolates.

Interestingly, the Fe-hydrogenase sequence determined for isolate 5895-C1 (HG008115) displayed 100% identity to the sequence JF681141 obtained for a trichomonad isolate from a Madagascar turtle-dove from Mahé, Seychelles (Lawson *et al*. 2011*a*; Chi *et al*. 2013). The sequence data from 5895-C1 also shared 100% identity with a captive Scottish budgerigar (B306472; Chi *et al*. 2013), implying that this might be the preponderant circulating strain in European budgerigars. This is supported by the observation that all trichomonads from eight budgerigars in the Grabensteiner *et al*. (2010) study seemed to be the same strain based on the two loci, ITS1-5.8S-ITS2 region and 18S rRNA gene. However, to investigate this hypothesis, further analyses using more isolates and multi-locus sequence analysis are required.

Finch trichomonosis is an emerging infectious disease now established across several western European countries. Besides chaffinches (Lawson *et al*. 2011*b*) and possible other passerine birds, which are hosts of the parasite, it is conceivable that columbiform species may also function as carriers of the finch epidemic *T. gallinae* strain. Chi *et al*. (2013) found that whilst sequence diversity exists amongst *T. gallinae* isolates from British columbiformes, the finch epidemic strain of *T. gallinae* was predominant. Importantly, the one feral pigeon from Austria, investigated in the present study, was also shown to be infected by the same strain which supports the hypothesis for parasite transmission between columbid and passerine populations, perhaps at shared food and water sources. This is not surprising since amongst columbiformes, *C. livia* is considered the primary reservoir of *T. gallinae* (Stabler, 1954; Bondurant and Honigberg, 1994). Some pigeons and doves are able to develop an immunity preventing harmful effects of virulent strains which may result in aclinical parasite carriage. Therefore, they act as reservoir hosts and can transmit the parasite to other birds (Kocan and Herman, 1971).

Although natural lethal infections of *T. gallinae* in passerine birds have not been frequently reported, localized outbreaks have been described in North America. In 2002, there was an outbreak of trichomonosis in Kentucky, USA, that involved approximately 200 wild house finches *Carpodacus mexicanus* and house sparrows (NWHC, 2002). Furthermore, an increased mortality of purple finches *Carpodacus purpureus* and American goldfinches *Carduelis tristis* due to trichomonosis was reported in the Canadian Maritime provinces (Nova Scotia, New Brunswick and Prince Edward Island) in 2007 and 2008 (Forzan *et al*. 2010). Due to the lack of sequence data of *T. gallinae* isolates from these outbreaks, no further comparison with the European finch trichomonosis epidemic strain could be made.

From 2001 to 2005, at the wildlife rehabilitation hospital at the Lindsay Wildlife Museum in Walnut Creek, CA, USA, several passerine birds including house finches, scrub jays *Aphelocoma californica*, crows *Corvus brachyrhynchos* and ravens *Corvurs corax* were screened for *T. gallinae* infection using wet mount preparations (Anderson *et al*. 2009). Sequence data of these parasite isolates based on the ITS1-5.8S-ITS2 region were identical to each other and to the European isolates collected from finch trichomonosis cases, however sequence data from other loci are not available for comparison.

From the present study it can be concluded that a single clonal strain of the parasite *T. gallinae* is the causative agent of the emerging infectious disease within passerine birds in Europe. The disease is continuing to spread southwards on the continent most likely mediated by bird migration. The strain retains infectivity to both passerine and columbiform species and it is likely that it can be transmitted between these populations. Furthermore, all the reports of trichomonosis outbreaks for which *T. gallinae* isolates show a 100% ITS1-5.8S-ITS2 sequence identity to the European wild finch isolates may hint to a wider distribution of this virulent parasitic strain. However, the results of the present work showed evidently that the resolution of the ITS1-5.8S-ITS2 region is insufficient to identify trichomonad strains and multi-locus analysis such as that performed in this study is therefore necessary to confirm the finch-trichomonad strain as the aetiological agent of any other outbreak of avian trichomonosis. Some of the sequence loci used in the present study, such as Fe-hydrogenase and 18S rRNA, already showed good typing potential for *T. gallinae* isolates, whereas this still needs further evaluation for the *rpb1* gene. In future, complete genome analyses may offer the gold standard for strain differentiation, but at the moment this remains expensive, time consuming and requires extensive interpretation. Therefore, effort should be focused to improve multi-locus approaches by further defining those loci that facilitate best resolution between *T. gallinae* strains.

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