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Research Note—

***Tetratrichomonas* and *Trichomonas* spp.-Associated Disease in Free-Ranging Common Eiders (*Somateria mollissima*) from Wellfleet Bay, MA and Description of ITS1 Region Genotypes**

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SUMMARY. During an outbreak of Wellfleet Bay virus (WFBV) in common eiders (*Somateria mollissima*) from the Cape Cod region of Massachusetts, several birds were diagnosed with trichomonosis consisting of multiple trichomonad species. Six birds were examined, with trichomonads found in ceca in four birds and associated typhlitis in three of these four birds. PCR and DNA sequencing utilizing trichomonad-specific primers targeting the ITS1 region of the ribosomal DNA (rDNA) revealed the presence of *Tetratrichomonas gallinarum* in the gastrointestinal tracts of five birds and *Trichomonas* spp. in the livers of two birds, one of which had a dual *Te. gallinarum*-*Trichomonas gallinae* infection. Sequence analysis revealed no variation between *Te. gallinarum* sequences whereas the ITS1 sequences obtained from the other *Trichomonas* spp. demonstrated the presence of multiple genotypes. One sequence had 100% identity to a *Trichomonas* sp. previously isolated from a Cooper's hawk (*Accipiter cooperii*) and the other sequence was 100% identical to a previously described *Tr. gallinae* isolate obtained from a Pacific Coast band-tailed pigeon (*Patagioenas fasciata monilis*). These findings suggest *Te. gallinarum* and other *Trichomonas* spp. possibly contributed to morbidity and mortality in this species. Furthermore, to the authors' knowledge, this is the first report of trichomonad-associated disease in a free-ranging sea duck.

RESUMEN. *Nota de Investigación.* Enfermedad asociada a *Tetratrichomonas* y *Trichomonas* spp. en eiders comunes (*Somateria mollissima*) mantenidos en semi libertad en la Bahía de Wellfleet Bay, Massachusetts y descripción de genotipos de la región ITS1.

Durante un brote del virus de la Bahía de Wellfleet (WFBV) en eiders comunes (*Somateria mollissima*) de la región de Cape Cod en Massachusetts, varias aves fueron diagnosticadas con tricomoniasis que consistía en múltiples especies de tricomonas. Se examinaron seis aves, con tricomonas encontradas en ciegos en cuatro aves y con tiftitis asociada en tres de estas cuatro aves. Mediante la técnica de PCR y por análisis de secuencias del ADN utilizando iniciadores específicos de tricomonas dirigidos a la región ITS1 del ADN ribosómico (rDNA) reveló la presencia de *Tetratrichomonas gallinarum* en el tracto gastrointestinal de cinco aves y *Trichomonas* spp. en los hígados de dos aves, una de las cuales sufría infección doble por *Te. gallinarum*-*Trichomonas gallinae*. El análisis de secuencias no reveló variación entre las secuencias de *Te. gallinarum* mientras que las secuencias ITS1 obtenidas de otras *Trichomonas* spp. demostraron la presencia de genotipos múltiples. Una secuencia tenía una identidad del 100% con una *Trichomonas* sp. previamente aislada de un halcón de Cooper (*Accipiter cooperii*) y la otra secuencia era 100% idéntica a la secuencia de *Tr. gallinae* previamente descrita y aislado de una paloma de collar (*Patagioenas fasciata monilis*) de la costa del Pacífico. Estos hallazgos sugieren que *Te. gallinarum* y otras *Trichomonas* spp. posiblemente contribuyeron a la morbilidad y mortalidad en esta especie. Además, de acuerdo al conocimiento de los autores, este es el primer informe de enfermedad asociada a tricomonas asociadas con enfermedad en patos en semilibertad rango libre.

Key words: common eiders, Protozoa, *Trichomonas gallinae*, *Tetratrichomonas gallinarum*, trichomonosis, typhlitis

Abbreviations: FFPE = formalin-fixed, paraffin-embedded; H&E = hematoxylin and eosin; ITS1 = internal transcribed spacer 1; *Te. gallinarum* = *Tetratrichomonas gallinarum*; *Tr. gallinae* = *Trichomonas gallinae*; WFBV = Wellfleet Bay virus

Certain members of the family *Trichomonadidae* (Order Trichomonadida) are recognized as important parasites of birds, causing mortality and morbidity in numerous avian species worldwide (6,8,11,14,15,21,34,35,38,39,40). These flagellated, microaerophil-

ic protozoa generally inhabit the avian digestive tract where they can cause a wide range of disease, from subclinical to fatal infections. Less frequently, trichomonads can cause lesions in the liver, brain, and nasal sinuses (15,40). Trichomonad pathogenicity varies depending on the trichomonad species or genotype and the avian host species' susceptibility. *Trichomonas gallinae*, the etiologic agent of avian trichomonosis, and *Tetratrichomonas gallinarum*, a common lower gastrointestinal tract parasite of anseriform and gallinaceous birds, are the most-frequently reported avian trichomonad species (16,22).

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Trichomonas gallinae is a well-documented and important pathogen of columbids (order Columbiformes), raptors (orders Falconiformes and Accipitriformes), and passerines (order Passeriformes). This parasite is transmitted to birds through various routes including crop feeding of nestlings, billing or feeding courtship rituals, congregation at birdfeeders or birdbaths, and consuming of infected birds (2,36,38). Once infected, birds typically develop ulcerated, caseous oral lesions which can lead to sepsis, dehydration, emaciation, or asphyxiation (15,40,41).

In contrast, the pathogenicity of *Te. gallinarum* in birds is controversial. Generally, *Te. gallinarum* is considered a nonpathogenic, commensal organism of the lower intestinal tract in anseriform and gallinaceous birds (22). Previous experimental infection studies in turkeys (order Galliformes) and other fowl have failed to produce consistent observable disease (4,3,23); however, a few reports have connected *Te. gallinarum* with pathologic changes in wild birds. *Tetratrachomonosis* has been associated with encephalitis, enteritis, and conjunctivitis in California mockingbirds (*Mimus polyglottos*) (5,32) as well as necrotizing hepatitis and splenitis in a Waldrapp ibis (*Geronticus eremita*) (26) and an American white pelican (*Pelecanus erythrorhynchos*) (7). Furthermore, trophozoites of *Te. gallinarum* were found by PCR and *in situ* hybridization in lesions of necrotizing typhlitis and typhlohepatitis in three captive ducks, a red-breasted merganser (*Mergus serrator*), a hooded merganser (*Lophodytes cucullatus*), and a common eider (*Somateria mollissima*), from a German zoologic collection (37). Collectively, these reports suggest *Te. gallinarum* can cause morbidity in a variety of bird species.

Common eiders (*S. mollissima*) are the largest duck species in the Northern Hemisphere and are found in both arctic and subarctic marine habitats (19). Common eiders nest on islands in large colonies and, during nonbreeding seasons, congregate in large populations in coastal and inshore locations. Eiders feed on mollusks and various crustaceans and routinely dive up to 20 m when feeding. Eider populations are stable but numbers vary by geographic location, and the presence of epornitics, including Wellfleet Bay virus (WFBV) as found in this population, may impact populations (1). Here we report the first documented occurrence of *Tr. gallinae-Te. gallinarum* coinfection in eiders from Cape Cod, MA.

MATERIALS AND METHODS

Necropsy and histopathology. As part of an investigation of seasonal, large-scale mortality, six moribund common eiders (four male, two female) were collected from the field in October 2010 along Jeremy Point, Wellfleet Bay, Cape Cod, MA, U. S. A. by United States Department of Agriculture (USDA) Wildlife Services personnel. Live birds were humanely euthanatized and carcasses were kept chilled during overnight transport. Necropsy was performed at the New Hampshire Veterinary Diagnostic Laboratory (Durham, NH). A standard set of tissues was taken for histopathology including brain, lung, liver, adrenal gland, gonad, spleen, kidney, trachea esophagus, lung, pancreas, proventriculus, ventriculus, intestine (duodenum, jejunum, and a distal section of ileum just proximal to the ileocecolic junction), ceca, cloaca, skeletal muscle, heart, and eye. These tissues were fixed in 10% neutral buffered formalin, dehydrated, and embedded in paraffin; 5- μ m sections were mounted on glass slides, stained with hematoxylin and eosin (H&E), and examined by light microscopy. Special stains and routine bacteriology were performed as indicated. Samples of liver and spleen were collected and submitted for virus isolation at a referral laboratory (Cornell Animal Health Diagnostic Center, Ithaca, NY).

PCR analysis and molecular characterization. Scrolls of formalin-fixed, paraffin-embedded (FFPE) tissue samples ($n = 12$) were submitted to the University of Tennessee Center for Wildlife Health (Knoxville, TN) for PCR and DNA sequence analysis. DNA was extracted using Qiagen DNA Extraction Mini kits (Qiagen, Valencia, CA) per the manufacturer's instructions. Extracted DNA was stored at -20°C until used for DNA amplification by PCR. The internal transcribed spacer 1 (ITS1) and partial 5.8S rDNA regions were amplified using order Trichomonadida family wide primers ITS1F (5'-AGCGCAATTTGCATTCAA-3') and ITS1R (5'-CGGTAGGTGAACCTGCCGTTGG-3') that were modified from previous reports (9,13). PCR components included 1–2 μ l of extracted DNA in a 25- μ l reaction containing Ready-to-go PCR beads (GE Scientific, Piscataway, NJ) and 20 pM of ITS1F and ITS1R primers. Cycling parameters for the amplification were 94 C for 2 min followed by 40 cycles of 94 C for 30 sec, 45 C for 30 sec, and 72 C for 2 min, with a final extension at 72 C for 15 min. A water control was included in DNA extraction, and water was used for all PCR reactions as a negative control to detect contamination. DNA isolated from a laboratory-propagated sample of *Tr. gallinae* was included as a positive control.

PCR amplicons were separated by gel electrophoresis using a 2% agarose gel, stained with ethidium bromide, and visualized with ultraviolet light. An approximate 200-bp amplicon was excised and the DNA purified using a QIAquick Gel Extraction kit (Qiagen). Sequencing of the amplicons was performed using the amplification primers at the University of Tennessee Genomics Core (Knoxville, TN).

Phylogenetic analysis of trichomonad ITS1 sequences. ITS1 sequences obtained from eider tissues and representative genotypes of *Tr. gallinae*, as well as from other closely related trichomonad species (Table 1), were aligned using MEGA7 (25). The phylogeny of the aligned sequences was generated using the neighbor joining method based on the Kimura (24) 2-parameter model with bootstrap test (1000 \times). The resulting phylogenetic tree (Fig. 3) was edited in FigTree v1.4.3 (<http://tree.bio.ed.ac.uk/software/figtree/>) and Canvas X (Canvas GFX, Inc., Plantation, FL).

RESULTS

Necropsy findings. At necropsy, all birds were in poor nutritional condition, with variable degrees of skeletal muscle wasting and loss of visceral and subcutaneous adipose tissue. Moderate to large numbers of acanthocephalan parasites were present in sections of small intestine (grossly consistent with *Polymorphus* spp.), with 1–2-mm firm, serosal nodules reflecting areas of parasitic mucosal attachment. Three birds had a few, 3–4-mm diameter, round to irregularly shaped, pale white foci on the surfaces of one or both hepatic lobes.

Microscopically, large numbers of oval to piriform protozoal organisms, approximately $4 \times 8 \mu\text{m}$, were present within cecal crypts of four birds, frequently infiltrating between and under intact enterocytes into the lamina propria (Fig. 1). These organisms had finely vacuolated, pale, basophilic cytoplasm and a single small, dense nucleus. Ulcerative lesions in intestine and ceca were seen in three birds, colonized by both mixed bacteria and protozoal organisms (Fig. 2). Small, unidentified trematodes were also present in ceca and intestinal lumina in multiple birds. Nodular, granulomatous inflammation was associated with sites of mucosal attachment and invasion by acanthocephalans. Erythrophagocytosis, histiocytic hemosiderosis in liver and spleen, and hepatocellular hemosiderosis were present in all birds. Grossly evident pale lesions on livers were shown to be randomly scattered foci of hepatocellular necrosis. No parasites or bacteria were identified in liver by routine

Table 1. Reference sequences utilized in the phylogenetic analysis of trichomonad ITS1 sequences. Examples from each of the known *Tr. gallinae* ITS region sequence groups with their corresponding references are provided.

Species	Host	Strain	Origin	ITS region type ^A	NCBI accession no.	Reference
<i>Trichomonas gallinae</i>	Mourning dove <i>Zenaida macroura</i>	6	Arizona, USA	A	EU215369	17
<i>Tr. gallinae</i>	House finch <i>Carpodacus mexicanus</i>		California, USA	A	EU290649	17
<i>Tr. gallinae</i>	Broad winged hawk <i>Buteo platypterus</i>	BWHA1	Florida, USA	B	EU215368	17
<i>Tr. gallinae</i>	Rock pigeon <i>Columba livia</i>	CGC	Colorado, USA	C	EU215362	17
<i>Tr. gallinae</i>	Eurasian collared dove <i>Streptopelia decaocto</i>	R20	Texas, USA	D	EU215364	17
<i>Tr. gallinae</i>	Eurasian collared dove <i>Streptopelia decaocto</i>	ECD858	Texas, USA	E	EU215363	17
<i>Tr. gallinae</i>	Common ground dove <i>Columbina passerina</i>	GD1321	Texas, USA	F	EU215358	17
<i>Tr. gallinae</i>	Common ground dove <i>Columbina passerina</i>	GD1	Texas, USA	G	EU215359	17
<i>Tr. gallinae</i>	White winged dove <i>Zenaida asiatica</i>	WWDO840	Texas, USA	H	EU215360	17
<i>Tr. gallinae</i>	White winged dove <i>Zenaida asiatica</i>	WWDO1159	Texas, USA	I	EU215361	17
<i>Tr. gallinae</i>	Mourning dove <i>Zenaida macroura</i>	M22	Texas, USA	J	EU215365	17
<i>Tr. gallinae</i>	Band tailed pigeon <i>Patagioenas fasciata</i>	BTPN2	California, USA	K	EU215367	17
<i>Tr. gallinae</i>	Coopers hawk <i>Accipiter cooperii</i>	COHA2	Arizona, USA	L	EU215366	17
<i>Tr. gallinae</i>	Stock dove <i>Columba oenas</i>	HT 2013 42	Germany	O	KX459442	30
<i>Tr. gallinae</i>	Turtle dove <i>Streptopelia turtur</i>	V15	Italy	Q	KX459510	30
<i>Tr. gallinae</i>	Turtle dove <i>Streptopelia turtur</i>	P24312	Spain	P	KF993705	28
<i>Tr. gallinae</i>	Racing pigeon <i>Columba livia</i> forma domestica	7895-C1	Austria	II	FN433474	20
<i>Tr. gallinae</i>	Nicobar pigeon <i>Caloenas nicobarica</i>	115 Nicobar	Great Britain	III	KC529665	10
<i>Tr. gallinae</i>	Canary <i>Serinus canaria</i> forma domestica	18087-C1	Austria	V	FN433477	20
<i>Tr. gallinae</i>	Bearded vulture <i>Gypaetus barbatus</i>	9361-C8	Czech Republic	VI	FN433478	20
<i>Trichomonas vaginalis</i>					TVU86613	13
<i>Trichomonas tenax</i>					TTU86615	13
<i>Tetratrichomonas gallinarum</i>	Baikal teal <i>Anas formosa</i>	AF	Czech Republic		AY245126	9
<i>Te. gallinarum</i>	Mallard <i>Anas platyrhynchos</i>	20-9-1	Czech Republic		AY245124	9
<i>Trichomonas foetus</i>	Domestic cat <i>Felis catus</i>	NCSU Tfs-1	North Carolina, USA		AF466749	27

^ASee Figure 3 for description of ITS region types.

H&E sections or by special stains (Gram stain, periodic acid Schiff stains). Bacterial cultures from liver and spleen of all birds showed no growth or light, sparse contaminants, with no commonality of isolates. Virus isolation was negative for all tissue pools.

PCR analysis and molecular characterization. All six eiders were positive for the presence of *Te. gallinarum* or *Trichomonas* sp. by PCR. Trichomonad DNA was detected in liver sections of two birds and cecal sections of five birds; a single bird was PCR-positive in both the liver and cecum (Table 2). From these, all PCR-positive samples were sequenced. When compared against GenBank,

sequences obtained from the ceca revealed a 97% maximum identity to the ITS1 region of two *Te. gallinarum* isolates, AF (GenBank accession no. AY245126) and 20-9-1 (GenBank accession no. AY245124). Sequence analysis of the ITS1-5.8S region revealed no genetic variation between the *Te. gallinarum* isolates. Trichomonad sequences obtained from liver tissues were identical either to the Cooper's hawk 2 (COHA2) isolate of a *Trichomonas vaginalis*-like *Trichomonas* sp. (GenBank accession no. EU215366) or the CA005882 isolate of *Tr. gallinae* (GenBank accession no. KC215387). Interestingly, these data revealed the bird with a

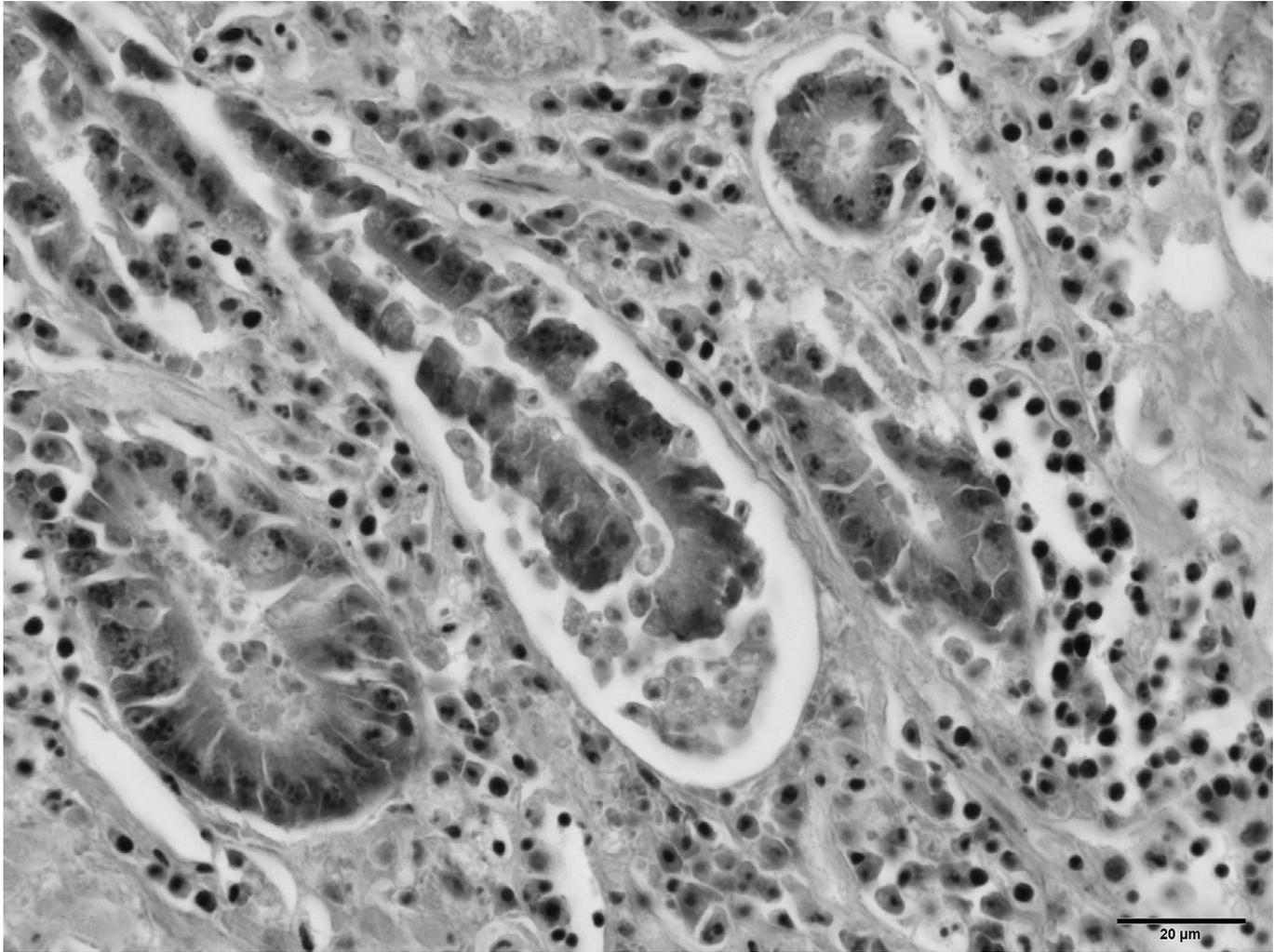


Fig. 1. Ceca. Numerous oval to piriform protozoal organisms, approximately $4 \times 8 \mu\text{m}$, within cecal crypts, and infiltrating between and under intact enterocytes into lamina propria.

PCR-positive liver and cecum had a dual infection with *Te. gallinarum* and *Tr. gallinae*.

DISCUSSION

The birds described here were collected during investigation of recurrent seasonal mortality in this population of common eider, now attributed to a novel orthomyxovirus, WFBV (1). Hepatic necrosis was a consistent finding in this wider study, with colocalization of virus in foci of hepatic necrosis. Birds in our study were collected at the same location, date, and time as those collected by Allison *et al.* (1) and, although virus isolation was negative, WFBV is suspected as the primary cause of death in these six birds (1); however, other factors may have been involved either as a potential primary cause or as coinfections.

Tetratrichomonas gallinarum DNA was amplified from FFPE tissue blocks containing intestine and ceca and were microscopically visible in cecal crypts and in a few foci of ulceration and necrosis. Evidence of invasion, crypt necrosis, and inflammatory aggregates containing tetratrichomonads suggests some degree of pathogenicity of this parasite. WFBV is not described as having significant effects on the intestine (1). Previous reports of *Te. gallinarum*-associated

mortality in eiders, housed in a zoologic collections with a turkey that harbored the parasite, suggested that eiders may be susceptible to *Te. gallinarum* infection. Furthermore, Cepicka *et al.* (9) suggested that *Te. gallinarum* may be a complex with cryptic species that may differ in virulence. At this point, it is unclear if the *Te. gallinarum* infection was solely responsible for the enteritis and typhlitis, but it does suggest that *Te. gallinarum* may be a contributing pathogen in this case. The co-occurrence of WFBV, heavy acanthocephaliasis, trematodiasis, and general debilitation and wasting may have contributed to the clinical signs and possibly influenced the *Te. gallinarum*-associated lesions. Further research is needed to determine how coinfection of WFBV may lead to parasitic diseases (and nonparasitic diseases) from organisms that are generally considered nonpathogenic or opportunistic in the immunocompetent host.

Trichomonas spp. and *Tr. gallinae* sequences were amplified from FFPE blocks containing kidney and liver from two birds. No organisms were visible in these tissues with routine stains, and the presence of these protozoa was not consistently associated with necrosis. In the previous report of hepatitis in ducks associated with *Te. gallinarum*, tetratrichomonads were clearly visible in foci of necrosis, supporting the hypothesis that hepatic lesions are primarily

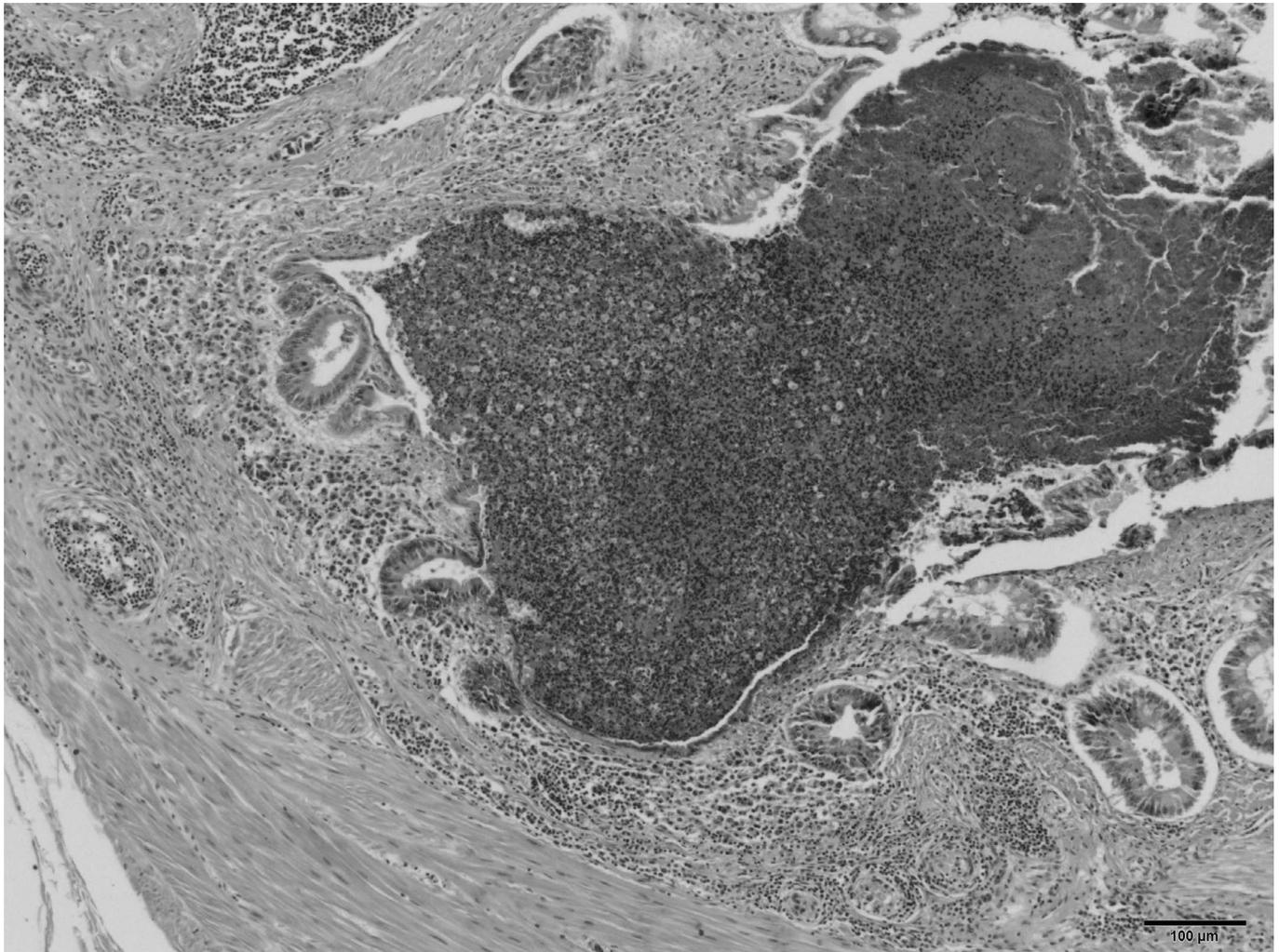


Fig. 2. Ceca. Ulcerative lesions colonized by both mixed bacteria and protozoal organisms.

viral in these eider (37). These findings support colonization of liver by *Tr. gallinae*, with questionable overall significance to the health of these birds. Further localization of the parasite in liver by immunostaining methods would be useful to determine parasite

load and possible association with necrosis or inflammation. The changes of erythrophagocytosis and hemosiderosis in liver and spleen are attributed to wasting and increased erythrocyte turnover rather than direct viral or parasitic infection.

Table 2. PCR and DNA sequence results of the trichomonad internal transcribed spacer region-1 of the ribosomal DNA with corresponding GenBank BLASTn analysis of resultant sequences obtained from common eider samples examined in this study.

Eider number	Tissue type	PCR results, positive/negative (P/N)	NCBI BLASTn max identification match			
			GenBank accession no. for closest sequence match	Parasite species for respective GenBank accession	Percent identity of eider sequence to GenBank accession	Percent query coverage of eider sequence to GenBank accession
1	Liver	N				
	Ceca	P	AY245126.1	<i>Tetratrichomonas gallinarum</i>	97	100
2	Liver	P	EU215366.1	<i>Trichomonas</i> sp.	100	100
	Ceca	N				
3	Liver	P	KC215387.1	<i>Trichomonas gallinae</i>	100	100
	Ceca	P	AY245124.1	<i>Te. gallinarum</i>	97	100
4	Liver	N				
	Ceca	P	AY245126.1	<i>Te. gallinarum</i>	97	100
5	Liver	N				
	Ceca	P	AY245124.1	<i>Te. gallinarum</i>	97	100
6	Liver	N				
	Ceca	P	AY245126.1	<i>Te. gallinarum</i>	97	100

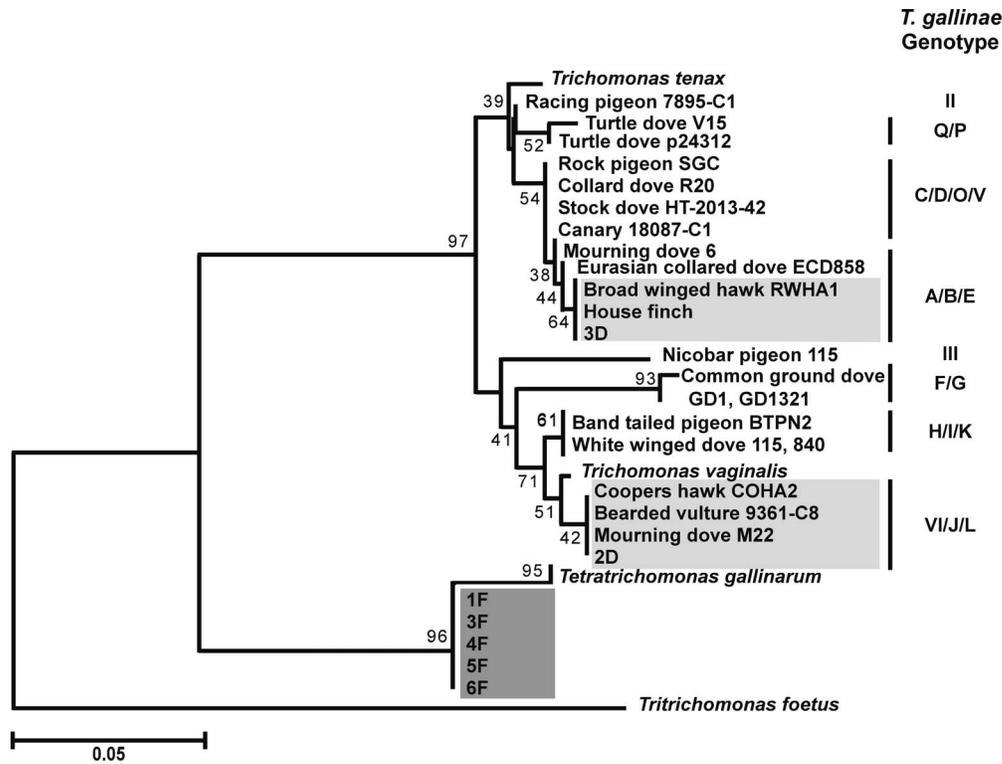


Fig. 3. Phylogeny of trichomonad ITS1 (122-bp) sequences isolated from eider liver and ceca tissues. Liver and ceca isolates are highlighted in dark and light grey, respectively. All *Tr. gallinae* reference sequences are denoted by their strain name and the host from which they were isolated. ITS1 region genotypes of *Tr. gallinae* reference isolates are listed on the right. *Tritrichomonas foetus* is included as an outgroup. The evolutionary history was inferred using the neighbor joining method based on the Kimura 2-parameter model. The tree is drawn to scale and the legend denotes substitutions per site. Bootstrap values (1000) greater than 35% are shown. All corresponding National Center for Biotechnology Information (NCBI) accession numbers and referenced literature are listed in Table 1.

Trichomonosis due to *Tr. gallinae* has caused substantial outbreaks in mourning doves (*Zenaida macroura*) and Pacific Coast band-tailed pigeons (*Patagioenas fasciata monilis*) (17,18,40). In 2005, *Tr. gallinae* was identified as an emerging pathogen in numerous British passerine species, leading to the eventual expansion to passerines in other European countries including central and southern Europe (2,31,33,38). The European passerine *Tr. gallinae* outbreaks were suspected to be associated with contaminated bird waterers and feeders, and recent studies have demonstrated that *Tr. gallinae* can persist in distilled water for at least 16 hr (36), indicating contaminated water can be a source of infection for birds. Furthermore, moribund birds may be easy prey for raptors and other opportunistic avian predators and scavengers, and trichomonosis has been reported in numerous raptor species (6,15,17,39,40). In this report, it is unknown how the eiders were infected with *Tr. gallinae*. Potentially, ingestion of contaminated water may have led to infection of these species. Further research is needed to understand transmission of this parasite in pelagic avian species.

The BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) analysis of the *Tr. gallinae* and the *Trichomonas* spp. ITS1 sequences disclosed a 100% identity to an isolate previously recovered from a band-tailed pigeon (*P. f. monilis*) from California or a Cooper's hawk (*A. cooperii*) from Arizona, respectively. Molecular epidemiology investigations of avian trichomonads have only occurred in the last 10 yr, and results indicate numerous circulating genotypes with variable sequence identities from multiple continents (5,12,17,29). Unfortunately, further molecular analysis using gene targets with

larger base pair PCR products, producing greater epidemiologic power (i.e., iron hydrogenase), was not possible due to the formalin fixation of tissue. As such, it is not possible to identify an epidemiologic link between the sequences in these eiders and other sequences available in GenBank or to speculate on the source of trichomonad-infected material the eiders ingested that led to infection. Future acquisition of fresh tissue or culture of trichomonad isolates obtained from eiders and other sea ducks would be valuable for further molecular studies utilizing full ITS1 and ITS2, 18S rDNA, and iron hydrogenase to elucidate the transmission of trichomonads to these duck species.

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