J. Parasitol., 94(6), 2008, pp. 1335–1341

MOLECULAR CHARACTERIZATION OF THE *TRICHOMONAS GALLINAE* MORPHOLOGIC COMPLEX IN THE UNITED STATES

Richard W. Gerhold, Michael J. Yabsley*, Autumn J. Smith†, Elissa Ostergaard‡, William Mannan§, Jeff D. Cann‖, and John R. Fischer

Southeastern Cooperative Wildlife Disease Study, College of Veterinary Medicine, The University of Georgia, Athens, Georgia 30602. *e-mail: rgerhold@uga.edu*

ABSTRACT: Forty-two *Trichomonas gallinae* isolates were molecularly characterized to determine whether isolates differed in genetic sequence of multiple gene targets depending on host species or geographical location. The 5.8S ribosomal RNA (rRNA) and flanking internal transcribed spacer (ITS) gene regions were amplified by polymerase chain reaction, and the sequences were analyzed phylogenetically. The results of the sequence analysis strongly suggest at least 2 species may exist within the *T. gallinae* morphologic complex. Based on ITS sequences, one group demonstrated high nucleotide identity to the 3 *T. gallinae* sequences available in GenBank, whereas the second group was more closely related to *T. vaginalis* (98%) than to *T. gallinae* (92%). Two common ground-dove (*Columbina passerina*) isolates shared a 95% identity with *T. vaginalis* and a 92% identity with *T. gallinae* and *T. tenax*. Sequence analysis of both the 18S rRNA and α-tubulin genes from a subset of the isolates supports the 5.8S-ITS sequence results. All of the *T. vaginalis*-like isolates originated from Arizona, California, or Texas, whereas *T. gallinae* isolates were found in all sampled states. Both *T. vaginalis*-like and *T. gallinae* isolates were involved in trichomoniasis outbreaks in California and Arizona.

Avian trichomoniasis, caused by the protozoan *Trichomonas gallinae*, is an acute and often fatal disease that is generally manifested as a caseous lesion within the upper digestive tract of affected birds. The rock pigeon (*Columba livia*) is an Old World species and is considered the ultimate source of *T. gallinae*. However, the protozoan may be pathogenic to both Old and New World avian species (Stabler, 1954). Trichomoniasis has been reported from several continents and is considered a major disease for numerous avian species in the Columbiformes and Falconiformes (Stabler, 1954; Forrester and Spalding, 2003; Villanua et al., 2006). In the southeastern United States, trichomoniasis is considered the most important disease of free-ranging mourning doves (*Zenaida macroura*) (Forrester and Spalding, 2003; Gerhold et al., 2007). During a 2-yr outbreak in multiple southeastern states, an estimated 50,000 to 100,000 mourning doves died of trichomoniasis in Alabama alone (Haugen and Keeler, 1952). The disease occurs annually in mourning doves and other columbids throughout North America, and large mortality events are reported frequently (Forrester and Spalding, 2003; Rosenstock et al., 2004; Gerhold et al., 2007). Recent population census information indicates mourning dove populations have declined over much of the United States, particularly in the eastern United States during the last few decades (Dolton and Rau, 2003), but it is unknown whether trichomoniasis is associated with the population decline.

Trichomoniasis may have an impact on several columbids, including the endangered pink pigeon from Mauritius (*Columba mayeri*), numerous passerine species, and a variety of raptors, including Cooper's hawk (*Accipiter cooperii*), barn owl (*Tyto alba*), and the endangered Bonelli's eagle (*Hieraaetus fasciatus*) (Work and Hale, 1996; Boal et al., 1998; Real et al., 2000; Pennycott et al., 2005; Bunbury et al., 2008). In Tucson, Arizona, approximately 85% of Cooper's hawk nestlings tested positive for *T. gallinae*, and trichomoniasis was the leading cause of death in nestlings and fledglings (Boal et al., 1998; Estes and Mannan, 2003).

Infectivity studies with *T. gallinae* have demonstrated a wide spectrum of virulence. With highly virulent isolates, columbids may succumb to infection within 14 days after inoculation with a single trichomonad, whereas with some avirulent isolates, birds may fail to seroconvert after inoculation with 1×10^6 parasites (Stabler and Kihara, 1954; Honigberg, 1979). Previous investigations with clinically virulent and avirulent isolates of *T. gallinae* suggest that virulence and pathogenicity are controlled genetically within the organism (Honigberg et al., 1971). However, virulent and avirulent isolates previously have not been analyzed genetically.

Recently, sequence analysis of the 5.8S ribosomal RNA (rRNA) and flanking internal transcribed spacer regions 1 and 2 (ITS1, ITS2) of organisms within *Tetratrichomonas* spp. disclosed unexpected diversity and host–parasite associations (Cepicka et al., 2005, 2006). The results indicated that at least 16 distinct monophyletic groups exist within the genus, and most of these monophyletic groups are host-specific (Cepicka et al., 2006). In contrast, sequence analysis of the ITS1, 5.8S, and ITS2 rRNA regions of 24 *T. gallinae* isolates from pink pigeons and Madagascar turtle doves (*Streptopelia picturata*) from the island of Mauritius disclosed no nucleotide polymorphisms among any of the isolates, and sequences were identical to *T. gallinae* isolates from 2 rock pigeons from the United States (Gaspar da Silva et al., 2007).

The goal of the present study was to analyze sequences of *T. gallinae* isolates from several avian species obtained from a wide-ranging geographic distribution in the United States to determine whether *T. gallinae* isolates differed genetically depending on host species or geographical location.

Received 1 February 2008; revised 7 April 2008; accepted 8 April 2008.

* D. B. Warnell School of Forestry and Natural Resources, The University of Georgia, Athens Georgia, 30602 and Southeastern Cooperative Wildlife Disease Study, College of Veterinary Medicine, The University of Georgia, Athens, Georgia 30602.

† Caesar Kleberg Research Institute-Texas A&M University, Kingsville, Texas 78363.

‡ Arizona Game and Fish Department, Tucson, Arizona 85745.

§ School of Renewable Natural Resources, University of Arizona, Tucson, Arizona 85721.

‖ California Department of Fish and Game, Monterey, California 93940.

MATERIALS AND METHODS

Parasite culture

Isolates collected during this study were acquired from hunter-killed birds, necropsy, and arbovirus-testing submissions to the Southeastern Cooperative Wildlife Disease Study (SCWDS), College of Veterinary Medicine, The University of Georgia, Athens, Georgia, and from birds captured in the field (Table I). Oral swabs were initially cultured in In-Pouch™ TF kits (BioMed Diagnostics, White City, Oregon), incubated at 37 C, and examined for trichomonad growth for 5 consecutive days. The In-Pouch TF kit has been shown to be as sensitive as tryptone/yeast extract/maltose (TYM) medium for isolating *T. gallinae*, and the kit has practical advantages for organism isolation, especially in field conditions (Bunbury et al., 2005). Subcultures were performed using TYM (pH 7.0), supplemented with 10% heat-inactivated horse serum (HIHS) (Sigma-Aldrich, St. Louis, Missouri) (Diamond, 1983). Initially, subcultures were supplemented with antibiotics as described previously (Diamond, 1983), but once axenic cultures were established, the use of antibiotics was discontinued. Axenic cultures in late logarithmic growth were harvested by centrifugation (750 *g* for 10 min) and cryopreserved in liquid nitrogen using HIHS supplemented with 8% dimethyl sulfoxide (Sigma-Aldrich) until further use. Three additional isolates, 2 from rock pigeons and 1 from a mourning dove, were purchased from the American Type Culture Collection (ATCC) (Manassas, Virginia).

Molecular characterization

In total, 5×10^6 late logarithmic-growth phase trichomonads were harvested by centrifugation (750 *g* for 10 min), and DNA was extracted using QIAGEN Mini kits (QIAGEN, Valencia, California) per the manufacturer's instructions. DNA amplification of the ITS1, 5.8S rRNA, and ITS2 regions was performed using trichomonad-specific primers TFR1 (5′-TGCTTCAGTTCAGCGGGTCTTCC-3′) and TFR2 (5′-CGGTAGGTGAACCTGCCGTTGG-3′) (Felleisin, 1997). Polymerase chain reaction (PCR) components included 1–2.5 µl of DNA in a 50-µl reaction containing 10 mM Tris-Cl (pH 9.0), 50 mM KCl, 0.01% Triton X-100, 1.5 mM $MgCl_2$, 0.2 mM each dNTP (Promega, Madison, Wisconsin), 0.75 U of *Taq* DNA polymerase (Promega, Madison, Wisconsin), and 0.5 µM primers TFR1 and TFR2. Cycling parameters for the amplification were 94 C for 2 min followed by 40 cycles of 94 C for 30 sec, 66 C for 30 sec, and 72 C for 2 min, and a final extension at 72 C for 15 min. For amplification of the 18S small subunit (SSU) rRNA, 1–2.5 µl of DNA was used in a 50-µl reaction containing the same PCR components as mentioned above, substituting primers 16S1 (5′-TACTTGGTTGATCCTGCC-3′) and 16SR (5′-TGATCCTTCTGCAGGTTCACC-3′) (Cepicka et al., 2006). Cycling parameters were the same as the 5.8S/ITS amplification, except for the use of 50 C annealing temperature. Amplification of the α-tubulin (α-tub) gene was performed using a nested PCR protocol with the primary reaction consisting of 5 µl of DNA in a 50-µl reaction using primers α-tubA (5′-RGTNGGNAAYGCNTGYTGGGA-3′) and α-tubB (5′-CCATNCCYTCNCCNACRTACCA-3′) and the secondary reaction consisted of 1 µl of DNA from the primary reaction placed into a 50-µl reaction using primers α-tubF1 (5′-TAYTGYYWNGARCAYGGNAT-3′) and α-tubR1 (5′-ACRAANGCNCGYTTNGMRWACAT-3′) (Edgcomb et al., 2001). The PCR components for the primary and secondary reactions were the same as stated above, and the cycling parameters were the same except for the use of 45 C annealing temperature. For all PCR extractions, a negative water control was included to detect contamination, and water controls were included in both primary and secondary reactions.

PCR amplicons were separated by gel electrophoreses using a 1% agarose gel, stained with ethidium bromide, and visualized with UV light. An approximate 350-base pair (bp) (5.8S and ITS regions), 1,600-bp (18S SSU rRNA), or 1,150-bp (α-tub) amplicon was excised and the DNA purified using a QIAquick® Gel Extraction kit (QIAGEN) per the manufacturer's instructions. Sequencing of the 5.8S and the flanking ITS regions was performed using amplification primers TFR1 and TFR2. Overlapping segments of the SSU rRNA were obtained using the external primers 16S1 and 16SR and the internal primers 514F (5′-GTGCCAGCMGCCGCGG-3′), 1055F (5′-CGGCCATGCACCACC-3′), 1055R (5′-CGGCCATGCACCACC-3′), and 1385R (5′-GATCCTAACATTGTAGC-3′) (Cepicka et al., 2006). Overlapping α-tub gene sequences were obtained using the external primers α-tubF1 and α-tubR1 and internal primers trichtubF1 (5′-CTCMTTCGGTGGTGG-3′) and trichtubR1 (5′-KGGGAAGTGGATACG-3′) (Edgcomb et al., 2001). Amplicons were sequenced at the Integrated Biotechnology Laboratories, The University of Georgia, Athens, Georgia. Sequences obtained from this study and from other trichomonads stored in GenBank were aligned using the multisequence alignment ClustalX program (Thompson et al., 1994). Phylogenetic analyses were conducted using Molecular Evolutionary Genetics Analysis (Center for Evolutionary Functional Genomics, Tempe, Arizona), version 3.1 program (Kumar et al., 1993) using the neighbor-joining and minimum evolution algorithms using the Kimura 2-parameter model and maximum parsimony using a heuristic search. Bootstrap values were constructed using Felsentein's bootstrap test (Felsentein, 1985). The GenBank accession numbers of sequences obtained in this study are listed in Table I.

RESULTS

Isolates were obtained from band-tailed pigeons (*Patagioenas fasciata*), a broad-winged hawk (*Buteo platypterus*), common ground-doves (*Columbina passerina*), Cooper's hawks, Eurasian collared-doves (*Streptopelia decaocto*), mourning doves, rock pigeons, white-winged doves (*Zenaida asiatica*), a house finch (*Carpodacus mexicanus*), and a captive ring-necked dove (*Streptopelia risoria*) from 9 states (Table I). An additional 3 isolates were obtained from ATCC.

Sequence analysis of the isolated disclosed that 1 group of 27 isolates (designated sequence groups A–E in Table I and Fig. 1) shared >98.8% identity to the ITS1, 5.8S, and ITS2 regions of 3 *T. gallinae* sequences (GenBank accessions EF208019, AY349182, and U86614) and a second group of 13 isolates (groups H–L) had >98.4% identity to *T. vaginalis* (GenBank accession AY871048) and ≤92.7% identity to *T. gallinae*. The 2 common ground-dove isolates (groups F and G) had an approximate 95% identity with *T. vaginalis* and an approximate 92% identity with *T. gallinae* and *T. tenax* (GenBank accession U86615), and they contained 15 nucleotides that were unique compared with all other sequences. Phylogenetic alignment of the ITS1, 5.8S, and ITS2 regions performed using 1 sequence per unique sequence group along with related organisms and *Tritrichomonas nonconforma* (as outgroup, AY886845) resulted in a 261-bp alignment, of which 157 were invariant, 62 variable characters were parsimony uninformative, and 42 of the 104 variable characters were parsimony informative. Robust neighbor-joining (bootstrap = 100%), minimum evolution (bootstrap = 100%), and maximum parsimony (bootstrap = 99%) values supported the separation of the 2 clades (Fig. 1).

Isolates belonging to the *T. gallinae* clade were obtained from all sampled states (Arizona, California, Colorado, Florida, Georgia, Kentucky, Pennsylvania, Tennessee, and Texas). Both *T. gallinae* and *T. vaginalis*-like isolates were obtained from Arizona, California, and Texas. Although a host–parasite association was noted for rock pigeons (*T. gallinae*), Eurasian collared-doves (*T. gallinae*), white-winged doves (*T. vaginalis*-like), and common ground-doves (*Trichomonas* sp.), no association was noted for isolates from Cooper's hawks, band-tailed pigeons, or mourning doves (Table I). *Trichomonas vaginalis*-like isolates were only detected in western states (Arizona, California, and Texas).

The 18S rRNA sequence analysis supported the 5.8S/ITS results. The *T. vaginalis*-like isolates showed a 98.5% sequence identity to *T. vaginalis* (accession AY338473) and only 95.2% (white-winged dove, 1,200) and 95.8% (mourning dove, 9) identities to the sequence of 1 isolate (Cooper's hawk, 4) be-

TABLE I. List of *Trichomonas* spp. isolates obtained from free-ranging birds (unless stated otherwise) and included in the molecular analyses.

Isolate/host	State of origin	ITS region sequence group*	GenBank accession no.		
			Internal transcribed regions	18S rRNA	α-Tub
Band-tailed pigeon 1	California	A	EU215369	nd†	nd
Band-tailed pigeon 2	California	K	EU215367	nd	nd
Band-tailed pigeon 3	California	K	EU215367	nd	nd
Band-tailed pigeon 4	California	K	EU215367	nd	EU215380
Broad-winged hawk 1	Florida	B	EU215368	EU215375	nd
Cooper's hawk 1	Arizona	L	EU215366	nd	nd
Cooper's hawk 3	Arizona	L	EU215366	nd	nd
Cooper's hawk 4	Arizona	A	EU215369	EU215372	EU215382
Eurasian collared-dove 829	Texas	D	EU215364	EU215374	EU215381
Eurasian collared-dove 858	Texas	E	EU215363	nd	nd
Eurasian collared-dove 1617	Texas	E	EU215363	nd	nd
Common ground-dove 1	Texas	G	EU215359	EU215371	EU215376
Common ground-dove 1321	Texas	F	EU215358	nd	nd
House finch 1	Kentucky	A	EU215369	nd	EU215382
Mourning dove ATCC 30095‡	Pennsylvania	A	EU215369	nd	nd
Mourning dove 2	Georgia	A	EU215369	nd	nd
Mourning dove 5	Kentucky	A	EU215369	nd	nd
Mourning dove 6	Arizona	A	EU215369	nd	nd
Mourning dove 9	Arizona	L	EU215366	EU215370	EU215377
Mourning dove 11	Georgia	A	EU215369	nd	EU215382
Mourning dove 18	Georgia	A	EU215369	nd	nd
Mourning dove 20	Georgia	A	EU215369	nd	nd
Mourning dove 22	Texas	J	EU215365	nd	EU215379
Ring-necked dove 3§	Tennessee	D	EU215364	nd	nd
Rock pigeon 8	Georgia	A	EU215369	nd	nd
Rock pigeon 11	Georgia	A	EU215369	nd	nd
Rock pigeon 14	Georgia	A	EU215369	nd	nd
Rock pigeon 15	Georgia	A	EU215369	nd	nd
Rock pigeon 20	Georgia	D	EU215364	EU215373	nd
Rock pigeon 22	Georgia	A	EU215369	nd	nd
Rock pigeon 27	Georgia	A	EU215369	nd	nd
Rock pigeon 28	Georgia	D	EU215364	nd	EU215382
Rock pigeon 32	Georgia	A	EU215369	nd	Nd
Rock pigeon 52	Georgia	D	EU215364	nd	nd
Rock pigeon ATCC 30230‡	Unknown	E	EU215363	nd	nd
Rock pigeon ATCC 30228 (SGC)‡	Colorado	C	EU215362	nd	nd
White-winged dove 840	Texas	H	EU215360	nd	nd
White-winged dove 947	Texas	L	EU215366	nd	nd
White-winged dove 1159	Texas	I	EU215361	nd	EU215378
White-winged dove 1200	Texas	L	EU215366	EU215370	nd
White-winged dove 1208	Texas	L	EU215366	nd	nd
White-winged dove 1323	Texas	L	EU215366	nd	nd

* Indicates isolates belonging to same ITS sequence group.
† Sequencing not performed on isolates.
‡ Obtained from ATTC.
§ Captive dove.

longing to the *T. gallinae* clade (no 18S rRNA sequence data from GenBank were available from *T. gallinae* for comparison). The 18S rRNA sequence of the common ground-dove isolates was 96.6% identical to *T. gallinae* and 97.7% identical to *T. vaginalis*. Alignment of the entire 18S rRNA region with related organisms and *Pentatrichomonas hominis* (as outgroup, DQ412643) resulted in an alignment of 1,005 bp, of which 892 were invariant, 62 variable characters were parsimony uninformative, and 47 were parsimony informative. Phylogenetic analysis of 2 *T. vaginalis*-like isolates demonstrated moderate to strong neighbor-joining (bootstrap = 73%), minimum evolution (bootstrap = 77%), and maximum parsimony (bootstrap = 99%) separation support from the sequences of the 4 *T. gallinae* isolates, *T. tenax* (accession D49495), and *T. canistomae* (accession AY247748) (Fig. 2). Although neighbor-joining and minimum evolution gave strong (bootstrap = 99%) support for the separation of the ground-dove isolate from the *T. gallinae* and *T. vaginalis*-like clades, maximum parsimony failed to resolve the ground-dove isolate as a separate clade.

The α-tub sequences of the *T. vaginalis*-like, *T. gallinae*, and common ground-dove isolates had a 98, 95, and 91% identity, respectively, to *T. vaginalis* (accession XM1323317). Align-

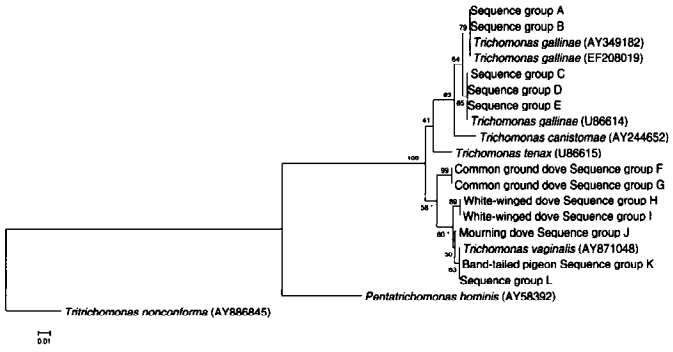

FIG. 1. Phylogenetic analysis of *Trichomonas* spp. isolates (1 isolate per unique sequence group) and other trichomonads based on sequencing of the 5.8S rRNA and flanking ITS regions. The tree was constructed using 261-bp aligned nucleotide positions using a neighbor joining algorithm with 500 replications in a Kimura 2-parameter model with *Tritrichomonas nonconforma* as an outgroup. Unique sequences from *Trichomonas* spp. isolates obtained in this study are designated by letters; see Table I for avian species and geographic location of each sequence group. Bootstrap values for neighbor-joining values are shown at nodes. Minimum evolution and maximum parsimony algorithms were done in addition. Minimum evolution values were highly similar to neighbor-joining. Asterisks indicate nodes in which maximum parsimony phylogenetic trees were not consistent with neighbor-joining and minimum evolution phylogenetic trees.

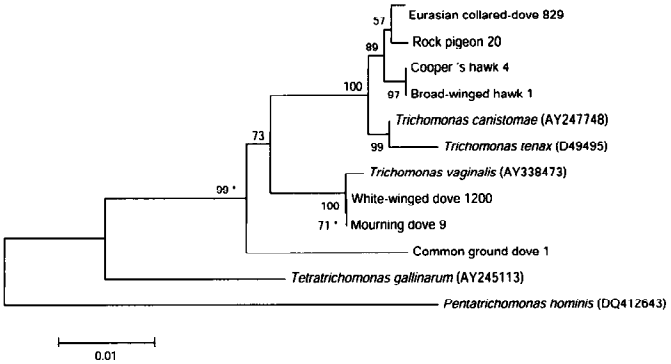

FIG. 2. Phylogenetic analysis of *Trichomonas* spp. isolates from this study and other trichomonads based on sequence alignment of overlapping 1,005-bp 18S rRNA gene. The tree was constructed using a neighbor-joining algorithm with 500 replications in a Kimura 2-parameter model with *Pentatrichomonas hominis* as an outgroup. Bootstrap values for neighbor-joining values are shown at nodes. Minimum evolution and maximum parsimony algorithms were done in addition. Minimum evolution values were highly similar to neighbor-joining. Asterisks indicate nodes in which maximum parsimony phylogenetic trees were not consistent with neighbor-joining and minimum evolution phylogenetic trees.

ment of the partial α-tub gene with related organisms and *Tritrichomonas foetus* (as outgroup, AY277785) resulted in an alignment of 705 bp, of which 543 were invariant, 53 variable characters were parsimony uninformative, and 108 were parsimony informative. Phylogenetic analyses gave strong neighbor-joining (bootstrap = 89%) and minimum evolution (bootstrap = 91%) support for separation of 4 *T. vaginalis*-like isolates from 6 *T. gallinae* isolates (Fig. 3). Maximum parsimony analysis failed to resolve the *T. vaginalis*-like isolates from the *T. gallinae* isolates (Fig. 3). The analysis also failed to resolve the common ground-dove isolate from either *Trichomonas* group or from 2 *Tetratrichomonas* species (Fig. 3).

DISCUSSION

Sequence and phylogenetic analysis of the ITS1, 5.8S, and ITS2 regions of the isolates gave strong support for 2 distinct clades within the *T. gallinae* morphologic complex. One clade was closely related to 3 *T. gallinae* sequences available in GenBank, and the second clade, exclusive of the 2 common ground-dove isolates, was more similar to *T. vaginalis* sequences (Fig. 1).

The isolates were initially analyzed by comparing the ITS and 5.8S rRNA sequences. The ITS regions are noncoding and hence evolve more rapidly. Previous investigations have demonstrated analysis of the ITS regions useful in molecular comparison and distinguishing intraspecific variations of trichomonads (Felleisen, 1997) and other protozoans, including *Perkinsus marinus* (Brown et al., 2004) and *Entamoeba* spp. (Som et al., 2000). To confirm the identification of multiple clades based on the 5.8S-ITS regions, we examined the sequences of 2 coding genes, the α-tub and 18S SSU rRNA genes, from a subset of isolates representing the 2 different clades. The analysis of both of these gene targets supported the separation of the *T. vaginalis*-like and *T. gallinae* isolates into separate clades (Figs. 2, 3). These findings suggest that there are at least 2 species within the *T. gallinae* morphologic complex.

The common ground-dove isolates contained considerable genetic polymorphisms in the 5.8S-ITS regions that were not present in any sequences from either the *T. gallinae* or *T. vaginalis*-like groups. Although the neighbor-joining (bootstrap = 58%) and minimum evolution (bootstrap = 62%) phylogenetic analysis of the 5.8S-ITS regions gave minimal support for the ground-dove isolates to be a subclade of the *T. vaginalis*-like clade (Fig. 1), maximum parsimony analyses failed to resolve the ground-dove isolates into either clade. Analysis of the 18S rRNA and α-tub genes supported the separation of the ground-dove isolates from the *T. gallinae* and *T. vaginalis*-like clades. Although only 2 isolates from common ground-doves were available for characterization, isolates from white-winged doves, Eurasian collared-doves, and a mourning dove collected in the same geographical localities as the ground-dove isolates were genetically distinct from the ground-dove isolates. It would be interesting to molecularly characterize trichomonad isolates from common ground-doves throughout their range to determine whether there is a distinct host–parasite association.

Few nucleotide polymorphisms have been reported during 3 previous studies on the 5.8S-ITS regions of *T. gallinae* (Felleisen, 1997; Kleina et al., 2004; Gaspar da Silva et al., 2007). Our findings of 2 distinct phylogenetic groups and multiple nucleotide polymorphisms are of particular interest given all 24 isolates originating from pink pigeons or turtle doves from Mauritius had nearly identical sequences to the 2 GenBank *T. gallinae* sequences that originated from rock pigeons in the United States (Gaspar da Silva et al., 2007). Because rock pigeons are considered the natural host for *T. gallinae* (Stabler, 1954; Tudor, 1991), intentional and accidental introduction of rock pigeons likely has contributed to the spread of *T. gallinae* worldwide. All rock pigeon isolates in our study belonged to the *T. gallinae* clade; however, a distinct host–parasite associ-

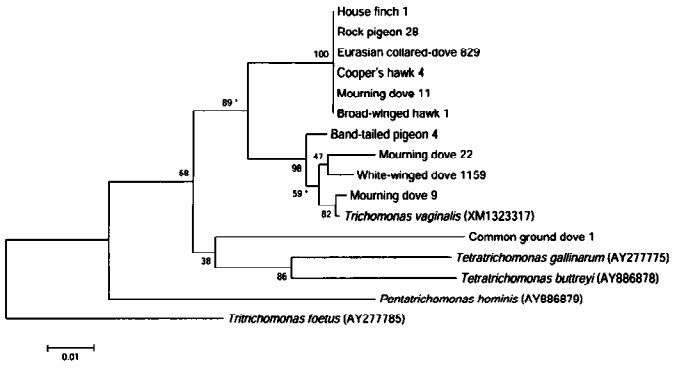

FIG. 3. Phylogenetic analysis of *Trichomonas* spp. isolates from this study and other trichomonads based on sequence alignment of overlapping 705-bp α-tub gene. The tree was constructed using a neighbor-joining algorithm with 500 replications in a Kimura 2-parameter model with *Tritrichomonas foetus* as an outgroup. Bootstrap values for neighbor-joining values are shown at nodes. Minimum evolution and maximum parsimony algorithms were done in addition. Minimum evolution values were highly similar to neighbor-joining. Asterisks indicate nodes in which maximum parsimony phylogenetic trees were not consistent with neighbor-joining and minimum evolution phylogenetic trees.

ation cannot be definitively concluded because, with the exception of 1 Colorado isolate, all rock pigeon isolates originated from Georgia. A more comprehensive examination of rock pigeon isolates from several geographical regions is needed to support a host–parasite association. Isolates from Eurasian collared-doves, an invasive species that is frequently reported in the southern United States and has expanded its range northward in recent years (Romagosa and Labisky, 2000), demonstrated a similar phylogenetic relationship as rock pigeon isolates (Figs. 1–3).

Isolates in the *T. vaginalis*-like clade originated from several avian species and included all isolates from white-winged doves (Fig. 1). Surveys of white-winged doves in Texas disclosed *T. gallinae* prevalences of nearly 100% (Glass et al., 2001), and surveys in Florida demonstrated similar high (97%) prevalences (Conti and Forrester, 1981). However, none of the isolates was genetically characterized. It is plausible, given the high prevalence of *T. gallinae* in white-winged doves and the data from this study, that white-winged doves are infected with a unique trichomonad species. With the exception of the band-tailed pigeon isolates from California, the *T. vaginalis*-like isolates originated from geographical regions associated with known white-winged dove populations. Molecular characterization of white-winged dove and band-tailed pigeon isolates in areas where the 2 species are sympatric and allopatric would be useful to clarify the epizootiology. Additionally, it would be useful to characterize isolates from white-winged doves throughout their entire range to determine whether a host–parasite relationship exists.

In this investigation, mourning dove, band-tailed pigeon, and raptor isolates belonged to both *T. gallinae* and *T. vaginalis*-like clades. Previous surveys of *T. gallinae* in mourning doves and band-tailed pigeons have disclosed prevalences ranging from 1 to 45%, with most reports indicating 5–15% (Stabler, 1951; Stabler and Herman, 1951; Locke and Herman, 1961; Donnelly, 1962; Carpenter et al., 1972; Barrows and Hayes, 1977; Conti and Forester, 1981; Rupiper and Harmon, 1988; Schulz et al., 2005). Conti and Forrester (1981) found that mourning doves sympatric with white-winged doves in Florida had a 17% *T. gallinae* prevalence, whereas in areas of Florida without white-winged doves, the prevalence in mourning doves was only 1%.

Interestingly, our data demonstrated both *T. vaginalis*-like and *T. gallinae* isolates were cultured during recent large trichomoniasis outbreaks involving Cooper's hawks and mourning doves in Arizona (Boal et al., 1998; Hedlund, 1998; Rosenstock et al., 2004) and from band-tailed pigeons in California (J. D. Cann, pers. obs.), indicating at least 2 separate trichomonad species were implicated in both of the outbreaks. If it is found that white-winged doves and rock pigeons are the natural hosts for different trichomonad species, it would support the prevalence data for mourning doves and band-tailed pigeons being infected by direct and indirect (sharing birdfeeders) exposure to white-winged doves and rock pigeons. Future molecular characterization of *Trichomonas* spp. isolates from both clinically affected birds as well as unaffected columbids may be useful in interpreting the epidemiology of trichomoniasis outbreaks.

Assignment of clinical virulence of our trichomonad isolates was not made in this study due to the lack of confirmatory histologic testing and inability to rule out other disease agents causing similar gross lesions. Additionally, clinical virulence must be viewed with caution. Previous investigations disclosed that domestic pigeons previously inoculated with a known avirulent isolate or surviving infection with a virulent isolate had protective immunity when challenged with a known virulent isolate (Stabler, 1948). Additionally, it has been demonstrated that clinically normal pigeons can harbor both avirulent and virulent isolates; however, naïve doves and pigeons challenged with a mixture of virulent and avirulent isolates will succumb to infection (Stabler, 1954). Thus, the actual virulence of the clinically avirulent isolates collected in this study is unknown. Experimental infection of naïve doves would aid in clarifying the actual virulence of the isolates.

To further characterize isolates from both clades and investigate intraspecific variation within each clade, additional molecular techniques, including random amplified polymorphic DNA analysis or amplified restriction fragment polymorphism, would be useful. In addition, cloning and molecular characterization of individual trichomonads from several isolates from both clades could determine whether isolates are composed of identical trichomonad organisms or a mixture of multiple species. Molecular and detailed morphologic analysis of additional samples collected from common ground-doves, white-winged doves, rock pigeons, and Eurasian collared-doves from a greater number of geographic localities would be useful to determine whether morphological differences are apparent in the different morphospecies and whether there are specific host–parasite relationships in the *T. gallinae* morphologic complex.

ACKNOWLEDGMENTS

We thank E. Howerth, A. Allison, D. Mead, W. Davidson, D. Stallknecht, G. Goekjian, P. Luttrell, and M. K. Keel of SCWDS; L. Shender of the Arizona Department of Game; D. Watson and E. Ciuzio of the Kentucky Department of Fish and Wildlife Resources; N. Nicholson, D. Mixon, C. George, D. McGowan, and K. Von Seggern of the Georgia Department of Natural Resources; M. Stromberg of the Hastings Reserve; and G. Gasper of South Carolina Center for Birds of Prey for

guidance, collection of isolates, and laboratory assistance. This investigation was supported primarily by the sponsorship of SCWDS by the fish and wildlife agencies of Alabama, Arkansas, Florida, Georgia, Kentucky, Kansas, Louisiana, Maryland, Mississippi, Missouri, North Carolina, Ohio, Puerto Rico, South Carolina, Tennessee, Virginia, and West Virginia. Support from the states to SCWDS was provided in part by the Federal Aid to Wildlife Restoration Act (50 Stat. 917) and Cooperative Agreement 2001-96130032-CA, Veterinary Services, APHIS, USDA; Cooperative Agreement 01ERAG0013, United States Geological Survey, Biological Resources Division, USDI. Additional support was given by Quail Unlimited's Dove Sportsman Society.

LITERATURE CITED

BARROWS, P. J., AND F. A. HAYES. 1977. Studies on endoparasites of the mourning dove (*Zenaida macroura*) in the southeast United States. Journal of Wildlife Diseases **13:** 24–28.

BOAL, C. W., R. W. MANNAN, AND K. S. HUDELSON. 1998. Trichomoniasis in Cooper's hawks from Arizona. Journal of Wildlife Diseases **34:** 590–593.

BROWN, G., K. L HUDSON, AND K. S. REECE. 2004. Genetic variation at the ITS and ATAN loci among and within cultured isolates of *Perkinsus marinus*. Journal of Eukaryotic Microbiology **52:** 258–270.

BUNBURY, N., D. BELL, C. JONES, A. GREENWOOD, AND P. HUNTER. 2005. Comparison of the InPouch® TF culture system and wet-mount microscopy for diagnosis of *Trichomonas gallinae* infections in the pink pigeon *Columba mayeri*. Journal of Clinical Microbiology **43:** 1005–1006.

———, C. JONES, A. GREENWOOD, AND D. BELL. 2008. Epidemiology and conservation implications of *Trichomonas gallinae* in the endangered Mauritian pink pigeon. Biological Conservation **141:** 153–161.

CARPENTER, J. W., J. C. LEWIS, AND J. A. MORRISON. 1972. *Trichomonas gallinae* (Rivolta, 1878) Stabler, 1938, in mourning doves, *Zenaida macroura*, in northwest Oklahoma. Proceedings of the Oklahoma Academy of Sciences **52:** 39–40.

CEPICKA, I., K. KUTISOVA, J. TANCHEZY, J. KULDA, AND J. FLEGR. 2005. Cryptic species within the *Tetratrichomonas gallinarum* species complex revealed by molecular polymorphism. Veterinary Parasitology **128:** 11–21.

———, V. HAMPL, J. KULDA, AND J. FLEGR. 2006. New evolutionary lineages, unexpected diversity, and host specificity in the parabasalid genus *Tetratrichomonas*. Molecular Phylogenetics and Evolution **39:** 542–551.

CONTI, J. A., AND D. J. FORRESTER. 1981. Interrelationships of parasites of white-winged doves and mourning doves in Florida. Journal of Wildlife Diseases **17:** 529–535.

DIAMOND, L. S. 1983. Lumen dwelling protozoa: *Entamoeba*, trichomonads and *Giardia*. *In* In-vitro cultivation of protozoan parasites, J. B. Jensen (ed.). CRC Press, Boca Raton, Florida, p. 65–111.

DOLTON, D. D., AND R. D. RAU. 2003. Mourning dove population status, 2003. U.S. Fish and Wildlife Service, Laurel, Maryland, 27 p.

DONNELLY, J. A. 1962. Trichomoniasis in the mourning doves. West Virginia Conservationist **26:** 13–14.

EDGCOMB, V. P., A. J. ROGER, A. J. B. SIMPSON, D. T. KYSELA, AND M. L SOGIN. 2001. Evolutionary relationships among "jakobid" flagellates as indicated by alpha- and beta-tubulin phylogenies. Molecular Biology and Evolution **18:** 514–522.

ESTES, W., AND W. R. MANNAN. 2003. Feeding behavior of Cooper's hawks at urban and rural nests in southeastern Arizona. Condor **105:** 107–116.

FELLEISEN, R. S. J. 1997. Comparative sequence analysis of 5.8S rRNA genes and internal transcribed spacer (ITS) regions of trichomonadid Protozoa. Parasitology **115:** 111–119.

FELSENSTEIN, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. Evolution **39:** 783–791.

FORRESTER, D. J., AND M. G. SPALDING. 2003. Parasites and diseases of wild birds in Florida. University Press of Florida, Gainesville, Florida, 1,132 p.

GASPAR DA SILVA, D., E. BARTON, N. BUNBURY, P. LUNNESS, D. BELL, AND K. TYLER. 2007. Molecular identity and heterogeneity of trichomonad parasites in a closed avian population. Infection, Genetics, and Evolution **7:** 433–440.

GERHOLD, R. W., C. M. TATE, S. E. GIBBS, D. G. MEAD, A. B. ALLISON, AND J. R. FISCHER. 2007. Necropsy findings and Arbovirus surveillance in mourning doves (*Zenaida macroura*) from the southeastern United States. Journal of Wildlife Diseases **43:** 129–135.

GLASS, J. W., A. M. FEDYNICH, M. F. SMALL, AND S. J. BENN. 2001. *Trichomonas gallinae* in an expanding population of white-winged doves from Texas. Southwestern Naturalist **46:** 234–237.

HAUGEN, A. O., AND J. E. KEELER. 1952. Mortality of mourning doves from trichomoniasis in Alabama during 1951. Transaction of North America Wildlife Conference **17:** 141–151.

HEDLUND, C. A. 1998. *Trichomonas gallinae* in avian populations in urban Arizona. M.S. Thesis, University of Arizona, Tucson, Arizona, 84 p.

HONIGBERG, B. M. 1979. Biological and physiological factors affecting pathogenicity of trichomonads. *In* Biochemistry and physiology of protozoa, M. Levandowsky and S. H. Hunter (eds.). Academic Press, New York, New York, p. 409–427.

———, M. C. LIVINGSTON, AND R. M. STABLER. 1971. Pathogenicity transformation of *Trichomonas gallinae* I. Effects on homogenates and of mixtures of DNA and RNA from a virulent strain on pathogenicity of an avirulent strain. Journal of Parasitology **57:** 929–938.

KLEINA, P., J. BETTIM-BANDINELLI, S. BONATTO, M. BENCHIMOL, AND M. BOGO. 2004. Molecular phylogeny of Trichomonadidae family inferred from ITS-1 5.8S rRNA and ITS-2 sequences. International Journal for Parasitology **34:** 963–970.

KUMAR, S., K. TAMURA, AND N. MASATOSHI. 1993. MEGA: Molecular evolutionary genetics analysis, version 1.01. The Pennsylvania State University, University Park, Pennsylvania.

LOCKE, L. N., AND C. M. HERMAN. 1981. Trichomonad infection in mourning doves (*Zenaida macroura*), in Maryland. Chesapeake Science **2:** 45–48.

PENNYCOTT, T., B. LAWSON, A. CUNNINGHAM, V. SIMPSON, AND J. CHANTREY. 2005. Necrotic ingluvitis in wild finches. Veterinary Record **157:** 360.

REAL, J., S. MANOSA, AND E. MUNOZ. 2000. Trichomoniasis in a Bonelli's eagle population in Spain. Journal of Wildlife Diseases **36:** 64–70.

ROMAGOSA, C. M., AND R. F. LABISKY. 2000. Establishment and dispersal of the Eurasian collared-dove in Florida. Journal of Field Ornithology **71:** 159–166.

ROSENSTOCK, S. S., M. J. RABE, C. S. O'BRIEN, AND R. B. WADDELL. 2004. Studies of wildlife water developments in southwestern Arizona: Wildlife use, water quality, wildlife diseases, wildlife mortalities, and influences on native pollinators. Arizona Game and Fish Department, Research Branch Technical Guidance Bulletin No. 8, Phoenix, Arizona, 15 p.

RUPIPER, D. J., AND W. M. HARMON. 1988. Prevalence of *Trichomonas gallinae* in central California mourning doves. California Fish and Game **74:** 239–240.

SCHULZ, J. H., A. J. BERMUDEZ, AND J. J. MILLSPAUGH. 2005. Monitoring presence and annual variation of trichomoniasis in mourning doves. Avian Diseases **49:** 387–389.

SOM, I., A. AZAM, A. BHATTACHARYA, AND S. BHATTACHARYA. 2000. Inter- and intra-strain variation in the 5.8S ribosomal RNA and internal transcribed spacer sequences of *Entomoeba histolytica* and comparison with *Entomoeba dispar*, *Entomoeba moshkovskii*, and *Entomoeba invadens*. International Journal for Parasitology **30:** 723–728.

STABLER, R. M. 1948. Protection in pigeons against virulent *Trichomonas gallinae* acquired by infection with milder strains. Journal of Parasitology **34:** 150–153.

———. 1951. A survey of Colorado band-tailed pigeons, mourning doves, and wild common pigeons for *Trichomonas gallinae*. Journal of Parasitology **37:** 471–472.

———. 1954. *Trichomonas gallinae*: A review. Experimental Parasitology **3:** 368–402.

———, AND C. M. HERMAN. 1951. Upper digestive tract trichomoniasis in mourning doves and other birds. Transactions of the North American Wildlife Conference **16:** 145–163

———, AND J. T. KIHARA. 1954. Infection and death of the pigeon resulting from the oral implantation of single individuals of *Trichomonas gallinae*. Journal of Parasitology **40:** 706.

THOMPSON, J. D., D. G. HIGGINS, AND T. J. GIBSON. 1994. Clustal-W: Improving and sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research **22:** 4673–4680.

TUDOR, D. C. 1991. Pigeon health and disease. Iowa State University Press, Ames, Iowa, 244 p.

VILLANUA, D., U. HOFLE, L. PEREZ-RODRIGUEZ, AND C. GORTAZAR. 2006. *Trichomonas gallinae* in wintering common wood pigeons (*Columba palumbus*) in Spain. Ibis **148:** 641–648.

WORK, T. M., AND J. HALE. 1996. Causes of owl mortality in Hawaii, 1992 to 1994. Journal of Wildlife Disease **32:** 266–273.