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

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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. galliniae morphologic complex. Based on ITS sequences, one group demonstrated high nucleotide identity to the 3 T. galliniae sequences available in GenBank, whereas the second group was more closely related to T. vaginalis (98%) than to T. galliniae (92%). Two common ground-dove (Columba passerina) isolates shared a 95% identity with T. vaginalis and a 92% identity with T. galliniae 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. galliniae isolates were found in all sampled states. Both T. vaginalis-like and T. galliniae isolates were isolated in trichomoniasis outbreaks in California and Arizona.

Avian trichomoniasis, caused by the protozoan Trichomonas galliniae, 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. galliniae. 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; Villanau 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 (Hau-gen and Keefer, 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; Dunbar et al., 2008). In Tucson, Arizona, approximately 85% of Cooper’s bird nests tested positive for T. galliniae, and trichomoniasis was the leading cause of death in nestlings and fledglings (Boal et al., 1998; Estes and Mannan, 2003).

Infectivity studies with T. galliniae 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 × 10⁶ parasites (Stabler and Kihara, 1954; Honigberg, 1979). Previous investigations with clinically virulent and avirulent isolates of T. galliniae 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 Tetraurachromonas 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. galliniae 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. galliniae 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. galliniae isolates from several avian species obtained from a wide ranging geographic distribution in the United States to determine whether T. galliniae isolates differed genetically depending on host species or geographical location.
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 1Y kit has been shown to be as sensitive as trypanoline/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, 1980). 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 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'-TGGCTTCAGTTACGGCGCTTTCC-3') and TFR2 (5'CG GTAGTGTCACGTCGGTTGG-3') (Fellaisis, 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, 0.1% Triton X-100, 1.5 mM MgCl₂, 0.2 mM each dNTP (Promega, Madison, Wisconsin), 0.75 U of Taq DNA polymerase (Promega, Madison, Wisconsin), and 0.3 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 16S (5'-TACTTGGTTGATCCTGCC-3') and 16Sr (5'-TAYTGYYWNGARCAYGGNAT-3') for the primary reaction consisting of 5 µl DNA in a reaction using primers α-tubFl (5'-RGTNGGNTRRTNGRTAC-3') and α-tubBr (5'-KGCGGGCTGGTGATACG-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 Felsenstein's bootstrap test (Felsenstein, 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 (Columba 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–I 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 II–L) had ≥98.4% identity to T. vaginalis (GenBank accession AY871048) and ≤92.7% identity to T. gallica. 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. gallica 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 Trichomonas nonconform (as outgroup, AY886485) 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. gallica), Eurasian collared-doves (T. gallica), 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.

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<th>Isolate/host</th>
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* Indicates isolates belonging to the same ITS sequence group.
† Sequencing not performed on isolates.
‡ Obtained from ATCC.
§ Captive dove.

Isolating to the *T. gallinæ* clade (no 18S rRNA sequence data from GenBank were available from *T. gallinæ* for comparison). The 18S rRNA sequence of the common ground-dove isolates was identical to *T. gallinæ* 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 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 from the sequences of the 4 *T. gallinæ* isolates, *T. tenax* (accession D49495), and *T. canis* (accession AY247748) (Fig. 2). Although neighbor-joining and minimum evolution gave strong support (bootstrap = 99%) support for the separation of the ground-dove isolate from the *T. gallinæ* 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. gallinæ*, and common ground-dove isolates had a 98, 95, and 91% identity, respectively, to *T. vaginalis* (accession XM1323317).
ment of the partial α-tub gene with related organisms and *Trichomonas 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 *Tetrastrichomonas* 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 (*Fellesen*, 1997) and other protozoans, including *Perkinus 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 these 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* (*Fellesen*, 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 assoc-
atation 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 (Romagnosa and Ihasky, 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 (87%) prevalences (Conti and Forrestor, 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; Burrows and Hayes, 1977; Conti and Forrestor, 1981; Rupiper and Harmon, 1988; Schulz et al., 2005). Conti and Forrestor (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. Caan, 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, naive 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.

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