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Diversity and temporal stability of bacterial communities in a model passerine bird, the zebra finch

CLARE McW. H. BENSKIN,* GLENN RHODES,† ROGER W. PICKUP,‡ KENNETH WILSON* and IAN R. HARTLEY*

*Lancaster Environment Centre, Lancaster University, Lancaster LA1 4YQ, UK, †Centre for Ecology and Hydrology, Lancaster Environment Centre, Library Avenue, Bailrigg, Lancaster LA1 4AP, UK, ‡Division of Biomedical and Life Sciences, School of Health and Medicine, Lancaster University, Lancaster LA1 4YQ, UK

Abstract

The composition and dynamics of the gastrointestinal bacterial communities in birds is determined by both host-specific and environmental exposure factors yet these are poorly understood. We selected the zebra finch, *Taeniopygia guttata*, as the host species to examine the diversity and temporal stability of the faecal microflora in a bird, owing to its importance as a model organism in avian ecology, neuroscience and evolution studies. The stability of the gut bacterial community of individual male and female zebra finches was assessed through repeat faecal sampling via culture and temperature gradient gel electrophoresis and partial sequencing of PCR-amplified eubacterial 16S rRNA gene products. Nineteen bacterial genera were detected across all samples (n = 99), with each bird carrying on average six operational taxonomic units. Using a novel statistical approach, we showed that bacterial assemblages and community richness varied between individual birds but remained stable over time within individuals. Neither the composition nor richness of bacterial communities differed significantly between the sexes. Our results show that zebra finches housed together under controlled conditions show consistent variation between individuals in their gut microflora that is not attributable to differences in host exposure to environmental microbial sources. Future studies could usefully explore the origin of this individual-specific variation and its consequences for host fitness and sexual selection.

Keywords: 16S rDNA, bacterial diversity and temporal stability, faecal microbial community analysis, passerine, PCR–TGGE

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Introduction

Host–microbe interactions can have important implications for individual host fitness (Backhed *et al.* 2005), which in turn can influence the structure of host communities and act as a driving force in the evolution of sex and other fundamental traits (Hudson *et al.* 2002). Infectious bacterial pathogens, such as some species of *Campylobacter* and *Salmonella*, may be important sources of mortality and morbidity in wild and domestic animal populations (Pennycott *et al.* 2002; Bull *et al.* 2008), whereas others, such as *Lactobacillus* and *Bifidobacterium*, may play an important role in nutrition, growth and protection from infection (Mead 1997; Backhed *et al.* 2005; Quigley 2010). Although previous work has explored the microbial ecology of the gastrointestinal flora in a number of mammal species (Simpson *et al.* 2000, 2002; McCracken *et al.* 2001; Zoetendal *et al.* 2004; Wei *et al.* 2007; Qin *et al.* 2010), remarkably little is known about the gut bacterial community of birds, with the exception of commercially bred poultry (Van der Wielen *et al.* 2002; Zhu *et al.* 2002; Kizerwetter-Świda & Binek 2008). These studies suggest that the gut microflora varies between breeds, suggesting potential genetic effects, and can also be influenced by environmental

Correspondence: I. R. Hartley, Fax +44 (0)1524593192;
E-mail: i.hartley@lancaster.ac.uk

factors, such as stocking density and food type (Apajalahti *et al.* 2001, 2004; Bjerrum *et al.* 2006). Moreover, individual chickens tend to have distinct intestinal bacterial communities (Van der Wielen *et al.* 2002) and a similar pattern is seen in mammals (Zoetendal *et al.* 1998; Simpson *et al.* 1999, 2000; Suchodolski *et al.* 2004), suggesting that host-specific factors are important in the establishment and maintenance of intestinal bacterial communities (Van der Wielen *et al.* 2002).

Most studies relating to the microbial ecology of wild birds are opportunistic investigations of epidemics of specific pathogens and are almost universally based on single samples from individuals (Benskin *et al.* 2009). Before we can develop our understanding of the interactions between hosts and gut pathogens, however, it is important to quantify the repeatability of the sampling method. A better understanding of the variation in microflora within and between individuals will underpin future studies of microbial diversity in wild birds.

In free-living wild birds, there are potentially many variables that could influence the gastrointestinal microflora, including the genotype or phenotype of the host, and differences in host exposure owing to variation in environmental sources (Lucas & Heeb 2005). One of the main phenotypic differences between individuals is sex. In many bird species, sexual selection has driven the evolution of sex differences in plumage, behaviour and parental care roles, and also differences in susceptibility to pathogens (Poulin 1996; McCurdy *et al.* 1998). Differences in susceptibility may be driven by hormonal effects on host immune systems, but also on differences in exposure (Zuk 2009). For example, in sexually size-dimorphic species, the larger sex may need to eat more food, so increasing exposure to diet-related pathogens (Moore & Wilson 2002). To understand better the host sex differences in gastrointestinal bacterial communities, we need to compare males and females living under similar environmental conditions.

The zebra finch, *Taeniopygia guttata*, is an important model organism, especially in avian neuroscience and behavioural ecology, owing to its easy husbandry and because it is sexually dimorphic, making it ideal for studies on sexual selection and bird song (e.g. Burley 1981; Lemon 1991; Royle *et al.* 2002; Birkhead *et al.* 2005; Feher *et al.* 2009). Moreover, the zebra finch is the first passerine to have its genome sequenced (Warren *et al.* 2010), greatly increasing the potential for studying the genetics of passerine birds (Birkhead *et al.* 2005; Santure *et al.* 2010). Thus, the zebra finch could serve as a new model organism for studying the genetic contribution to variation in the passerine gut microflora and the effects of gut bacteria on avian song, learning, mate-choice, sperm competition, sexual ornamentation and life history traits. However, future studies of this kind are potentially constrained by a number of factors, including the reliability and biases inherent in the analytical methods used, and the repeatability of bird bacterial profiles is especially important for field studies based on single faecal samples.

Thus, in an attempt to quantify some of these constraints, this study uses both culture and molecular methods to characterize the faecal bacterial community composition, species richness and dynamics in captive zebra finches in a controlled environment. Our aim was to address the following questions: (i) In a constant environment, do individuals exhibit distinct faecal bacterial communities, i.e. bacterial 'fingerprints'? (ii) Do these fingerprints remain stable over time? and (iii) Are there consistent sex differences in the faecal bacterial community? To answer these questions, individual male and female zebra finches, housed under the same controlled and constant conditions, were sampled repeatedly over a 10-week period. A novel statistical approach was used to test whether bacterial assemblages within individual birds were distinct and temporally stable and whether there was any sex difference in faecal bacterial communities.

Materials and methods

Sample collection

The zebra finch population used in the current study is largely derived from that which has been maintained at the University of Sheffield since 1985 (Birkhead *et al.* 1988; Santure *et al.* 2010). Outbreeding and pedigree data have been maintained since the Lancaster University population was established in 1989 (Royle *et al.* 2002). To address the three questions outlined above, faecal samples were collected from twenty healthy, nonsibling zebra finches (10 males and 10 females) housed in single-sex stock cages (width = 240 cm, depth = 110 cm, height = 310 cm) in a temperature-controlled room at 20 °C, under full-light spectrum, artificial light (Bennett *et al.* 1996) on a 16h:8h light:dark regime. Stock cages were located so that no physical contact was possible between sexes. When in stock cages, birds were provided daily with *ad libitum* seed (Foreign Finch Seed; Johnston & Jeff Ltd, Gilberdyke, East Yorkshire, UK), cuttlebone (Cuttlefish Bone; Juicy Pet Products, Southport, UK), grit (Birdgrit; Jondo Ltd, Dudley, UK) and drinking water in containers. Additionally, the birds were provided weekly with a vitamin, mineral and amino acid supplement (Prime®; Rolf C. Hagen Ltd, Castleford, UK) added to the drinking water, charcoal (Granulated charcoal; The Hatchwell Co. Ltd, Blackburn, UK) and bathing water.

Samples were collected over a period of 10 weeks at 14-day intervals, comprising a total of 100 samples. Birds were placed individually in holding cages (600 × 400 × 450 mm) for faecal collection. The bottoms of the holding cages were covered in Benchkote paper (Scientific Laboratory Supplies Ltd, Nottingham, UK) to provide a nonabsorbent layer from which to collect faeces. The paper was cleaned with 70% ethanol prior to introduction of birds to each cage. Faeces were collected in sterile microcentrifuge tubes and stored at 2–4 °C for <24 h prior to analysis. Throughout, we follow previous authors by assuming that differences in the bacterial community of faecal samples reflect differences in that of the intestinal tract (Savage 1977; Vaahtovuo *et al.* 2001).

An additional 200 mg faecal material was collected from two male and two female zebra finches for bacterial culture on full and half-strength nutrient agar (Oxoid, UK), and R2A agar (Oxoid, UK), at both 20 and 37 °C. Colonies displaying unique morphologies were purified by streak plating on nutrient agar. Amplicons of the partial 16S rRNA gene from cultured isolates were sent to Qiagen Genomic Services (Hilden, Germany) for purification, and single sequencing runs using primer pE (Lane *et al.* 1985; Table 1).

Nucleic acid extraction and PCR amplification

Total community DNA was isolated from avian faeces using the QIAamp DNA Stool Mini kit (Qiagen Ltd, Crawley, UK) incorporating minor modifications similar to those adopted by Wehausen *et al.* (2004). Briefly, faecal samples were suspended in 1.4 mL of the initial extraction buffer provided, added to FASTPREP lysing matrix B tubes (MP Biomedicals, UK) and homogenized using a FASTPREP machine (MP Biomedicals, UK) for 45 s at 6.5 m/s. Samples were centrifuged to pellet beads and faecal detritus, and supernatant was decanted and used subsequently according to the manufacturer's instructions.

For extraction of DNA from cultures, isolates and *Escherichia coli* K12 No. 10214 (CCAP, UK) were grown in 10 mL nutrient broth and incubated on a shaking platform at 30 °C for 24 h. DNA was extracted from 1 mL of each isolate suspension using the UltraClean Microbial DNA isolation kit (Mo Bio Laboratories, Inc., USA) in accordance with the manufacturer's instructions.

All PCR amplifications were carried out in a PX2 thermal cycler (Thermo Electron, UK). Bacterial 16S rRNA genes were amplified by nested PCR using primer pair pA/pH′ (Edwards *et al.* 1989; Table 1), followed by amplification with the universal primers F984/R1378 with the forward primer incorporating a 34-bp GC clamp (Heuer *et al.* 1997). PCR amplifications for each nested PCR were optimized using the Failsafe PCR system (EpiCentre, USA) in 50 μL volumes, such that subsequent reactions received the following: 25 μL 2× Failsafe premix buffer E, 1 μL (20 pmol) of each primer, 0.5 μL Taq polymerase mixture, 12.5 μL sterile molecular biology grade water (Sigma) and 10 μL of DNA. Nested reactions differed only in receiving 2× Failsafe premix buffer F, 1 μL DNA and the volume was made up to 50 μL with water. Positive controls contained genomic DNA from *E. coli* K12 in place of sample DNA, while negative controls contained water. Initial denaturation was carried out for 4 min at 94 °C, and cycling was performed as follows: 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min for 35 cycles. Nested reactions were denatured for 4 min at 94 °C, and cycling was performed at 94 °C for 1 min, 60 °C for 45 s and 72 °C for 2 min for 35 cycles. All amplifications were completed with a final elongation at 72 °C for 10 min.

Temperature gradient gel electrophoresis (TGGE) and DNA sequencing

16S amplicons were distinguished by TGGE using the TGGE Maxi system (Whatman, Biometra). Electrophoresis was performed on 3 μL of each amplification

Table 1 Primers used in PCR and sequencing

Primer	Sequence (5′–3′)	Position*	Primer target	Reference
pA (8F)	AGAGTTTGATCCTGGCTCAG	8–28	Eubacterial 16S rRNA gene	Edwards *et al.* (1989)
pH′ (1520R)	AAGGAGGTGATCCAGCCGCA	1542–1522	Eubacterial 16S rRNA gene	Edwards *et al.* (1989)
pE	AAACTCAAAGGAATTGACGG	908–928	Eubacterial 16S rRNA gene	Lane *et al.* (1985)
F984	AACGCGAAGAACCTTAC	968–984	Eubacterial 16S rRNA gene	Heuer *et al.* (1997)
F984GC	CGCCCGGGGCGCGCCCCGGGCGGG GCGG- GGGCACGGGGGGAACGCG AAGAACCTTAC	968–984	Eubacterial 16S rRNA gene	Heuer *et al.* (1997)
R1378	CGGTGTGTACAAGGCCCGGGAACG	1401–1378	Eubacterial 16S rRNA gene	Heuer *et al.* (1997)

**Escherichia coli* numbering—corresponds to the positions in *E. coli* 16S rRNA gene

product in polyacrylamide gels (6% polyacrylamide, 7 M urea, 10% formamide and 2% glycerol) with 1× TBE buffer (40 mM tris-borate-EDTA, pH = 8.0), using a multistep program. Step one lasted for 10 min at 300 V with a constant temperature of 20 °C across the gel, to enable samples to migrate into the gel. The second step consisted of establishing a temperature gradient from 39.5 to 47.5 °C without applying voltage, to allow the temperature gradient to stabilize prior to electrophoresis. Electrophoresis was then performed for 3 h at 300 V, with the temperature gradient established in step two. Gels were subsequently stained with SYBR Gold (Invitrogen) for 30 min and visualized on a UV transilluminator (320 nm).

For DNA sequencing, bands were excised from gels under UV using a sterile scalpel blade, placed into individual sterile Costar Spin-X centrifuge tube filters (Corning Inc., USA) containing 40 μL elution buffer (QIAamp DNA Stool Mini kit), and DNA was extracted by centrifugation at 16 000 *g* for 10 min. SYBR gold stain was removed from extracted DNA by ethanol precipitation, and purified DNA (20 μL) was re-amplified with primers F984/R1378, using the PCR conditions described above.

The QIAquick PCR purification kit (Qiagen Ltd, Crawley, UK) was used to purify PCR products, of which, 3 μL was cloned into the pDrive cloning vector using the QIAGEN A-addition kit and the QIAGEN PCR Cloning kit. Plasmid DNA was extracted from bacterial clones using the QIAprep Spin Miniprep kit, according to the manufacturer's instructions. The presence of cloned inserts was confirmed by restriction digestion using the enzyme *Eco*RI (Abgene) followed by electrophoresis for 1 h at ~100 V in 0.7% w/v agarose gels. The DNA sequence from these 16S clones was determined by Qiagen Genomic Services (Germany) by single read, using universal primer T7 (Wallace *et al.* 1981). Sequences were compared with those in the GenBank database with the BLASTN search program (Altschul *et al.* 1990; http://www.ncbi.nlm.nih.gov/blast/Blast.cgi?PAGE=Nucleotides).

Estimation of microbial richness

The positions of bands on TGGE gels were normalized by using the molecular weight ladder Lambda *Eco*RI/*Hind*III (Advanced Biotechnologies, Surrey) as an external reference standard to permit comparisons between gels. Bacterial richness in each sample was measured by counting the number of bands found in each lane, assuming that each band represents different sequences, and therefore different operational taxonomic units (OTUs; Atlas & Bartha 1998). A binary matrix was produced for each sample by noting OTU presence/absence.

Data analysis

The community compositions of faecal bacteria were quantified and compared using Jaccard similarity matrices (Jaccard 1908) generated from the TGGE-derived binary data, using the statistical software package SPSS 16.0 (SPSS Inc., Chicago, IL, USA). A dendrogram was constructed using the SPSS hierarchical classification system (Fig. 1), with Jaccard between-groups linkage, where the Jaccard similarity matrix was used to determine the relative similarity between samples and to group them accordingly. Dendrograms based on other selection criteria (Sorensen-Dice's coefficients and Anderberg's coefficients) generated qualitatively similar results (C. Benskin, unpublished analysis).

To statistically quantify patterns in the zebra finch bacterial community structure, we follow several recent studies (Lucas & Heeb 2005; Ruiz-Rodríguez *et al.* 2009) and apply Mantel's test (Mantel 1967). Thus, we determined the correlation between the similarity matrix generated by the pair-wise comparisons of TGGE profiles (as described earlier) to similar (binary) matrices generated for each of the potential variables explaining variation in those profiles, i.e. bird identity, sampling period and sex (such that, for example, when pairs of faecal samples came for the same bird or same sampling period, the cell value was 1, otherwise it was 0). Mantel correlations between matrices were computed in S-PLUS 8.0 using the *mantel.fcn* function (Reynolds & Bolker 2001), and significance levels were obtained following 10 000 permutations. To test for the effects of sampling period and sex, partial Mantel correlations were determined after controlling for between-bird effects, following the method of Reynolds & Bolker (2001).

Although Mantel's test provides a useful statistical method for gauging the correlation between pairs of similarity matrices, it cannot easily be extended to multiple interacting factors. We therefore explored the potential utility of a second statistical approach to this problem using a much more flexible generalized linear modelling framework. To do this, we used the constructed dendrogram to assign faecal samples into cluster groups sharing similar bacterial community fingerprints. This was carried out by 'cutting' the dendrogram at a range of Jaccard distance measures so as to generate clusters whose members shared a minimum level of similarity (based on Jaccard distances); depending on where the dendrogram is cut, this would generate between 1 and n clusters (where n is the number of samples represented in the dendrogram). Next, we constructed a generalized linear model (GLM) with a Poisson error distribution and a log link function (McCullagh & Nelder 1989), in which the dependent

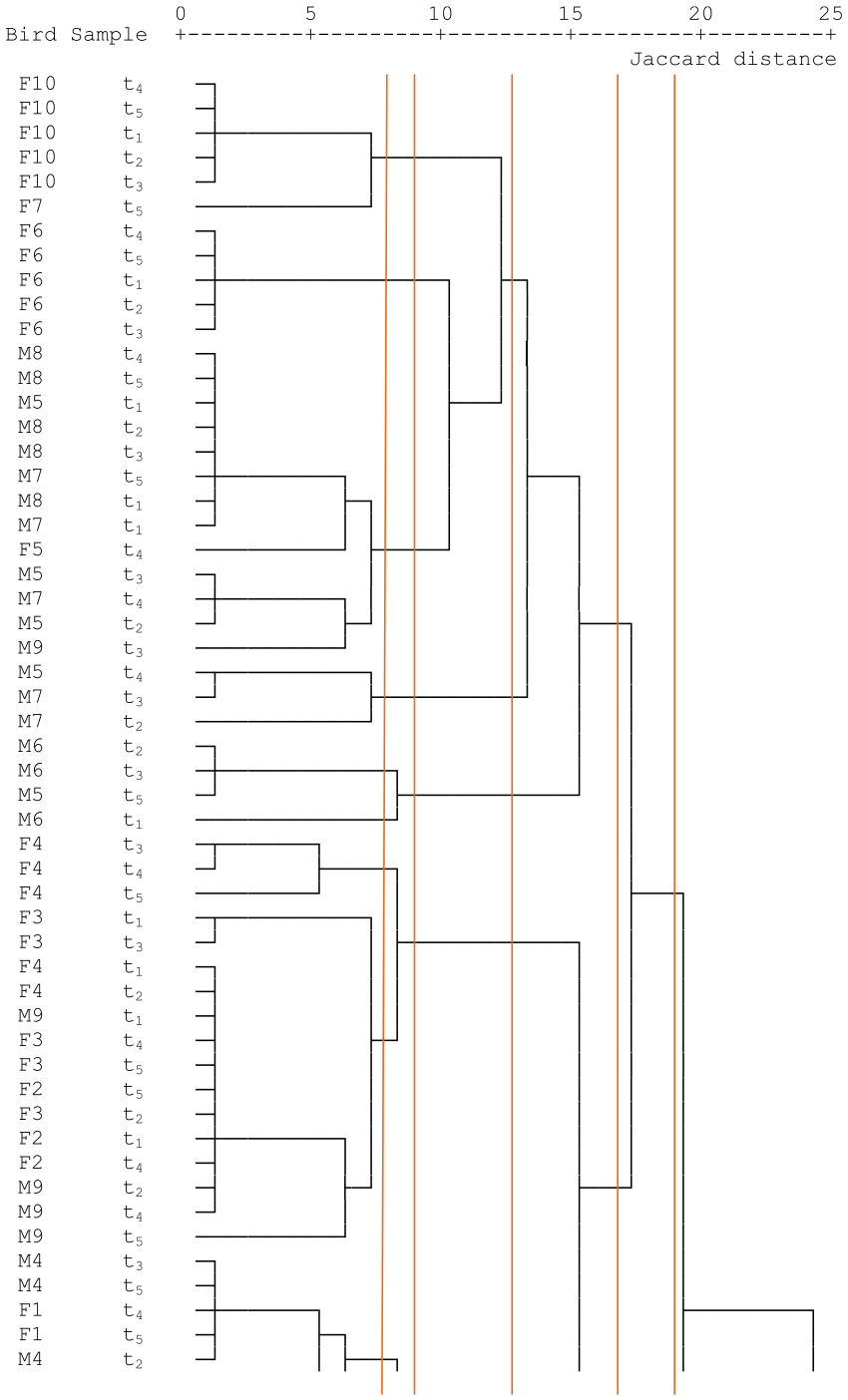

Fig. 1 Relationships between faecal bacterial communities from ten captive male (M1–M10) and female (F1–F10) zebra finches, taken at five different time points (t_1–t_5), illustrated by a between-groups linkage dendrogram, generated from Jaccard distance matrices calculated from temperature gradient gel electrophoresis banding profiles. The scale represents the Jaccard distance measure. Lines a–e represent the cut-off points for 23, 18, 9, 5 and 4 grouping levels, respectively.

variable was the cluster group (*Group*) and the explanatory term was the identity of the individual birds that were sampled (*Bird*). A significant result here would indicate that there was more variation *between* birds in bacterial community composition than there was *within*, i.e. that bacterial cluster group membership was repeatable. To allow for possible overdispersion, an empirical dispersion parameter was used and significance levels

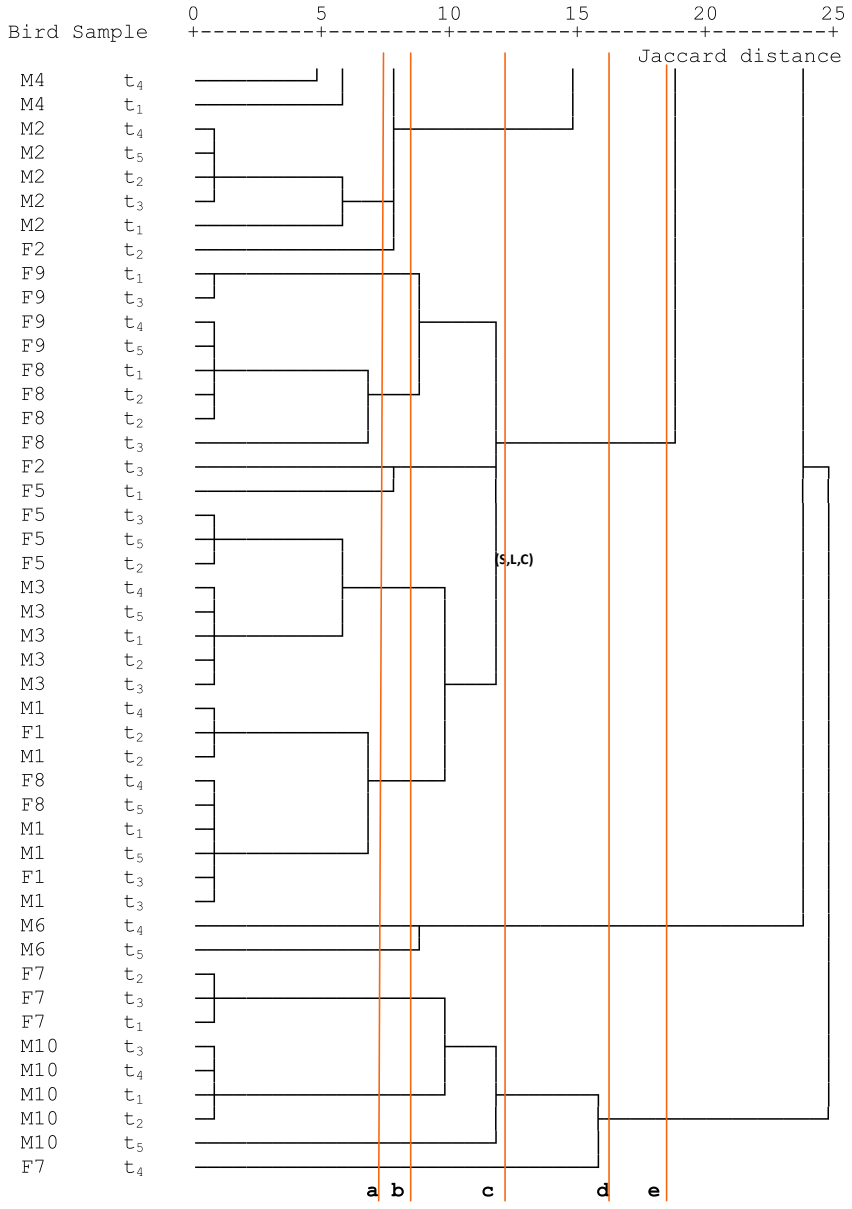

Fig. 1 (Continued)

were determined using *F*-tests rather than chi-squared tests (Wilson *et al.* 1996; Wilson & Grenfell 1997). To test the robustness of this result, we conducted a permutation test in which *Group* was randomly sampled without replacement 10 000 times (using the *sample* function in S-PLUS) and the residual deviance of the resultant model determined to generate a null distribution for the model. This was then compared to the residual deviance generated by the original data to produce a robust estimate of the probability of generating the observed result by chance alone (Hartley *et al.* 1999; Wilson & Hardy 2002). Finally, to determine whether there was consistent variation in the community composition of individual birds over time, or between the sexes, we used a generalized linear mixed model (GLMM) with Poisson errors, using the penalized quasi-likelihood (PQL) method. The dependent variable was again *Group*, and *Bird* was included in the model as a random term; both the sex of the bird (*Sex*) and the sample number (*Sample*) were included as potential explanatory terms.

To determine whether the *number* of bacterial OTUs detected in zebra finch faeces varied consistently between individuals, the two sexes and over sampling periods, GLM and GLMMs were conducted, with the total number of TGGE bands detected per sample

(*Richness*) as the dependent variable, which was again assumed to follow a Poisson distribution. All analyses were conducted using the S-PLUS 8.0 (Insightful Corp., Seattle, WA, USA) statistical package.

Results

Analysis of cultured isolates

Nineteen morphologically distinct bacterial isolates, designated culture clone (CC) 1–19, were recovered from four zebra finches through culture on full and half-strength nutrient agar and R2 agar. 16S rRNA genes from all cultured isolates were successfully sequenced and attributed with accession numbers AB576081 through to AB576099. Sequences showed ≥97% similarity with a known sequence in the GenBank database (Table 2), with the exception of CC6 and CC14, which were unique. CC1, CC15 and CC17 all shared the closest homology with *Bacillus mojavensis* JF-2, which is ubiquitous in nature; CC13 and CC16 corresponded to entities closely related to the uncultured bacterium clone D5; and the closest homologue of both CC5 and CC11 was *Klebsiella* species TNT1. CC3 and CC8 both shared the closest homology with *Enterobacter* species USC7, while CC2 and CC18 showed homology to other *Enterobacter* sequences. Members of the genus *Enterobacter* are normally associated with the gut microflora and are also commonly found in water and soil. CC7 and CC19 showed sequence homologies to separate *Staphylococcus* species, a genus commonly found in soil. CC12 showed closest homology with *Bacillus licheniformis*, which has been associated with feather and body condition (Burtt & Ichida 1999; Goldstein *et al*. 2004; Gunderson *et al*. 2009), both of which influence mate-choice (Bakker & Pomiankowski 1995; Burley & Fostera 2006), and could be introduced to the gut during preening. The sequence homologues for the remaining isolates did not overlap at the genus level (Table 2).

Bacterial community analysis using TGGE and sequencing

One sample was excluded from all analyses owing to its failure to produce a visible TGGE banding profile. Across the remaining 99 samples from twenty birds, 22

Table 2 Bacterial identification of culture clones (CC) from zebra finch faeces and of TGGE clones (TC), by sequencing of excised temperature gradient gel electrophoresis (TGGE) bands, as identified by partial 16S rRNA. Cultured clones were grown on full and half-strength nutrient agar (NA and 1/2NA, respectively) and R2A agar

Clone	Medium	Closest 16S homologue in GenBank	Identity scores (% similarity)	Accession no.
CC1, CC15, CC17	R2A, NA, R2A	*Bacillus mojavensis* JF-2	100	AY436360.1
CC2	R2A	*Enterobacter cloacae*	99	EU545406.1
CC3, CC8	R2A, NA	*Enterobacter* sp. USC7	98	FJ890898.1
CC4	R2A	*Pseudomonas* sp. S27	99	EU747694.1
CC5, CC11	R2A, 1/2NA	*Klebsiella* sp. TNT1	99	DQ229100.1
CC6	1/2NA	Uncultured bacterium	95	AB460952.1
CC7	1/2NA	Uncultured *Staphylococcus* sp. clone 87–5	99	AF467420.1
CC9	NA	*Micrococcus* sp. TUT1210	99	AB188213.1
CC10	NA	*Rothia nasimurium* str. CCUG 35957	99	NR_025310.1
CC12	1/2NA	*Bacillus licheniformis* str. MS5–14	99	EU718490.1
CC13, CC16	R2A, 1/2NA	Uncultured bacterium clone D5	100, 99	EU556993.1
CC14	R2A	*Arthrobacter* sp. VTT E-052923	95	EF093129.1
CC18	NA	*Enterobacter* sp. XW122	99	EU545406.1
CC19	NA	*Staphylococcus aureus* subsp. *aureus* USA300_TCH1516	100	CP000730.1
TC1, TC2, TC4	–	*Campylobacter lari* RM2100	100	GQ167657.1
TC3	–	*Pseudomonas* sp. RF-58	99	GQ205100.1
TC6, TC8, TC9, TC13, TC20	–	*Lactobacillus aviarius*	98, 99, 99, 81, 99	AB001836.2
TC12	–	*Catellicoccus phocoenae*	97	AJ854484.1
TC15	–	*Pseudomonas* sp. M2L4	100	FJ560470.1
TC16	–	*Erwinia tasmaniensis* str. ET1/99	99	CU468135.1
TC17, TC18	–	*Atopobium parvulum*	95, 97	AF292372.1

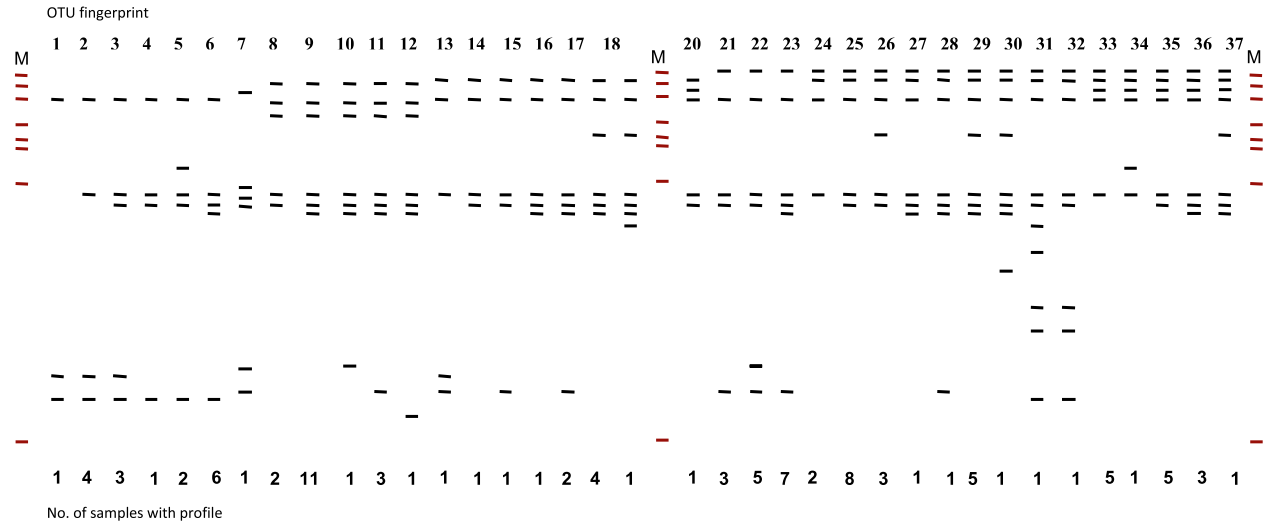

Fig. 2 Schematic representation of the range of 16S rRNA operational taxonomic unit fingerprints (n = 37) detected by temperature gradient gel electrophoresis across 99 samples. M = λ*Hind*III*Eco*R1.

distinct bands were identified by TGGE, comprising 37 distinct OTU fingerprints (Fig. 2), with each bird displaying, on average, 6 ± 1 OTUs (mean = 5.7; range = 3–10; variance = 1.5; Fig. 3). Several separate TGGE bands were identified as sharing the same closest homologue in the GenBank database (Table 2). Of the 22 bands excised from TGGE gels, designated TGGE clone (TC) 1–22, 14 were successfully re-amplified, cloned and sequenced and attributed with accession numbers AB576140 through to AB576153. These all showed ≥97% identity with a known sequence in the GenBank database (Table 2), with the exception of clones TC13 and TC17, which were unique (81% and 95% similarity, respectively). Five clones (TC6, TC8, TC9, TC13 and TC20) shared the closest homology with *Lactobacillus aviarius*, a bacterium previously associated with the intestines of chickens (Fujisawa *et al.* 1984), while clones TC1, TC2 and TC4 were homologous to *Campylobacter lari* RM2100, a bacterium commonly found in a variety of environmental and avian sources (Waldenström *et al.* 2002; Matsuda & Moore 2004). TC3 and TC15 corresponded to entities closely related to *Pseudomonas* species, TC17 and TC19 were homologous to *Atopobium parvulum* (formerly *Streptococcus parvulus*; Collins & Wallbanks 1992), and the remaining clones, TC12 and TC16, had closest homology to *Catellicoccus phocoenae*, a bacterium found in gull faeces (Lu *et al.* 2008), and *Erwinia tasmaniensis*, which is commonly associated with fruit trees (Kube *et al.* 2008).

Sequencing of excised TGGE bands revealed that several bacteria genera or species were represented by more than one band (Table 1), as noted in other studies (Fogel *et al.* 1999; Dahllöf *et al.* 2000; Dahllöf 2002).

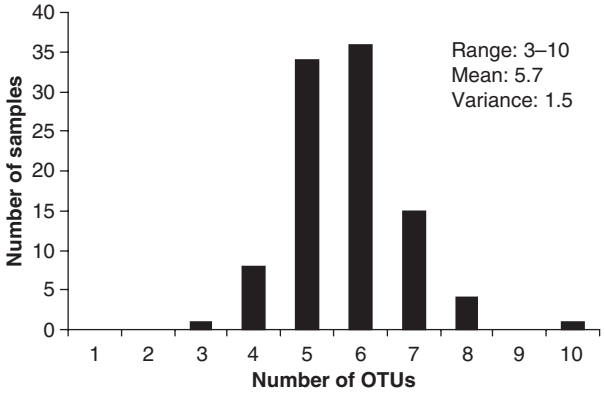

Fig. 3 The prevalence of operational taxonomic units detected by temperature gradient gel electrophoresis in faecal samples (n = 99) from 20 captive zebra finches.

Consequently, the subsequent statistical analysis of banding profiles below relates to 16S rRNA gene sequence diversity rather than to species richness within the bacterial community.

Statistical assessment of bacterial community richness

Mantel's tests indicated that significant variation in bacterial profiles could be explained by host identity (Mantel's correlation, based on 10 000 permutations, *Bird*: r = 0.354, n = 99, $P < 0.0001$), but not by sampling period (*Sample*: partial r = −0.033, n = 99, $P > 0.88$) or the sex of the bird (*Sex*: partial r = −0.004, n = 99, $P > 0.59$).

To examine the utility of the GLM statistical approach, we divided the faecal samples into 18 cluster groups (*Group*) by 'cutting' the dendrogram at a Jaccard distance

of 16 (line *d* in Fig. 1). This point was chosen because the number of clusters it generates is closest to the number of birds that were repeat sampled ($n = 20$), but statistical analyses based on fewer or greater numbers of clusters (4, 5, 9, 23) generated similar results (K. Wilson, unpublished analysis). There was highly significant variation between individual birds in their bacterial communities, as determined by the repeatability of their cluster group membership (GLM with Poisson errors, *Bird*: $F_{19,79} = 22.52$, $P < 0.0001$; probability determined by 10 000 permutations: $P < 0.0001$). However, there was no significant difference between the two sexes in the composition of their bacterial communities (GLMM with Poisson errors with *Bird* as a random effect, *Sex*: $F_{1,18} = 0.12$, $P = 0.73$) and no consistent variation across the five sampling points (*Sample*: $F_{4,75} = 0.15$, $P = 0.96$). These results are similar to those generated using Mantel's test and suggest that individual birds carried distinct faecal bacterial assemblages that remained stable over time and that males and females did not differ significantly in the composition of their bacterial communities.

Bacterial richness (as estimated by the number of OTUs detected in the faeces) varied significantly between individual zebra finches (GLM: *Bird*; $F_{19,79} = 8.24$, $P < 0.0001$). Moreover, as with bacterial community composition, there was no consistent variation in bacterial richness associated with either the sex of the bird or the sampling period (GLMM with Poisson errors and *Bird* as a random factor: *Sample*: $F_{4,75} = 1.43$, $P = 0.12$; *Sex*: $F_{1,18} = 1.19$, $P = 0.29$).

Discussion

To our knowledge, this is the first longitudinal study to both statistically characterize the structure of the faecal bacterial communities of captive birds and to quantify temporal variation of the bacterial assemblages within and between individuals.

Methodological approaches

The combination of culture and molecular methods in this study allowed a more comprehensive evaluation of the constituent bacterial genera in the faeces of captive birds than could be achieved by using either method in isolation. The detection by PCR of every target sequence in every population is not guaranteed, and consequently, a subset of organisms may remain undetected (Felske *et al.* 1997). What we present here is likely, therefore, to be an underestimation of the actual species richness within the samples and instead represents a less rich community comprising the numerically dominant components, or those preferentially amplified by PCR (Muyzer *et al.* 1993; Murray *et al.* 1996; Muyzer & Smalla 1998; Zhao *et al.* 2005). Culture also has significant limitations (Stolp 1988), and the three culture media used here (full and half-strength nutrient agar and R2 agar) were chosen to try to maximize growth of a diverse culturable population, but cannot be regarded as exhaustive. It was clear from the identification of amplified 16S rDNA here that, with the exception *Pseudomonas* spp., there was little overlap between the genera identified by the two methods. While both approaches have their limitations, this finding supported the importance of combining the two techniques and highlighted the disparity of using either alone.

Statistically quantifying bacterial community structure and richness

TGGE, a comparable method to denaturing gradient gel electrophoresis (DGGE), is a long-established technique for the determination of bacterial diversity within environmental samples (Muyzer 1999). Using the TGGE-based method in this study, we have shown that the bacterial communities of individual zebra finches remained stable over time, that individual birds had distinct intestinal bacterial assemblages and that there was no significant difference between sexes in their bacterial richness. We were able to statistically quantify these effects by employing Mantel's tests and a novel GLM approach to the analysis of these data. Unlike most previous studies, which have assessed community structure simply by visually inspecting the dendrograms generated from the similarity matrices, or inferring structure from the raw similarity coefficients or banding patterns (e.g. Zoetendal *et al.* 1998; Simpson *et al.* 1999, 2002; Wei *et al.* 2007), we used the dendrogram to quantify distinct groups of samples sharing similar bacterial communities and used these groups as the dependent variable in generalized linear (mixed) models with Poisson errors. In so doing, we were able to quantify the factors correlated with similarities and differences in bacterial community between individual samples, including host identity, host sex and sampling period. Because our analysis explicitly assumed that the error distribution was approximately Poisson, we also conducted a permutation test (randomization without replacement) to generate a null distribution against which the observed distribution of data could be tested, so relaxing this assumption.

As far as we are aware, this statistical approach has not been used previously to analyse these kinds of data. However, some recent studies have used Mantel's test (Lucas & Heeb 2005; Ruiz-Rodríguez *et al.* 2009), which was originally developed to analyse spatial clustering of disease (Mantel 1967), and in ecology is mostly used to analyse the spatial correlations between, for example,

geographical distance and the genetic similarity of spatially separated communities (Fielding 2007). Mantel's test has the advantage that it examines correlations between variables across the whole range of distance similarities, whereas the GL(M)M approach employed here assesses correlations at a single threshold distance similarity and so effectively partitions the similarity matrix data into values above and below that threshold similarity. However, while Mantel's test is extremely useful and powerful for simple analyses like the one presented here, it is not available in most current statistical packages and is unwieldy for more complex analyses (although partial Mantel coefficients can be calculated in some software packages). In contrast, the GLM approach employed here uses the dissimilarity matrix to define clusters in the metric of interest which can then be used as the dependent variable in standard GLM or GLMM models, allowing multiple predictors to be assessed simultaneously and for random effects to be included; a clear advantage for more complex datasets like those generated under field conditions. Comparison of the results using both methods suggests that they are broadly comparable for the current dataset.

Intrinsic and extrinsic determinants of bacterial community structure

In the present study, housing conditions and the composition of feed were standardized for all birds throughout the study. Despite this, the composition of bacterial communities showed significant variation between birds and remained consistent within birds over a 10-week period, indicating that individuals had distinctive faecal bacterial communities. This is consistent with findings from several previous studies showing the composition of bacterial communities in humans, dogs, pigs, captive pandas and chickens to be both host-specific and relatively stable over time (Zoetendal *et al.* 1998; Simpson *et al.* 1999, 2002; Lu *et al.* 2003; Wei *et al.* 2007), although in most of these cases, specificity and stability were not statistically quantified in any way. In contrast, a recent study of cross-fostered nestling blue tits and great tits showed that environmental factors (rearing nest) had a greater influence on the gut microflora than did host-specific factors (species identity), although this could be a consequence of the fact that very young birds were used (Lucas & Heeb 2005). Given the absence of environmental differences in our study, host-specific factors, such as homeostatic mechanisms that relate to nutrient supply, pH control and host secretions (Simpson *et al.* 2002), may account for the naturally occurring differences in the composition and species richness of bacterial communities detected in the zebra finches.

Previous studies on a range of animal taxa have identified significant sex differences in the prevalence, intensity and/or diversity of parasites (including bacteria), especially in sexually dimorphic species (Poulin 1996; Schalk & Forbes 1997; McCurdy *et al.* 1998; Moore & Wilson 2002). In the present study, however, we found no significant differences between the sexes in their bacterial communities, despite the fact that zebra finches are sexually dimorphic (Zann 1996) and males and females were housed in separate aviaries, so preventing physical contact between the sexes (although they were exposed to the same environment because the aviaries were in the same room). This lack of a sex difference in bacterial communities may reflect the importance of behaviour in generating sex-specific differences in bacterial infections (Moore & Wilson 2002).

Relevance of bacteria identified in faeces

The predominant bacterial genera identified by both sequence analysis of TGGE-derived bands and through culture were *Atopobium*, *Bacillus*, *Campylobacter*, *Catellicoccus*, *Enterobacter*, *Lactobacillus*, *Pseudomonas* and *Staphylococcus* (Table 2). *Enterobacter* species, which were detected through culture only, have been sporadically isolated from healthy captive birds, including psittacines (parrots, macaws and parakeets) and raptors (Bangert *et al.* 1988a,b). In general, *Bacillus* and *Lactobacillus* species are rarely considered pathogenic (Sneath *et al.* 1986) and have both previously been detected in the digestive systems and faeces of healthy birds (Byrd *et al.* 2001; Lan *et al.* 2004; Barbosa *et al.* 2005; Lu *et al.* 2008). They are thought to help prevent colonization of the avian gastrointestinal tract by pathogenic bacteria (Lu *et al.* 2003), and spores of *Bacillus subtilis* have recently been introduced as probiotic feed additives in the poultry industry to enhance the gut microflora and reduce colonization by pathogenic bacteria (Barbosa *et al.* 2005; Cartman *et al.* 2008). Common to numerous samples in this study was a TGGE band that was homologous to the bacterial genus *Catellicoccus*. Using molecular techniques, this bacterium was shown to be ubiquitous in gull faeces, although it was suggested that the organism had restricted host specificity, as it was not detected in any other faecal samples, including those of several other avian species (Lu *et al.* 2008). *Catellicoccus* is closely related to *Lactobacilli* (Sneath *et al.* 1986), which have been shown to have beneficial effects for the host, including promotion of gut maturation, antagonism against pathogens and immune modulation (Jin *et al.* 1998; Lan *et al.* 2005).

Potentially pathogenic bacteria detected in this study included *Campylobacter lari*, which was first identified in the 1980s, when isolated from gulls (Benjamin *et al.* 1983), and has since been isolated from a variety of

environmental and animal sources, including several wild bird species (Lastovica & Skirrow 2000; Moore *et al.* 2002; Waldenström *et al.* 2002; Matsuda & Moore 2004). Although a potential pathogen *C. lari* has frequently been isolated from apparently healthy avian carriers (Kapperud & Rosef 1983; Pacha *et al.* 1988; Waldenström *et al.* 2002) and was isolated from the majority of zebra finches in this study, which were outwardly healthy at the time of sample collection. Staphylococci are relatively common disease agents of domestic poultry (Droual *et al.* 1997; White *et al.* 2003), and infections with *Staphylococcus aureus* are frequently secondary to impairment of the host defence mechanisms (Wobeser & Kost 1992). While *Pseudomonas* species can be potentially pathogenic to birds (Levesque *et al.* 2000; Walker *et al.* 2002; White *et al.* 2003), they have been isolated from the feathers of apparently healthy individuals (Shawkey *et al.* 2005).

Conclusions

Application of TGGE enabled the variability and temporal stability of bacterial communities within and between individual birds to be reliably monitored and provided robust evidence that individual birds exhibit a consistent bacterial community, as demonstrated by stable and repeatable banding patterns. By demonstrating that, in the absence of environmental variation, the composition of bacterial assemblages is both host-specific and stable over time, bacterial community patterns in populations of wild birds may be evaluated with greater confidence. In particular, we have shown that single faecal samples can be used to provide a repeatable indication of an individual bird's current gut microflora. Given that repeatability sets the upper limit to heritability (Falconer & Mackay 1996), the current study also provides a first step in characterizing the genetic contribution to determining the bacterial community of a passerine species that is an important model organism in avian sexual selection and behavioural ecology studies (e.g. Burley 1981; Lemon 1991; Royle *et al.* 2002; Birkhead *et al.* 2005; Feher *et al.* 2009).

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CB is an ecologist with research interests in the fields of disease epidemiology, animal behaviour and sexual dimorphism. GR is a microbial ecologist with research interests in all aspects of bacterial survival and adaptation. RP is a microbial ecologist whose main research interest is in environment and health. KW is an evolutionary ecologist whose main research interest is in host-parasite interactions. IH is a behavioural ecologist with an interest in bird family conflicts.
