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Molecular identity and heterogeneity of trichomonad parasites in a closed avian population

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Abstract

Columbids (pigeons and doves) are the primary host of *Trichomonas gallinae*, the flagellate protozoon which causes avian trichomoniasis, a widespread, often lethal disease. Although predominantly apathogenic, the organism is paradigmatic for the study of strain-specific virulence, with some strains causing greater than 75% mortality and epizootic die-offs in wildlife populations. In recent years, research on this important emerging pathogen has been neglected and genetic variation within the parasite has not hitherto been investigated. The pink pigeon (*Columba mayeri*), endemic to Mauritius and one of the world's rarest pigeons, suffers high levels of nestling/fledgling mortality from trichomoniasis. As a closed oceanic island population with recorded life-history parameters for all birds, this species represents a unique resource for the study of this host–parasite interaction. To investigate genetic variation within *T. gallinae* in Mauritian columbids, isolates were collected from pink pigeons and another widespread species, the Madagascar turtle-dove (*Streptopelia picturata*). Comparison of the 5.8S region of rDNA and surrounding internally transcribed spacer regions (ITS) showed no sequence variation between isolates or with an unrelated but previously sequenced *T. gallinae* isolate (Genbank). This confirmed all 24 isolates as *T. gallinae*, and defined this section of the genome as a good species marker. In contrast, Random Amplified Polymorphic DNA (RAPD) analysis of the isolates revealed considerable genotypic variation between isolates. RAPD genotypes appeared to correlate with geographic distribution and host species, suggesting inter-species transmission and rapid host adaptation by the parasite.

Keywords: *Trichomonas gallinae*; Endangered species; Molecular epidemiology; Molecular evolution; Pink pigeon

1. Introduction

Trichomonas gallinae is the causative agent of avian trichomoniasis, or canker, an often lethal and epizootic disease. Although columbiform birds (pigeons and doves) are considered to be the primary hosts of *T. gallinae*, infection occurs in many other avian orders worldwide, including both wild and domestic birds (Stabler, 1954). Because it is not known whether *T. gallinae* is a single species or a group of species and whether virulent strains are simply the result of shifts in one or two key virulence determinants, or represent entirely different genotypes which could be described as separate species or subspecies; this study set out to assess the degree of genetic diversity between isolates of this pathogen.

Trichomonas gallinae is widespread in columbiforms but is often apathogenic, with strains varying markedly in their virulence, and this has been attributed to phenotypic features such as haemolytic activity. Differential virulence has been demonstrated for other trichomonads, such as *T. vaginalis*, and is strongly associated with genetic strain variability (Vanacova et al., 1997). Virulent strains of *T. gallinae* cause large economic impacts through the loss of avian livestock, and pose problems for wild avian species. For instance, *T. gallinae* has been implicated in recent die-offs of greenfinches *Carduelis chloris* in the UK (Holmes and Duff, 2005; Lawson et al., 2006). Historically it has been speculated that *T. gallinae* may have played a role in the final demise of the extinct passenger

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pigeon, *Ectopistes migratorius* (Stabler, 1954; Hoefle et al., 2004).

The oceanic island of Mauritius has seen a number of avian extinctions since human colonisation, including that of the dodo, *Raphus cuculatus*. The last remaining endemic columbid species, the pink pigeon, *Columba mayeri,* declined to just 15–20 individuals in the 1970s (Durrell, 1984; Jones, 1987). An integrated species and habitat recovery programme has successfully increased the total pink pigeon population size to around 350 birds in 5 subpopulations. As a result of intensive monitoring, all birds have been individually marked with numbered and coloured leg rings and long-term data on life-history parameters, such as reproductive success and survivorship, have been collected (Jones, 2004). In addition to the endemic pink pigeon, other species such as the Madagascar turtle-dove, *Streptopelia picturata,* are now widespread across Mauritius and may be acting as reservoir hosts for *T. gallinae* infection (Bunbury, 2006). The pink pigeon population has fluctuated between 300 and 370 individuals since 2000 and trichomoniasis has been highlighted as a major mortality factor (Swinnerton et al., 2005; Bunbury, 2006).

Strain variation as an explanation of differential virulence for avian trichomonads is well established (BonDurant and Honigberg, 1994) and may contribute to the fluctuating levels of infection, morbidity and mortality observed within pink pigeon subpopulations. Virulence also depends on other factors such as previous exposure to the pathogen (protective immunity) and individual immunocompetence. The latter can be affected by factors such as age, concurrent disease, genetic heterogeneity and food availability (Stabler and Braun, 1975; Swinnerton et al., 2005).

In the present study, we used molecular techniques (amplification and sequencing of the ITS1/5.8S/ITS2 region) to confirm that the parasites found in the crop of the Mauritian pink pigeon and Madagascar turtle-dove are *T. gallinae* and then investigate the genetic variability between these isolates using Random Amplified Polymorphic DNA (RAPD) analysis. We also relate *T. gallinae* genotype to life-history parameters available for those individuals from which the parasite isolates were collected to investigate the genetic heterogeneity of *T. gallinae* in Mauritius.

2. Material and methods

2.1. Parasites

Samples were derived from primary (crop) cultures incubated in InPouch TF culture kits (BioMed Diagnostics, San Jose, Ca, USA) from 24 *Trichomonas*-infected, Mauritian columbids including 19 pink pigeons of known sex, age and subpopulation and 5 Madagascar turtle-doves (for further details on sampling technique see Bunbury et al., 2005).

Each sample consisted of 25–50 μl of primary culture suspended in 1 ml DNAzol® stored at room temperature for no more than 1 month prior to processing. Samples were collected from birds in all five pink pigeon subpopulations; Combo, Bel Ombre, Ile aux Aigrettes, Pigeon Wood and Plaine Lievre (Fig. 1). To determine whether isolates were species-specific, samples were also collected from infected Madagascar turtle-doves, sampled at a sixth location (Petrin) in Mauritius. Details of the life-history of all birds sampled are compiled in Table 1.

2.2. DNA preparation

Genomic DNA was isolated from each DNAzol® homogenised sample using protocol detailed at www.mrcgene.com/dnazol.htm with slight modifications. Samples were centrifuged at 10,000 × *g* for 10 min at room temperature and 500 μl of 100% ethanol was added to the resulting supernatant. Samples were mixed by inversion and left to stand at room temperature for 3 min. Further centrifugation of 10,000 × *g* for 10 min at room temperature, was used to separate out DNA by sedimentation. The DNA pellet was washed twice, using 75% ethanol. Following each addition of ethanol, the pellet was resuspended and sedimented by 10 min centrifugation at 10,000 × *g*. The DNA pellet was air-dried, solubilised in

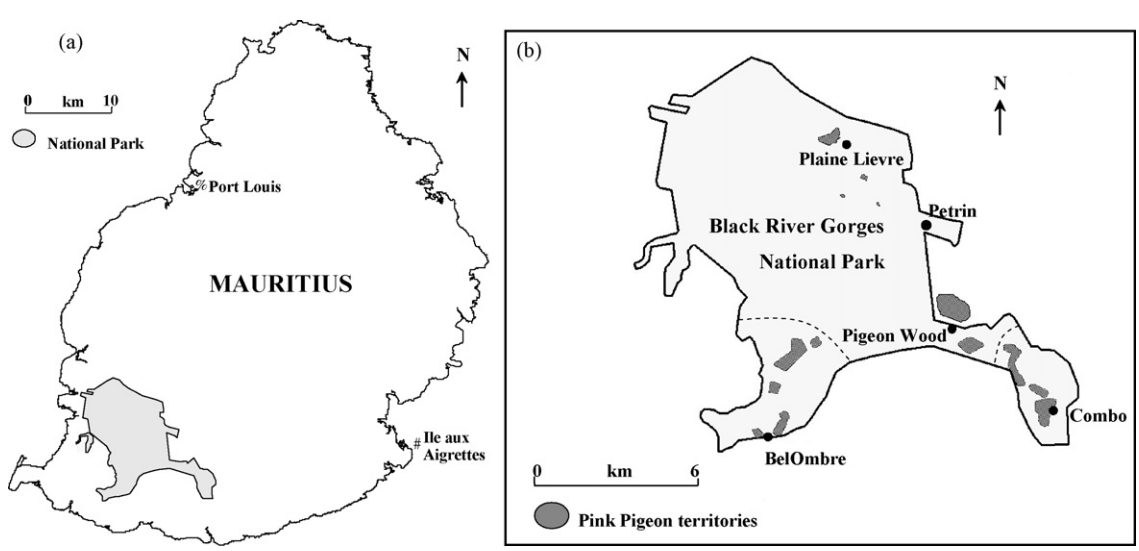


Fig. 1. Map of Mauritius Island, showing the location of the 5 pink pigeon populations. Taken from Bunbury (2006) with consent.

Table 1
Summary of information from life-histories for each columbid

#	ID	Species	Age (years)	Sex	Capture site	Hatch site
1	0729	CM	7	F	BO	Captive
2	0731	CM	7	F	BO	Captive
3	6–84263	CM	1	F	PW	PL
4	6–84255	CM	1	F	PW	PL
5	6–23079	CM	2	M	PL	PL
6	R226	SP	n/a	n/a	PETRIN	n/a
7	R232	SP	n/a	n/a	PETRIN	n/a
8	R239	SP	n/a	n/a	PETRIN	n/a
9	R241	SP	n/a	n/a	PETRIN	n/a
10	R244	SP	n/a	n/a	PETRIN	n/a
11	A157	CM	8	M	PW	PW
12	A202	CM	9	M	BO	BO
13	6–23000	CM	3	M	IAA	IAA
14	6–23176	CM	0	F	IAA	IAA
15	6–23181	CM	0	M	IAA	IAA
16	0732	CM	7	F	BO	Captive
17	6–23024	CM	4	M	COMBO	COMBO
18	6–22891	CM	5	M	COMBO	IAA
19	6–22938	CM	2	F	BO	BO
20	6–22949	CM	2	F	COMBO	COMBO
21	6–23034	CM	3	M	COMBO	COMBO
22	6–23051	CM	4	M	PL	PL
23	6–23107	CM	4	M	BO	PW
24	6–23127	CM	3	M	COMBO	PW

CM: *Columba mayeri*, SP: *Streptopelia picturata*, F: female, M: male, BO: Bel Ombre, PW: Pigeon Wood, IAA: *Ile aux Aigrettes*, PL: Plaine Lievre, n/a: not available data.

100 μl distilled water and stored at 4 °C. DNA concentrations for each isolate were obtained by spectrophotometry, based on absorbance readings at 260 nm.

2.3. PCR amplification of the ITS1/5.8S/ITS2 region

Amplification of the ITS1/5.8S/ITS2 region was adapted from Felleisen (1997). Oligonucleotide primers, TFR1 (TGCTTCAGTTCAGCGGGTCTTCC) and TFR2 (CGGTAGGTGAACCTGCCGTTGG) were obtained from Qiagen (Quigen Ltd., Crawley, UK). PCR reactions were undertaken using Taq DNA polymerase reagent kit (Invitrogen Ltd., Paisley, UK). Fifty microlitres reaction volumes were used as described in the accompanying protocol, with slight modifications: 1× buffer, 0.5 mM $MgCl_2$, 80 pmol dNTP mix (Bioline Ltd., London, UK), 1–4 μl DNA, 1.25 U Taq, 0.05% W-1 detergent and autoclaved distilled water to 50 μl. Following 5 min initial denaturation at 94 °C the following profile was applied: 35 cycles of denaturation for 1 min at 94 °C, annealing for 30 s at 67 °C, extension for 1 min at 72 °C and a final extension step at 72 °C for 5 min. The amplified product was observed under UV light after ethidium bromide staining of a 1.5% agarose gel.

2.4. Sequencing and phylogenetic analysis of the ITS region

Amplified PCR products were purified at room temperature using the QIAquick PCR purification kit (Qiagen Ltd.) Sequencing was carried out using ABI capillary DNA sequencer (John Innes Genome Laboratory, Norwich, UK), applying the same primers (TFR1 and TFR2) used for amplification. Results in both directions were aligned and reverse complemented using lasergene analysis software seqman and editseq (DNAstar Inc, Maddison, WI). Sequences were also manually trimmed using the same software. Chromatograms were manually checked and edited using Chromas 2.3 (www.technelysium.com.au). Sequences were submitted online to Genbank using BankIt (identifier: bankit872251 accession number: EF208019).

Homology between available *T. gallinae* strains at this locus was compared using ClustalW software (available in http://www.ebi.ac.uk/clustalw/#). In addition to the 24 newly determined sequences, 13 ITS-1/5.8S/ITS-2 rRNA nucleotide gene sequences from Trichomomadidae were obtained from NCBI Genbank™ (Table 2).

ClustalW version 1.82 was used to generate a pairwise and then multiple alignment algorithm containing the Mauritius consensus nucleotide sequence and the 13 sample sequences. The neighbor-joining method (Saito and Nei, 1987) was used to generate this alignment and a corresponding phylogenetic tree.

Table 2
List of species and additional GenBank™ information used for comparisons with Mauritian isolates

Species	GenBank™ accession number	Strain	Host	Length oflTS-1/5.8S1TS-2 region (bp)
Trichomonas gallinae	AY349182	G7	Pigeon	300
Trichomonas gallinae	U8B614	TG	Pigeon	369
Trichomonas canistomae	AY244652	FJRIXI	Dog	328
Trichomonas sp.[a]	AJ784785	24 K	Dog	373
Trichomonas tenax	U86615	Hs-4	Human	368
Trichomonas vaginalis	AY349185	JT	Human	297
Trichomonas vaginalis	AY349184	TO63	Human	297
Tetratrichomonas gallinarum	AY244649	1-11-M2	Chicken	328
Tetratrichomonas gallinarum	AY244648	Z26/7	Chicken	328
Trichomonas sp.	AF236105	–	Pekin duck	372
Tritrichomonas foetus	AF466751	NCSU-TCa	Cat	1395
Tritrichomonas foetus	AF4B6749	NCSU Tfs-1	Cat	1395
Tritrichomonas augusta	AY245163	HYLA	Common tree frog	325

[a]Trichomonad species not determined.

2.5. RAPD analysis

Ten nanograms genomic DNA was randomly amplified in 20 μl final reaction volume, containing 1 × PCR buffer, 2.5 mM $MgCl_2$, 0.05U/μl *Taq* from *Taq* DNA polymerase reagent kit (Invitrogen Ltd, Paisley, UK) plus 0.25 μmol primer. The primers used for this analysis, OPD1, OPD2, OPD3, OPD4, OPD5, OPD6, OPD7, OPD8, OPD9, OPD10, were obtained from Operon Technologies Inc. (Alameda, California). PCR was performed in 0.2 ml eppendorf tubes in a thermocycler using the following conditions: 5 min initial denaturation at 94 °C, 40 cycles of denaturation at 94 °C for 1 min, annealing at 36 °C for 1 min, extension at 72 °C for 2 min and a final extension of 15 min at 72 °C. A no template control was used for each reaction. PCR products were separated using 10% polyacrylamide gels, stained with ethidium bromide, visualised under UV light and digitally recorded using a Multi Doc-it imaging system (UVP, Inc.).

Amplification was repeated at least twice for each primer, in order to confirm the obtained pattern.

2.6. Heterogeneity analyses

The presence or absence of each band was visually scored (single investigator blinded) and a taxon/character matrix was constructed. The program FreeTree was used for all computations (Pavlicek et al., 1999). The data was analysed using the Dice similarity coefficient (Dice, 1945), based on band-sharing between all possible pairs in an analysis group, and a matrix of similarity was constructed. The similarity data was then used for unweighted pair group method analysis (UPGMA) (Sneath and Sokal, 1962), and bootstrap values (for 1000 replicates) were computed. The phenon line marked on the UPGMA indicates the point of reference for dividing the organisms into separate groups.

3. Results

3.1. ITS1/5.8S/ITS2 amplification and sequencing

Amplification of all 24 isolates produced fragments of 216 base pairs, comprising all of the 5.8S region (158bp) and partial ITS1 (20bp) and ITS2 (38bp) regions. Sequencing of these fragments revealed that all sequences were identical within the region amplified in this study. The consensus sequence (EF208019) was compared to previously known trichomonad ITS sequences. Within the Genbank™ database, sequences corresponding to this region of trichomonad DNA are meagre, with only two *T. gallinae* sequences available. Alignments of these and the Mauritian consensus sequence, revealed the

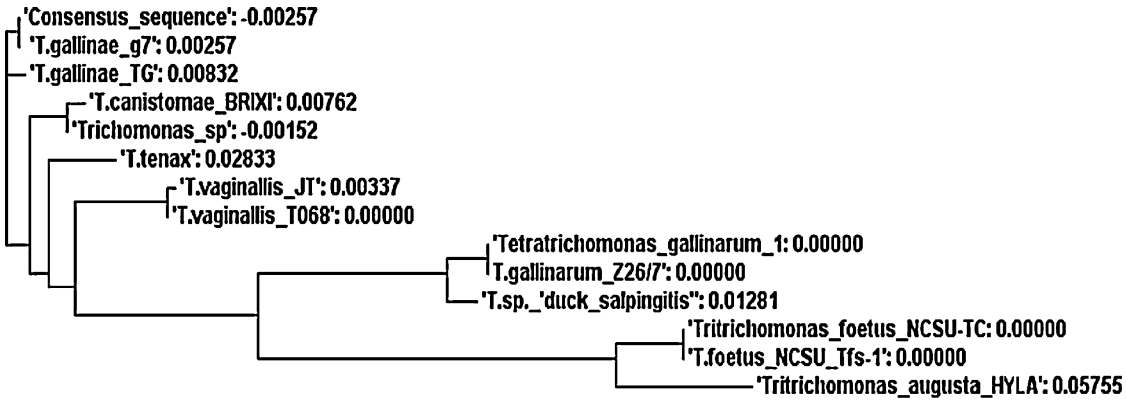


Fig. 2. Phylogenetic tree determining genetic relationship and evolutionary distance between related Trichomonad species. Mauritian *T. gallinae* DNA corresponding to partial ITS-1/5.8S/partial ITS-2 sequence was analysed against other members of the Trichomonad order. Data for tree construction obtained from http://www.ncbi.nlm.nih.gov/. The length of each branch is proportional to the amount of evolutionary distance between the different species.

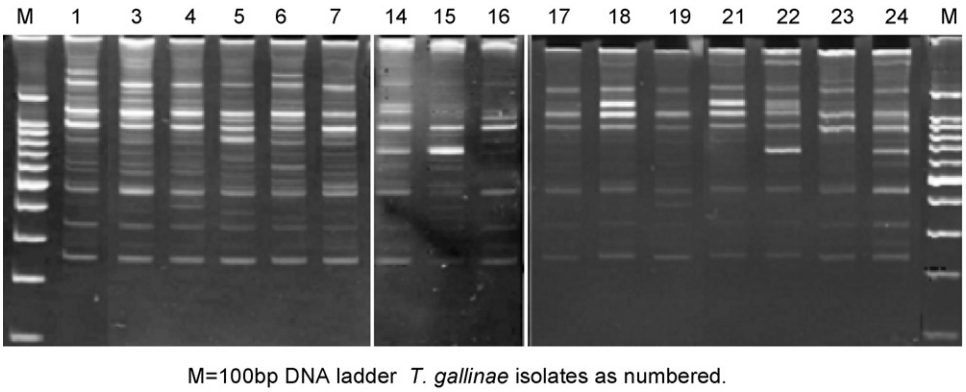


Fig. 3. Representative RAPD pattern obtained for the isolates. An EtBr stained 10% polyacrylamide gel separating OPD5 obtained pattern is shown.

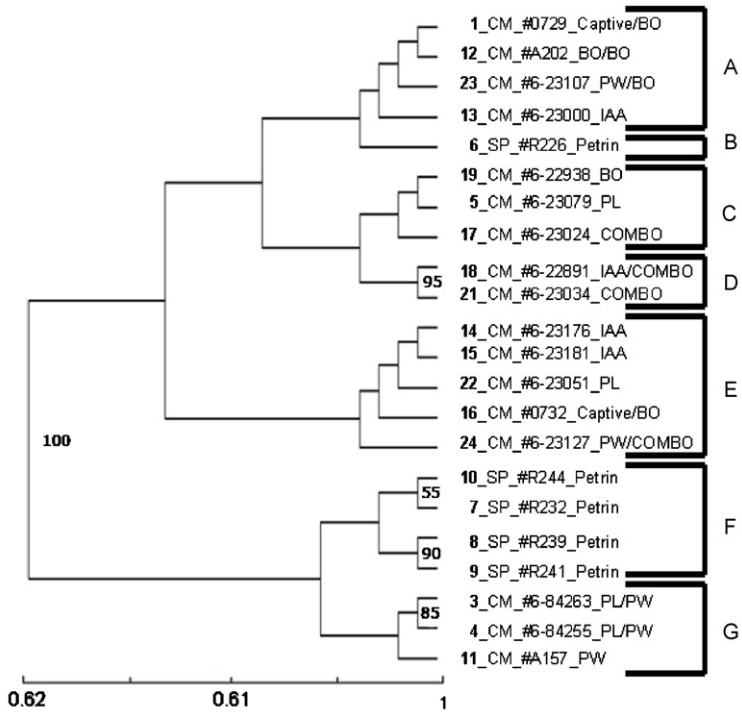
Mauritian isolates were identical to the *T. gallinae* Brazilian G7 strain sequence (Kleina et al., 2004), and differed at three bases from the other *T. gallinae* isolate available from the Rivolta 1878 strain. One of these base changes was found within the ITS1 sequence, and the other two within the 5.8S sequence. To determine the length of inferred evolutionary change that has occurred between the sampled *T. gallinae* and other members of the Trichomonad order, a phylogenetic tree was constructed (Fig. 2), which clearly segregates *T. gallinae* sequences from other trichomonad species.

3.2. Heterogeneity

The RAPD technique was used to assess intraspecific variability within *T. gallinae*. Amplification of the DNA samples was tested using 10 random primers. Of these, four resulted in a pattern useful for heterogeneity analysis: OPD3 (GTCGCCGTCA), OPD5 (TGAGCGGACA), OPD7 (TTGGCACGGG) and OPD8 (GTGTGCCCCA). Two pink pigeon samples were excluded from the analysis due to weak and inconsistent amplification. Fig. 3 is a representative example of patterns obtained for the other samples. Of the bands obtained, 22 were considered consistent and reliable (reproducing the same band pattern in at least three independent amplifications) and were therefore scored for the different isolates. Of these, eight bands were common to all individuals while 14 were polymorphic. The phenogram divided the isolates into seven distinct groups (Fig. 4). All *T. gallinae* genotypes from the Madagascar turtle-dove were clustered within two groups, B and F, whilst the pink pigeon isolates were more polymorphic, comprising five groups, with some geographical correlation. Genetic heterogeneity was found within the *T. gallinae* isolates, indicating strain variation within these two columbid species in Mauritius, and demonstrating species-specific and geographical correlations.

3.3. Correlation with geographical distribution and host species

Trichomonas genotypes varied between pink pigeon subpopulations (Fig. 4). By UPGMA analysis of the genotypes two lineages are discerned with a high degree of confidence, with four out of five of the Madagascar turtle-dove derived isolates clustering together within one group (F) of lineage I. Geographically, all samples collected from the Pigeon Wood subpopulation clustered together in group G. In group A, three of the four birds sampled were from Bel Ombre, while in group D, both birds were from Combo. Group E was the most heterogeneous group, comprising birds from the Ile aux Aigrettes, Bel Ombre, Combo and Plaine Lievre subpopulations. Isolates 14 and 15 were from the Ile aux Aigrettes subpopulation clustered closely, whilst the other isolate from the Ile aux Aigrettes, is in group A, together with Bel Ombre isolates. In the case of Group F it is worth further considering that parasites specific to the Madagascar turtle-dove were drawn from a distinct geographical location at Petrin.



CM = *Columba mayeri*, SP = *Streptopelia picturata*, BO= Bel Ombre, PW= Pigeon Wood, IAA = *Ile aux Aigrettes*, PL= Plaine Lievre.
When two locations are indicated, the first is the hatch site and the second is the capture site of the bird.

Fig. 4. Phenogram for *T. gallinae* isolates based on the RAPD data. UPGMA Dice bootstrapping for 1000 replicates was used; the distance matrix was calculated with dice coefficient based on RAPD data from OPD3, OPD5, OPD7 and OPD8 primers, totalling 22 bands.

4. Discussion

Trichomonas gallinae is an economically important pathogen, since it affects avian livestock; and poses a considerable threat to the conservation of threatened species of columbid, their avian predators and also to small passerine species such as finches. Species definition and diagnosis of *T. gallinae* to date remains the product of traditional microbiological methods; trichomonads found in the upper digestive tract of birds are typically named as *T. gallinae*, whilst those from the lower digestive tract and caecum are assumed to be *Tetratrichomonas gallinarum*, another important pathogen of poultry. These two trichomonads are morphologically (and genetically) distinct, allowing for simple differential diagnosis by microscopic examination.

Trichomonas gallinae is a ubiquitous pathogen of columbids; infection is usually asymptomatic but periodically virulent strains *of T. gallinae* cause high mortality among affected pigeons and doves. *T. gallinae* has been recognised as a good model for the study of virulence on the basis of these characteristics (Stabler and Braun, 1975; Narcisi et al., 1991) but these studies did not use molecular techniques to guage the degree of actual genetic variation. Thus, at the outset of the present study it was unclear whether *T. gallinae* is a single or group of species and whether virulent strains are simply the result of shifts in one or two key virulence determinants (such as haemolytic activity), or represent entirely different genotypes which could be described as separate species or subspecies. To our knowledge, this is the first attempt to assess the degree of diversity within this pathogen.

The ITS1/5.8S/ITS2 region is commonly used to investigate polymorphism within organisms, at both interspecific and intraspecific levels (Cupolillo et al., 1995; Snipes et al., 2000; Walker et al., 2003). The rDNA sequences from a given organism can be important in identifying levels of genetic variability and may, therefore, be potentially useful in characterising different strains or species, being specially recommended for epidemiological studies (Zingales et al., 1999) Phylogenetic studies of the trichomonadidae family (Felleisen, 1997; Kleina et al., 2004) have revealed variation between species in the ITS1/5.8S/ITS2 region. These latter studies did not examine intraspecific variation per se, but previous analysis of the 5.8S sequence from the human trichomoniasis agent, *T. vaginalis*, found complete homology amongst strains sequenced for this region (Katiyar et al., 1995; Snipes et al., 2000)

Our comparison shows that all wildlife isolates of *T. gallinae* sequenced to date at this locus (ITS1/5.8S/ITS2) have identical sequences which differ markedly from sequences of other *Trichomonas* species, pointing this region as a strong species marker and confirming previous morphological and pathological observations in field studies on Mauritius which have identified parasites found in the crops of pigeons and doves as *T. gallinae*. The Rivolta 1878 strain showed polymorphism at three positions within this region. This strain had been in long-term culture, so it is not clear whether some degree of natural polymorphism exists at these nucleotides or whether this was an artefact of extended culture. Clearly a larger study with diverse isolates would be desirable to evaluate this further.

An interesting point arising from our analysis of this locus is that the *T. gallinae* sequence is quite distinct from that of another avian trichomonad, *Tetratrichomonas gallinarum,* but genetically similar to the canine pathogen *Trichomonas caninistomae*, which causes superficial infection of the canine oral mucosa. Pigeons form part of the prey spectrum of dogs, raising the possibility that *T. gallinae* may have infected (carnivorous) mammals in the past. Although apparent morphological differences argue against a recent species jump, it is clear that *T. gallinae* pathogenesis is not entirely restricted to birds since it is long established as being able to induce pathology in laboratory infected murine models (Honigberg, 1961). Moreover, an analogous species jump for *T. gallinarum* to the human oral mucosa has recently been documented (Cepicka et al., 2005; Kutisova et al., 2005).

Worldwide, there may be more heterogeneity at this locus in *T. gallinae* than is apparent from our small and localised study. If this is supported by subsequent work, the 100% conservation of the ITS1/5.8S/ITS2 observed among the Mauritian *T. gallinae* isolates may be due to the parasite having been introduced relatively recently into the closed island ecosystem. This is believed to have occurred with the introduction of exotic columbid species in the 18th and 19th century (Swinnerton et al., 2005). The absence of heterogeneity amongst Mauritian isolates at the ITS1/5.8S/ITS2 prompted the subsequent use of another procedure for further investigation of strain variation in *T. gallinae*.

The RAPD technique has been found to produce suitable markers for intraspecific analysis of a variety of different organisms including trichomonadidae (Vanacova et al., 1997; Pavlicek et al., 1999; Snipes et al., 2000; Sedinova et al., 2003). RAPD patterns are mainly due to point nucleotide substitutions and allow for phylogenetic relationships to be inferred between strains. However, as most nucleotide changes are selectively neutral, this does not necessarily imply phenotypic variation (Jukes and Kimura, 1984; Williams et al., 1990).

It is likely that interspecies transmission was the route by which *T. gallinae* was introduced into the current pink pigeon population following the bottleneck. The genetic heterogeneity of the isolates uncovered by our RAPD analysis suggests that this was unlikely to be a single event followed by clonal transmission through the species. It is possible that variation has been introduced by multiple instances of inter-specific transmission and that the apparent geographical correlation reflects this. pink pigeons appear to be more susceptible to infection with *T. gallinae* than the exotic columbid species present in Mauritius (Swinnerton et al., 2005; Bunbury, 2006) which may reflect a relatively short period of exposure and host adaptation to the parasite.

Pigeon Wood is the site where the last remaining wild birds, whose infection status is unknown, were found in the 1970s and isolates from this site are closely related to the Madagascar turtle-dove isolates collected at the nearby Petrin location (Fig. 1).

Birds 3 and 4 both hatched in the Plaine Lievre subpopulation before later dispersal to Pigeon Wood. The strain carried by these two birds was the same as that carried by bird 11 which originated at Pigeon Wood, which suggests acquisition of a local strain and provides evidence for horizontal transmission within the pink pigeon population.

Within the pink pigeon population as a whole, movement between subpopulations, mate-switching and the possibility of simultaneous infections with different strains may explain the non-geographical pattern observed for some isolates such as group C. It is important to note that the presence of multiple infecting strains in an individual could confound the analysis by yielding apparently complex genotypes. The number of samples used in this study, corresponding to 5–6% of the total pink pigeon population, precluded statistically valid correlation between genotypic features, host-specificity and limited geographic correlations. A more wide-ranging analysis, with a larger number of birds and multiple time-points, is planned.

In summary, this study, the first evaluation of *T. gallinae* genotypic heterogeneity, finds no evidence of multiple subspecies of *T. gallinae* in Mauritian columbids. It does, however, confirm that the ITS1/5.8S/ITS2 is an effective species marker and also that there is significant and apparently rapidly evolved genetic heterogeneity between isolates detectable through RAPD analysis, which appears to correlate with host species and geography. Further studies in Mauritius and worldwide, on this neglected pathogen, are strongly warranted.

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