Molecular Ecology (2001) **10**, 2319–2327

Host specificity and incidence of *Trypanosoma* in some African rainforest birds: a molecular approach

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Abstract

Studies of host–parasite interactions in birds have contributed greatly to our understanding of the evolution and ecology of disease. Here we employ molecular techniques to determine the incidence and study the host-specificity of parasitic trypanosomes in the African avifauna. We developed a polymerase chain reaction (PCR)-based diagnostic test that amplified the small subunit ribosomal RNA gene (SSU rRNA) of *Trypanosoma* from avian blood samples. This nested PCR assay complements and corroborates information obtained by the traditional method of blood smear analysis. The test was used to describe the incidence of trypanosomes in 479 host individuals representing 71 rainforest bird species from Cameroon, the Ivory Coast and Equatorial Guinea. Forty-two (59%) of these potential host species harboured trypanosomes and 189 individuals (35%) were infected. To examine host and geographical specificity, we examined the morphology and sequenced a portion of the SSU rRNA gene from representative trypanosomes drawn from different hosts and collecting locations. In traditional blood smear analyses we identified two trypanosome morphospecies, *T. avium* and *T. everetti*. Our molecular and morphological results were congruent in that these two morphospecies had highly divergent SSU rRNA sequences, but the molecular assay also identified cryptic variation in *T. avium*, in which we found seven closely allied haplotypes. The pattern of sequence diversity within *T. avium* provides evidence for widespread trypanosome mixing across avian host taxa and across geographical locations. For example, *T. avium* lineages with identical haplotypes infected birds from different families, whereas single host species were infected by *T. avium* lineages with different haplotypes. Furthermore, some conspecific hosts from geographically distant sampling locations were infected with the same trypanosome lineage, but other individuals from those locations harboured different trypanosome lineages. This apparent lack of host or geographical specificity may have important consequences for the evolutionary and ecological interactions between parasitic trypanosomes and their avian hosts.

Keywords: birds, disease prevalence, host–parasite interactions, host specificity, PCR detection method, *Trypanosoma*

Received 7 January 2001; revision received 19 April 2001; accepted 19 April 2001

Introduction

Protozoa of the genus *Trypanosoma* infect populations of birds world-wide (Apanius 1991). In humans, *Trypanosoma cruzi* causes Chagas' disease, which can cause serious heart dysfunction, and *Trypanosoma brucei* causes African sleeping sickness which affects about 300 000 persons each year (WHO report 1998). Trypanosomiasis is not as well studied in birds, but the parasite does affect the growth and fitness of highly infected individuals (for review see Joshua 1983; Molyneux *et al*. 1983; Apanius 1991). Studies of host–parasite interactions in natural bird populations have most often focused on the haematozoa *Plasmodium* and *Haemoproteus* (e.g. Atkinson & van Riper 1991; Bennett *et al*. 1993), but greater attention to *Trypanosoma* seems

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warranted given this parasite's broad host range, wide geographical distribution and documented effects on fitness.

At least 96 trypanosome species that infect birds have been described (Bennett *et al*. 1982). However, the legitimacy of many of these species is in doubt since they were described based on the assumption that each trypanosome species is host-specific (Bennett *et al*. 1994). Experimental evidence has shown that one avian trypanosome can be transmitted to a variety of host bird species (Bennett 1961; Woo & Bartlett 1982), thus refuting the host-species specificity hypothesis. In addition, based on morphological criteria, Bennett *et al*. (1994) found that many bird species from different families in natural sub-Saharan populations are infected with trypanosomes that are morphologically indistinguishable. However, due to the highly pleiomorphic nature of these protozoa, an accurate morphology-based classification of *Trypanosoma* at the species level has proven elusive (Bennett *et al*. 1994). In addition, *T. avium* is designated as one species although it is found in Africa, North America and Europe, and differs substantially in pertinent morphological criteria (Kirkpatrick *et al*. 1986; Bennett *et al*. 1994). In this study, we employ molecular biology techniques to help clarify the pattern of host-specificity in avian trypanosomes.

The most common vectors for the transmission of trypanosomes in birds are louseflies of the family Hippoboscidae (Olsen 1974). In addition, culicine mosquitoes and dermanyssid mites have been identified as avian trypanosome vectors (Molyneux 1977). In humans and other mammals, the prevalence of trypanosomes is directly proportional to the density of the vector population, and has been shown to vary with seasonal rainfall and temperature (e.g. Ahmed & Dairri 1987; Rogers 1988; Rogers & Randolph 1991; Rogers & Williams 1993; Woolhouse *et al*. 1993; Rogers *et al*. 1994). The epizootiology of avian trypanosomiasis has been less well documented (Molyneux 1977; Apanius 1991). In a study on birds at a single site in Cameroon, about 2% of the birds studied were infected with trypanosomes (Kirkpatrick & Smith 1988). In North America, 3.9% of birds were infected (Greiner *et al*. 1975), but another study showed that 28% of breeding redstarts (*Phoenicurus phoenicurus*) were infected with trypanosomes in Finland (Rintamäki *et al*. 1999). However, since trypanosomes are generally rare in peripheral blood but are typically much more abundant in bone marrow, the method of determining the prevalence of trypanosomes can strongly influence the results of prevalence assays (Apanius 1991). Nonetheless, data on the prevalence of trypanosomes in birds have been applied to tests of the Hamilton–Zuk hypothesis of parasite-driven sexual selection (Potti & Merino 1996), the hypothesis of a development rate/immune function trade-off in birds with different developmental periods (Ricklefs 1992), and the hypothesis of a relationship between maternal effort and pathogen prevalence (Merino *et al*. 1996). More detailed information on trypanosome prevalence and host-specificity will help to address these and other questions about avian host–parasite relationships and their ecological and evolutionary effects.

In order to detect the presence of trypanosomes in birds, we employed the highly sensitive polymerase chain reaction (PCR). PCR has been used to detect trypanosomes and other parasites in a wide variety of mammalian species (for examples see Centurion-Lara *et al*. 1994; Britto *et al*. 1995; Katakura *et al*. 1997; Noyes *et al*. 1999). Since avian erythrocytes are nucleated, nonspecific amplification is common when using PCR to detect pathogens in avian blood (Perkins *et al*. 1998). Thus, we developed a nested PCR protocol that uses two rounds of DNA amplification in order to optimize sensitivity to the target trypanosome locus. We developed the protocol using ribosomal genes, since they are a widely employed marker for trypanosomal molecular systematics, their gene products are essential, and they have both conserved and variable sequences (Feldman *et al*. 1995; Stevens & Gibson 1999). We compare the PCR method with the standard but more laborious method of blood smear analysis by microscopy.

The objectives of this study were: (i) to develop a trypanosome-specific nested PCR parasite assay for use on avian blood samples; (ii) to compare traditional blood smear scanning with the PCR method for the detection of trypanosomes; (iii) to use the two detection methods to assess the prevalence of avian trypanosomes at sampling localities in Cameroon, the Ivory Coast and Equatorial Guinea; and (iv) to conduct a preliminary survey of sequence diversity in the amplified PCR product to assess the relationships between parasite lineages, host lineages and geographical locations.

Materials and methods

Blood and extraction

The blood samples used in this study were collected opportunistically as part of an ongoing study of avian evolution in Central Africa (Smith *et al*. 1997, 2000). All birds are placed into families based on the taxonomy of Sibley & Monroe (1990). Samples were collected over a period of 10 years (between 1990 and 2000) from various habitats, and over several seasons, in Cameroon, Equatorial Guinea and the Ivory Coast. The dates and locations for each sample are available upon request. Birds were captured in mist nets and then released after a small amount of blood (~50 μL) had been taken via brachial venepuncture (see Smith *et al*. 1997). Blood smears were made on site and air-dried (Kirkpatrick & Smith 1988). Blood samples for molecular assays were collected in

lysis buffer (10 mM Tris-HCl pH 8.0, 100 mM ethylenediaminetetraacetic acid, 2% sodium dodecyl sulphate). To obtain total DNA, the blood was either extracted following a DNeasy kit protocol (Qiagen), or with phenol–chloroform followed by ethanol precipitation (Kocher *et al*. 1989).

PCR

To amplify a trypanosome-specific DNA product, we used primers described by Maslov *et al*. (1996). Two rounds of PCR were employed in a nested protocol to maximize sensitivity. In the first round of PCR, we used the primers S-762 (GACTTTTGCTTCCTCTA(A/T)TG) and S-763 (CATATGCTTGTTTCAAGGAC), which anneal to the conserved 5′- and 3′-end regions of the small subunit ribosomal RNA (SSU rRNA) gene, in the following protocol: initial denaturation at 95 °C for 5 min followed by five cycles at 95 °C for 1 min, 45 °C for 30 s, 65 °C for 1 min, and 35 cycles at 95 °C for 1 min, 50 °C for 30 s, 72 °C for 1 min and a final extension at 65 °C for 10 min. This initial round amplified a 2-kb fragment. For the second round of PCR, 1 μL of the previous reaction was added to 24 μL of a PCR reaction and amplified using the primers S-755 (CTACGAACCCTTTAACAGCA) and S-823 (CGAA(T/C)AACTGC(C/T)CTATCAGC) at 96 °C for 3 min, followed by 35 cycles at 96 °C for 30 s, 58 °C for 1 min, 72 °C for 30 s, and a final extension at 72 °C for 7 min. Each 25 μL reaction mixture contained 10 mM Tris-HCl pH 8.3, 50 mM KCl, 4.0 mM $MgCl_2$, 0.001% gelatine, 200 μM of each dNTP, each primer at 1.25 μM, and 1 unit Amplitaq (Cetus-Perkin-Elmer). This second round of amplification targeted a 326-bp fragment that was subsequently run on 2% agarose gels that were stained with ethidium bromide for UV-light visualization. Samples were scored positive for trypanosomes when bands of the appropriate size (326 bp) were detected. Representative birds known to be infected with parasites unrelated to *Trypanosoma* (*Plasmodium*, *Leucocytozoon* and nematode microfilaria) showed no amplification. In all the PCR tests, two types of positive controls were always included; the first, purified *Trypanosoma brucei* DNA, and the second, the DNA of several birds that were known to be infected with trypanosomes as determined by microscopy.

Sequencing and analysis

PCR products were purified using a Qiagen® kit following the manufacturer's instructions. Bi-directional sequencing of the 326-bp PCR fragments was performed using the primers S-755 and S-823 in an ABI Prism 377 automated sequencer (Applied Biosystems, Inc.). We used double-stranded cycle sequencing with dye–terminator fluorescent labelling, and electrophoresed sequenced products through a 5% Long Ranger gel. Sequences are deposited in GenBank™ with the accession numbers AF361423–AF361430. Published sequences for the rRNA genes of trypanosomes were obtained from GenBank™ using the following accession numbers: U39578 (*T. avium*), U22316 (*T. vivax*), U22319 (*T. congolense*), AJ012413 (*T. minasense*), AJ009168 (*T.* H25 isolate), AJ005278 (*T. grayi*), AJ223566 (*T. lewisi*), AJ223568 (*T. musculi*), AJ012417 (*T. rangeli*), M31432 (*T. cruzi*), AJ009152 (*T. dionisii*), AJ009166 (*T. vespertilionis*), M12676 (*T. brucei*), AJ009158 (*T. microti*), AJ131958 (*T. cyclops*), AJ009164 (*T. theileri*), AJ012411 (*T. conorhini*), AJ012412 (*T. leeuwenhoeki*), AJ223562 (*T. bennetti*) and X07773 (*Leishmania donovani*). These sequences were identified using an NCBI BLAST-search. Sequences were aligned using the program SEQUENCHER version 3.0. Further alignment was done by eye, and this final alignment is archived in the EMBL alignment database (accession number Align_000110). We conducted phylogenetic analyses of the sequences using parsimony, distance, and maximum-likelihood techniques implemented in PAUP 4.0b3a (Swofford 1999). A small number of nucleotide substitutions separated most of the avian trypanosome SSU rRNA haplotypes, however, and this relative lack of nucleotide variation resulted in little well-supported hierarchical structure in our tree-based phylogenetic reconstructions, with six of the eight novel haplotypes obtained for this study forming a polytomy in most reconstructions. Owing to this lack of phylogenetic resolution, we therefore interpret our results primarily by examining the presence or absence of distinct haplotypes across host species and across geographical sampling locations.

Blood smear analysis

Following fixation in methanol, smears were stained with 3% Giemsa for 20 min, and examined using an Olympus BH compound microscope at ×200, ×400 and ×1000 for 20–50 min. Presence and intensity of parasites were recorded. Using the morphological criteria given in Bennett *et al*. (1994), we identified the parasites from 11 individuals, representing four bird species, as *T. avium*. One *T. everetti* isolated from one individual Little Green Bulbul (*Andropadus virens*) was identified based on the description given in Molyneux (1973).

Results

Development of the PCR test

We have designed a nested PCR protocol specifically to detect trypanosomes in avian peripheral blood. A representative diagnostic gel demonstrating this technique is shown in Fig. 1(A), which depicts the results of three independent amplifications of infected avian blood and

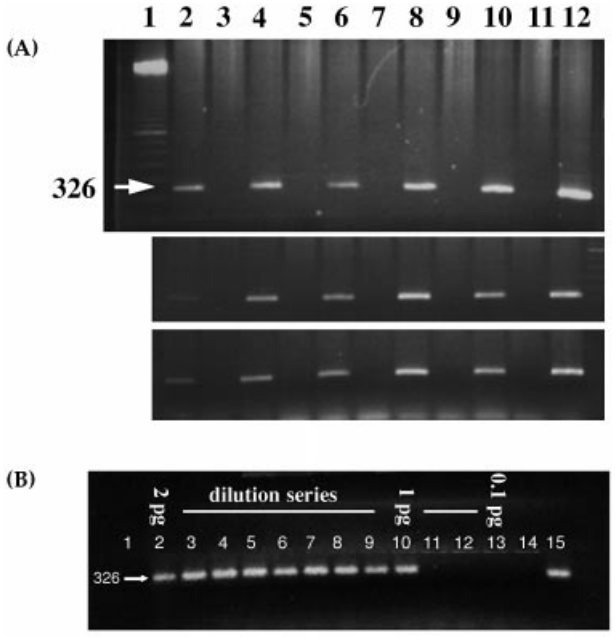

Fig. 1 (A) Diagnostic PCR gel. The gel shows several infected birds (lanes 2, 4, 6, 8, 10 and 12). Noninfected birds are in lanes 3, 5, 7, 9 and 11. Lane 1 is a molecular 100-bp ladder. The figure shows three independent experiments to demonstrate reproducibility of the PCR assay. (B) Results of a serial dilution experiment using purified *Trypanosoma brucei* genomic DNA. Lane 1 is a molecular 100-bp ladder, lane 2 is approximately 2 ng of *T. brucei* DNA, lane 3 = 1 ng, lane 4 = 200 pg, 5 = 100 pg, 6 = 20 pg, 7 is 10 pg, 8 = 5 pg, 9 = 2 pg, 10 = 1 pg, 11 = 0.5 pg, 12 = 0.2 pg, 13 = 0.1 pg, lane 14 is 100 ng of *Plasmodium falciparum* genomic DNA, and lane 15 is 2 ng of *T. brucei* DNA again (a repeat of lane 2).

thereby illustrates the reproducibility of the protocol. Six samples known to be infected based on blood smear analysis, and five negative samples were each tested three times. The gel shows the results of the second round of amplification of DNA; the first round of amplification produced a 2-kb fragment that is not visible by simple agarose gel electrophoresis. The second round of the nested PCR protocol produced the 326-bp DNA fragment consistently present only in the positive samples.

To evaluate the detection limit of the test, PCR was conducted with a dilution series of purified *Trypanosoma brucei* DNA (Fig. 1B). The purified DNA starting concentration was 100 μg/mL. The dilution series tested 1 μL solutions of a 1 : 50 dilution (~2 ng), 1 : 100 (1 ng), 1 : 500 (200 pg), 1 : 1000 (100 pg), 1 : 5000 (20 pg), 1 : 10 000 (10 pg), 1 : 20 000 (5 pg), 1 : 50 000 (2 pg), 1 : 100 000 (1 pg), 1 : 200 000 (0.5 pg), 1 : 500 000 (0.2 pg), and 1 : 1 000 000 (0.1 pg). A strong product was visible in all dilutions up to and including 1 : 100 000 (1 pg). The product is of equal intensity in all cases because it is the primary round of PCR that is limiting. Only at dilutions greater than 1 : 100 000 was product from the first round insufficient to seed the second round. One picogram of DNA represents approximately 9.8×10^8 bp (Cavalier-Smith 1985). The diploid genome of *T. brucei* is about 7×10^7 bp (El-Sayed *et al*. 2000), and that of *T. congolense* is about 4×10^7 bp (Katakura *et al*. 1997). Assuming avian trypanosomes have a similar genome size, the genome weight is between 0.04 and 0.07 pg DNA/organism. Thus at the concentration of 1 : 100 000, we estimate a nested PCR protocol detection threshold of 14–25 organisms.

To further estimate the sensitivity of the PCR assay, we measured the parasitaemia on blood smears, and then extracted DNA from the corresponding blood sample and performed the PCR test. For example, on some slides, two trypanosomes were found in a smear of approximately 20 μL of blood. Thus, when an equivalent amount of the corresponding blood diluted in lysis buffer was extracted, and a PCR product was detected, the sensitivity implies a detection limit of two parasites.

Although the test is very sensitive, in several samples the amounts of trypanosome DNA were near the lower threshold of detection. In an experiment to address reproducibility for these samples with low trypanosome parasitaemias we tested eight samples, each with eight replicated, independent PCR assays (data not shown). The negative control sample (DNA of uninfected blood) was consistently negative, and the positive control (*T. brucei*-purified genomic DNA) produced bright positive bands in all eight replicates. Two additional infected avian blood samples suspected of having moderate parasitaemias also scored positive across all eight replicated assays. The final three blood samples were chosen a priori to represent samples suspected of having low trypanosome parasitaemias. These samples appear to have parasitaemias near the detection threshold of our assay, as each scored positive in approximately half of the replicated assays and negative in the other half. This experiment suggests that our assay will assign false negatives to some samples where trypanosomes are present at very low levels. It also suggests that replicated tests on individual samples will help to detect individuals with parasitaemias near this detection threshold.

Verification of the PCR test

The total number of birds examined by blood smear analysis and the number found positive for trypanosome infection are shown in Table 1. Out of 193 samples analysed by both methods, 43 (22%) were identified positive by blood smear scanning, and 59 (31%) by PCR. Eighty of the 193 samples were tested at least twice using the PCR assay. Of the samples analysed by microscopy, all of the slides that scored positive also scored positive using the nested PCR method. In six instances (3%), however, slides that were scored positive by microscopy were initially scored negative by PCR. In these cases, DNA

Table 1 Prevalence of trypanosomes diagnosed by PCR and blood smear

Test	No. positive	Total	%	No. discrepant
Smear	43	193	22	0 (6*)
PCR	59	193	31	16

$\chi^2 = 6.711$, DF = 1, $P = 0.0096$ ($P < 0.05$).
*Initially six positives were not detected by microscopy, but were found to be positive after reanalysis.

was re-extracted from the blood samples, and upon re-amplification, the samples scored positive. The parasitaemia levels are very low in these samples, and it is probable that some aliquots of blood are void of trypanosomes, and thus not detectable by PCR, as discussed above. Similarly, we suspect that many blood smears that scored negative by microscopy for trypanosomes were actually positive, since the PCR method detected trypanosomes in a higher number of individuals (16 additional positives). In six cases, blood smears initially scored negative by microscopy were re-examined after the PCR test had identified them as positive, and upon re-analysis under the microscope they were confirmed positive. These data suggest that although both methods assign false negatives to some samples, the error rate associated with the PCR assay is probably considerably lower than the error rate associated with traditional blood smear analysis.

Epizootiology of trypanosomes in African birds

We surveyed the incidence of trypanosomes in 71 species of birds from 17 sites in Cameroon, Equatorial Guinea and the Ivory Coast (Table 2). Forty-two (59%) of the bird species harboured trypanosomes. Birds of the family Nectariniidae had relatively high levels of infection. Species in this group included the Olive Sunbird (*Nectarinia olivacea*) (70 of 204 individuals tested were infected; 29%), the Blue-throated Brown Sunbird (*N. cyanolaema*) (nine of nine were infected; 100%), and the Olive-backed Sunbird (*N. verticalis*) (six of nine infected; 67%). The Yellow-throated Leaf-love (*Chlorocichla flavicollis*) (eight of 11 infected; 73%) in the Pycnonotidae, and the White-tailed Ant-thrush (*Neocossyphus poensis*) (all four infected; 100%) in the Muscicapidae also exhibited relatively high levels of infection. Kingfishers of the family Alcedinidae, including the Pigmy Kingfisher (*Ceyx picta*) (three of 28 infected; 11%), had relatively lower levels of infection. The total infection rate was 35%, or 187 of 479 birds.

Host–parasite interactions

To verify that the PCR product in fact was the expected 326-bp fragment of the SSU rRNA gene, we sequenced the amplification products from 17 positive samples representing seven bird species from four families and six collection sites. These included three contiguous rainforest sites, two savanna–rainforest ecotone sites and one montane site (see Smith *et al*. 1997, 2000). Eight SSU rRNA haplotypes were found among the 17 sequences. Of these, six haploytpes (haploytpes I–VI) formed an effective polytomy in all phylogenetic reconstructions, one (haplotype VII) was similar to these six polytomous lineages, and one (haplotype VIII) was highly divergent. Figure 2 graphically illustrates the network of relationships among host species, collection sites and trypanosome haplotypes.

Table 2 Incidence of trypanosomes in 71 bird species

Family	No. species examined	No. indiv. examined	No. infected	%
Alcedinidae	5	36	5	14
Cisticolidae	6	13	7	54
Coliidae	1	1	0	0
Columbidae	2	2	0	0
Corvidae	6	21	4	19
Dacelonidae	1	7	1	14
Fringillidae	1	1	0	0
Indicatoridae	1	3	0	0
Lybiidae	5	9	2	22
Muscicapidae	9	36	15	42
Nectariniidae	5	225	88	39
Passeridae	9	40	10	25
Picathartidae	1	1	1	100
Picidae	1	1	1	100
Pycnonotidae	13	67	27	40
Sylviidae	5	16	6	38
Total	71	479	167	35

Sequences were aligned with published SSU rRNA DNA sequences of *Trypanosoma*. Of the 20 published sequences examined, the sequences of seven of the eight haplotypes we identified most closely resembled the SSU rRNA gene of *T. bennetti*, a trypanosome initially found in the American Kestrel (*Falco sparverius*) in New Jersey, USA. Although none of our samples was identical to the published *T. bennetti* sequence, this sequence grouped into the effective polytomy with haplotypes I–VI. The mean number of base changes between these six haplotypes and the published *T. bennetti* sequence (AJ223562) was 1.83. Between these six samples and the published sequence of *T. avium* (U39578), the mean number of base changes was 10.71. One haplotype (VII in Fig. 2) exhibited considerable divergence from the primary cluster of haplotypes, with a mean number of base changes of 8.83. The sequence of haplotype VIII was highly divergent from the others, with a mean of 23.0 base changes between it and the other seven haplotypes, a greater magnitude of divergence than that between haplotypes I and VII and many *Trypanosoma* species isolated from mammals or reptiles (i.e. *T. cruzi*, *T. brucei*, *T. grayi* and *T. theileri*).

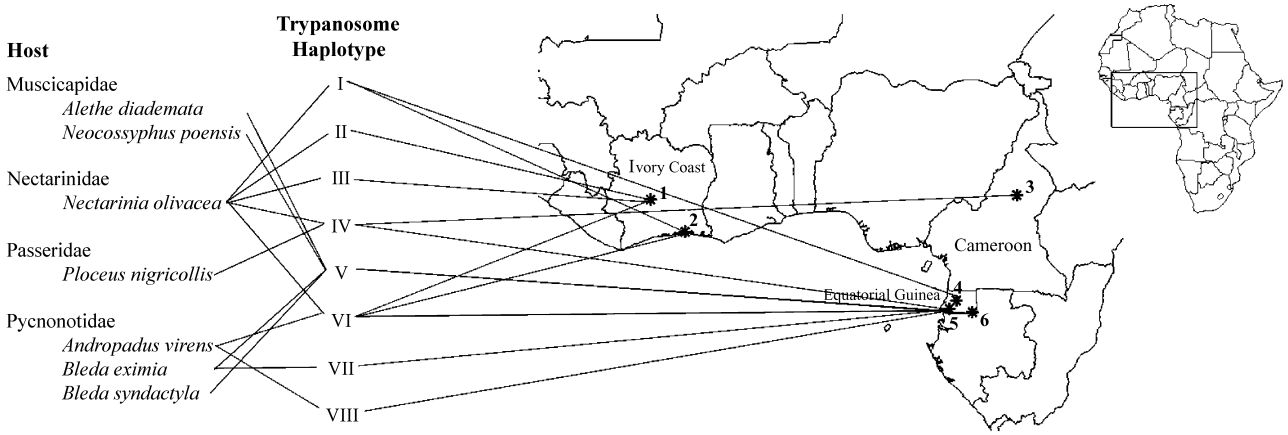

Fig. 2 Schematic representation linking the eight determined SSU rRNA haplotypes with bird species in four families, and six different collection sites. Results show that a single haplotype can be found in locations that are geographically distant, and also two haplotypes can be found in a single location. In addition, a single haplotype can be found in more than one bird family, and also two haplotypes can be found in a single bird species. Haplotypes I–VII were found in the morphospecies *Trypanosoma avium*. Haplotype VIII was found in *T. everetti* and differs considerably from I–VII. Collection sites were as follows: 1, Marahoue, Ivory Coast (ecotone); 2, CSRS, Ivory Coast (rainforest); 3, Wakwa, Cameroon (ecotone); 4, Mount Alen, Equatorial Guinea (montane); 5, Ncoho, Equatorial Guinea (rainforest); 6, Mokula, Equatorial Guinea (rainforest). The number of samples per haplotype was as follows: I (n = 2), II (n = 1), III (n = 1), IV (n = 2), V (n = 5), VI (n = 4), VII (n = 1), VIII (n = 1).

Using traditional blood smear analysis, we were able to locate and determine the morphospecies of 10 of the 17 sequenced samples. The nine samples corresponding to haplotypes I–VII contained trypanosomes that were striated, and exhibited mensural characteristics typical of *T. avium* (Bennett *et al*. 1994). The one sample in which we found haplotype VIII harboured a short stumpy trypanosome that was readily identified as *T. everetti* (Bennett *et al*. 1994). None of the trypanosomes exhibited the small slender phenotype characteristic of *T. bennetti* (Kirkpatrick *et al*. 1986).

The seven haplotypes found among the sequences of the 10 samples identified as *T. avium* (I–VII, in Fig. 2) were found in seven different bird species collected from five different locations. Given our limited sequence data, we found no predictable pattern linking haplotypes with families or collection sites. These results suggest that avian trypanosomes are highly vagile, both in terms of host use and geography.

Discussion

Development of a PCR assay to detect avian trypanosomes

This work details the development of a reliable and sensitive method for the detection of trypanosomes in avian blood samples. Although there have been several PCR-based tests to detect trypanosomes in mammals and in vector organisms (for examples see Centurion-Lara *et al*. 1994; Britto *et al*. 1995; Katakura *et al*. 1997; Morlais *et al*. 1998; Clausen *et al*. 1998; Gomes *et al*. 1998; Kabiri *et al*. 1999; Noyes *et al*. 1999), to our knowledge this is the first PCR-based technique that specifically identifies avian trypanosomes. The PCR protocol is specific to trypanosomes, since we did not detect PCR products from noninfected individuals, nor from blood samples infected with other parasites such as *Plasmodium*, *Haemoproteus*, or nematode microfilaria. In addition, sequence data verified that the PCR products were indeed trypanosomal in origin. Alternative techniques of estimating the sensitivity of this protocol provide differing results, but together suggest that this technique has a higher sensitivity than the traditional and time-consuming method of blood-smear analysis.

Using a combination of this PCR protocol and traditional blood smear analysis, we surveyed the prevalence of trypanosomes in a wide array of African birds. Of 71 avian species, 42 (59%) harboured trypanosomes. Many of these potential host species were represented by a small number of individuals, and we predict that a greater number of species would test positive with an increase in sample size. In addition, samples were collected over several years and seasons, and rainfall and temperature effects could also influence the levels of infection. However the observed patterns are congruent with previous studies that have found trypanosome parasites in most African passerine families (Bennett *et al*. 1994), and that have found trypanosome prevalences as high as 70% in some North American bird populations (Apanius 1991). Nonetheless, the overall prevalence of 35% in our study is relatively high, particularly compared to a previous study on similar African birds that reported only a 2% infection rate (Kirkpatrick & Smith 1988). One possible reason for this discrepancy is

that Kirkpatrick & Smith (1988) scanned slides for approximately 10 min, whereas in the present study, the time was increased to 20–50 min per slide. The detection levels of trypanosomes are generally low on blood smears (Apanius 1991), and can increase significantly when microhaematocrit tubes are used for centrifugation prior to analysis (Bennett 1962). Furthermore, Kirkpatrick & Suthers (1987) found that 40% of birds were found to be positive for trypanosome infection using blood culture methods, while only 6% of the same birds scored positive by blood smear analysis. We suggest here that the nested PCR method is more sensitive than blood smear analysis, and suspect that it may reflect prevalence levels closer to those that would be obtained by blood culture.

In this study, six of the 43 samples scored positive for trypanosomes by microscopy were initially scored negative by PCR, but after re-extracting blood for DNA and re-amplification, they tested positive. In addition, several samples in a reproducibility test tested positive by PCR ~50% of the time. Thus, false negatives do occur using our PCR assay, and we therefore recommend replicated tests on individuals that might exhibit low parasitaemias. Initially 22 samples tested positive by PCR that were negative by blood smear analysis, but in six cases, samples that initially scored negative were re-examined, and found to be positive. Re-analysis of the remaining 16 slides did not detect trypanosomes. We caution that although we believe our PCR protocol to be more sensitive than blood smear analysis, accurate quantification of levels of parasitaemia cannot be obtained with this PCR method due to the nature of the nested-PCR protocol. However, accurate levels of parasitaemia cannot be assessed by blood smear analysis either, due to the disproportionately low levels of trypanosomes in peripheral blood as compared to bone marrow (Apanius 1991). Future studies will determine if the protocol can be used on bone marrow samples, with the intention of comparing prevalences in blood vs. bone marrow.

Studies on host–parasite–geography relationships

We found eight SSU rRNA haplotypes among the 17 samples that were sequenced. These haplotypes were found in seven bird species representing four families. However, no predictable pattern emerged that linked the haplotypes with bird species, or even families. Although we cannot make any conclusive statements about the levels of diversification of African avian trypanosomes, these data do provide the first molecular-based evidence that a single morphospecies of *Trypanosoma avium* can infect several bird species, as we found haplotypes associated with the *T. avium* morphospecies in seven bird species. This pattern is consistent with morphological and biochemical data that assert the same hypothesis of low host-specificity for *T. avium* (Kirkpatrick *et al*. 1986; Bennett *et al*. 1994). Morphological studies have shown that, for example, *T. avium* parasitizes at least 13 passeriform bird species in sub-Saharan Africa (Bennett *et al*. 1994). Our study also provides the first molecular evidence that a single bird species can be infected with more than one species of trypanosome. For example, two individuals of the Little Green Bulbul (*Andropadus virens*) were infected with trypanosomes with highly divergent SSU rRNA sequences (Fig. 2, haplotypes VI and VIII representing *T. avium* and *T. everetti*).

This pattern of low host-specificity of *Trypanosoma* differs substantially from that seen in some other avian parasites. For example, a recent study on the haematozoa parasites *Plasmodium* and *Haemoproteus* found high levels of host specificity, with only one of 17 cytochrome *b* haplotypes found in more than a single host species (Bensch *et al*. 2000). Of the eight haplotypes in this study, three were found in more than one bird species representing different families. The cause of the low host specificity in avian trypanosomes is unknown, but we speculate that it may stem from low host specificity of the vectors. Apparently, perhaps due to the modes of transmission of these parasites, avian trypanosomes have a significantly lower degree of host specificity.

Although through microscopy most of the trypanosomes in our study were identified as *T. avium*, the SSU rRNA haplotypes of these trypanosomes more closely resembled the published sequence of *T. bennetti*, a trypanosome identified in New Jersey from the American Kestrel (Kirkpatrick *et al*. 1986). The published sequence of *T. avium* was obtained from a trypanosome in the Eurasian Rook (*Corvus frugilegus*) from the Czech Republic (Maslov *et al*. 1996). We conclude that the sequence of the SSU rRNA of this European *T. avium* differs substantially from the African forms we sequenced. Furthermore, we found seven distinct haplotypes in the samples infected with the *T. avium* morphospecies. This high level of diversity suggests that this morphospecies harbours considerable genetic diversity, and that morphological criteria may underestimate the taxonomic diversity of this group. Further sequence-based surveys using longer and more variable sequences are likely to increase the number of identifiable *Trypanosoma* lineages and could provide a more robust phylogenetic framework for addressing taxonomic issues.

The blood samples collected for this study were obtained from a variety of habitats including contiguous rainforest, fragmented rainforest sites in the ecotone, the transition zone between the rainforest and grassland (Smith *et al*. 1997) and one montane site (Smith *et al*. 2000). However, we found no concordance between SSU rRNA haplotypes and habitat type. This suggests that a single trypanosome species infects birds in both the Ivory Coast and in mainland Equatorial Guinea at sites separated by many hundreds of kilometres.

The work presented here raises many questions about the interactions of trypanosomes and their avian hosts. One hypothesis about host–parasite interactions asserts that vector specificity may create trypanosome specificity within wild bird communities (Apanius 1991). We have little knowledge about the vectors specific to these bird populations, but do find that the haplotypes do not vary significantly over large distances, and find little host–trypanosome specificity altogether. In concert with sequence-based studies, the PCR assay reported here is likely to prove useful in studies of host–parasite interactions at several levels, including identifying the specific vectors that transmit avian trypanosomes among host individuals and species, assaying levels of trypanosome gene flow among host taxa and geographical regions, and further elucidating the phylogenetic relationships among avian trypanosomes.

Acknowledgements

We are grateful to Dr Irby Lovette for many significant contributions to this work. We are also indebted to Dr Karen Holder, Dr Catherine Graham, Dr Sonya Clegg, Dr Hans Slabbekoorn, Kristin Ruegg, and other members of the Center for Tropical Research for invaluable help on this manuscript. We thank Jeff Roberts in the laboratory of Dr Nina Agabian at UCSF for purified *Trypanosoma brucei*. Dr Sehgal was supported by a 'Research Infrastructure in Minority Institutions' award from the National Center for Research Resources with funding from the Office of Research on Minority Health, National Institutes of Health #5 P20 RR11805. This work was also funded by the grants NSF-DEB9726425 and NSF-IRCEB9977072.

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