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Article

Characteristics of Wild Bird Resistomes and Dissemination of Antibiotic Resistance Genes in Interconnected Bird-Habitat Systems Revealed by Similarity of bla_{TEM} Polymorphic Sequences

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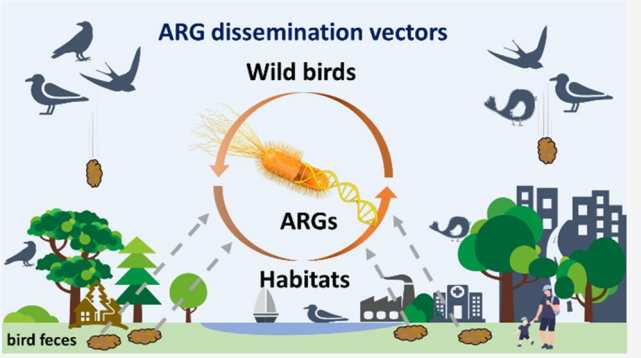
ABSTRACT: Wild birds are known to harbor and discharge antibiotic-resistant bacteria (ARB) and their associated antibiotic resistance genes (ARGs). However, assessments of their contribution to the dissemination of antibiotic resistance in the environment are limited to culture-dependent bacterial snapshots. Here, we present a high-throughput sequencing study that corroborates extensive ARG exchange between wild bird feces and their habitats and implies the need to scrutinize high-mobility birds as potential vectors for global propagation of ARGs. We characterized the resistome (281 ARGs) and microbiome of seven wild bird species and their terrestrial and aquatic habitats. The resistomes of bird feces were influenced by the microbial community structure, mobile genetic elements (MGEs), and residual antibiotics. We designated 33 ARGs found in more than 90% of the bird fecal samples as core ARGs of wild bird feces, among which 16 ARGs were shared as core ARGs in both wild bird feces and their habitats; these genes represent a large proportion of both the bird feces (35.0 ± 15.9%) and the environmental resistome (29.9 ± 21.4%). One of the most detected β-lactam resistance genes (bla_{TEM}, commonly harbored by multidrug resistant "superbugs") was used as molecular marker to demonstrate the high interconnectivity of ARGs between the microbiomes of wild birds and their habitats. Overall, this work provides a comprehensive analysis of the wild bird resistome and underscores the importance to consider genetic exchange between animals and the environment in the One Health approach.



KEYWORDS: *wild birds, microbiome, resistome, antibiotic resistance genes, ARG dissemination*

INTRODUCTION

Dissemination of antimicrobial resistance is one of the most serious global issues threatening public health. While the spread of antibiotic resistance genes (ARGs) within clinical settings has been extensively considered, a growing number of studies have quantitatively addressed how the widespread use of antibiotics in the livestock, poultry and aquaculture industries[1,2] and the associated ARG discharges with animal feces contribute to the development of the environmental resistome.[1,3−7] Prior studies have shown that ARG abundance and diversity in the feces of farmed animals could be significantly influenced by the use of antibiotics in animal agriculture.[8] Moreover, vast amounts of ingested antibiotics (between 30% and 90%) are excreted unchanged,[9] which have been suggested to exert selective pressure for ARG maintenance and propagation in receiving environments.[10−13] In particular, domestic animals dwelling with human beings (e.g., pets) contribute to the pool of clinically relevant ARGs and antibiotic-resistant bacteria.[14,15] Notably, ARGs may be transferred across human, animals, and environmental microbiomes, which represent interdependent ecosystems of potential relevance to public health.[16,17]

Growing urbanization and increasing fragmentation of natural habitats may fortuitously expand the role of wildlife on ARG propagation, including invertebrates,[18] avian,[19] and mammals,[20,21] since animals increasingly forage on food sources and water contaminated by residual antibiotics and bacteria harboring ARGs.[22−25] Wild birds that frequently inhabit ARG-contaminated environments have been postulated as sentinels, reservoirs, and potential spreaders of antibiotic resistance.[26−28] Some migratory bird species may even expand

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ARG dissemination to faraway locations, which would accelerate the globalization of antimicrobial resistance.[16]

Several studies have characterized resistant bacteria isolated from wild birds, including pigeons,[29] ducks and geese,[30−32] cormorants,[33] gulls,[34−39] passerines[40] and rooks.[41] Of most concern are bacteria carrying multidrug resistance ARGs such as New Delhi metallo-β-lactamase encoding gene *NDM-1*[3] and colistin resistance *mcr*-1,[42,43] which respectively confer resistance to clinically relevant β-lactams and colistin.[39,44−50] Several studies have documented that wild birds living in environments affected by human activities generally harbor more ARGs than those in remote areas.[19,51] Moreover, bird gut microbiota are influenced by host phylogeny, age, sex, health, diet and other environmental factors,[52] resulting in diverse and dynamic fecal ARG profiles. Birds are exposed to microbes at their foraging or nesting sites and dispose of fecal microbes broadly, representing a potentially important link in ARG exchange and dissemination. It is not yet clear, however, whether extensive ARG exchange occurs between wild animals and their habitats and how this exchange shapes the birds' and environmental resistomes, which are important knowledge gaps to advance the One Health approach.[16]

Sporadic reports based on culture-dependent approaches and ARG sequence homology suggest that *mcr-1* and β-lactamase genes carried by birds are linked to their habitat resistome.[3,43] This suggests their value as molecular markers to assess ARG transfer between wild birds and their habitats. However, due to limitations of culture-based approaches, such results provide only snapshots of potential ARG exchange between bird gut and indigenous habitat microbiomes. It is important to assess microbiome interconnectivity between wild birds and their habitats, based on a larger data set of DNA sequences that includes unculturable bacteria, to inform the significance of wild birds in facilitating global ARG dissemination.

Episodes of natural selection for a given gene may leave molecular "footprints" in DNA sequences or adjacent genomic regions that could theoretically be used to assess gene transfer between different habitats. These unique signatures are gene polymorphisms generated from combinations of basic evolutionary processes such as genetic drift and mutation.[53] Previous studies of aminoglycoside 6′-*N*-acetyltransferase AAC(6′)-Ib polymorphisms[54] revealed that AAC(6′)-Ib polymorphic diversity is closely associated with their specific ecological niche. Polymorphisms of the class A β-lactamase gene bla_{TEM} have been implicated in the extended spectrum of antibiotic resistance.[55] A previous study found that 5411 TEM β-lactamase protein sequences obtained from the NCBI GenBank database exhibited variations in critical residues that were related to their broader resistance spectra.[54] The occurrence of nearly identical ARG nucleotide sequences (more than 99% nucleotide identity) in different bacterial species was shown to be an indicator of recent horizontal gene transfer (HGT).[56,57] This encouraged us to consider nearly identical nucleotide sequences of bla_{TEM} sequences (an ubiquitous ARG in bird feces and the environment[42,58]) to assess ARG interconnectivity between wild birds and their habitats.

In this study, we used quantitative PCR (qPCR) to characterize the resistome of fresh feces from seven bird species (i.e., pigeon (*Columba livia*), sparrow (*Passer domesticus*), chough (*Pyrrhocorax pyrrhocorax*), swallow (*Hirundo rustica*), black-headed gull (*Larus ridibundus*), snowy owl (*Bubo scandiacus*), and common buzzard (*Buteo buteo*)) to assess interspecies variability. We screened 292 genes (16S rRNA, ARGs and MGEs) to investigate the wild bird fecal resistome and compared them to those of their habitats (e.g., soil for terrestrial birds and water for waterfowl). Wild bird fecal microbial community structures were analyzed using 16S rRNA gene amplicon sequencing. High-throughput sequencing was used to investigate ARG interconnectivity by analyzing high-similarity DNA sequences (>99% nucleotide identity)[57] of the common β-lactam resistance gene, bla_{TEM}, in both bird feces and their interconnected habitats. Analysis of antibiotic residues in bird feces were also conducted to help interpret the data. Overall, this study provides a comprehensive analysis of the wild bird fecal resistome and provides supplementary evidence for extensive ARG dissemination between wild birds and their habitats to implicate migratory birds as potential vectors for ARG dissemination in the environment.

MATERIALS AND METHODS

Sampling. A total of 35 samples of fresh bird feces were collected within minutes to hours of deposition, including four fecal samples from pigeon (PI1−PI4), 12 from sparrow (SP1−SP12), two from chough (CH1−CH2), five from swallow (SW1−SW5), 10 from black-headed gull (BH1−BH10), one from snowy owl (SO1), and one from common buzzard (CB1). Ten soil samples within a 1 km radius from the feces of several terrestrial birds (pigeon, sparrow, swallow, and chough) and eight water samples from habitats of waterfowl black-headed gulls were also collected and analyzed to represent the environments that were associated with the corresponding birds. Detailed information, including sampling strategies, time, and sites, is provided in the Supporting Information (Text S1 and Table S1 and S2). The fecal samples, soil samples, and water samples were collected in sterile plastic tubes and shipped to the lab on ice immediately. Subsequently, the fecal samples and soil samples were lyophilized and homogeneously mixed followed by storage at −20 °C for further use. Water samples were stored at 4 °C and filtered within 48 h. Total DNA was extracted from all the fecal/soil/water samples using a QIAamp PowerFecal DNA Kit (Qiagen, Germany)/DNeasy PowerSoil Pro Kit (Qiagen, Germany)/E.Z.N.A. Water DNA kit (Omega Biotek, America) according to the manufacturer's instructions.

Quantification of ARGs and Bacterial Communities. The qPCR analysis was performed to quantify the 281 ARGs, 10 MGEs (eight primer pairs for transposase genes, one primer pair for class 1 integron-integrase gene− *IntI1* class1, and one primer pair for "clinical" class 1 integron-integrase gene−clinical *IntI1*[59]), and 16S rRNA gene (reference gene) in the samples as previously described.[60] All of the primers used in this study are provided in Table S3. The relative ARG copy number was calculated as the relative ARG copy number = $10^{(Ct(ARG)-Ct(16S))\ (10/3)}$, where Ct(ARG) and Ct(16S) refer to the cycle thresholds for ARGs and the 16S rRNA gene in the qPCR amplification, respectively. For Illumina sequencing, the V3−V4 region of the bacterial 16S rRNA gene was amplified using the universal primers 338F and 806R. Sequencing and library construction were performed by Beijing Biomarker Technologies Co. Ltd., and the Biomarker biocloud platform (www.biocloud.org) was applied for the bioinformatics analysis. Details about the amplification and sequence analysis are provided in Text S2.

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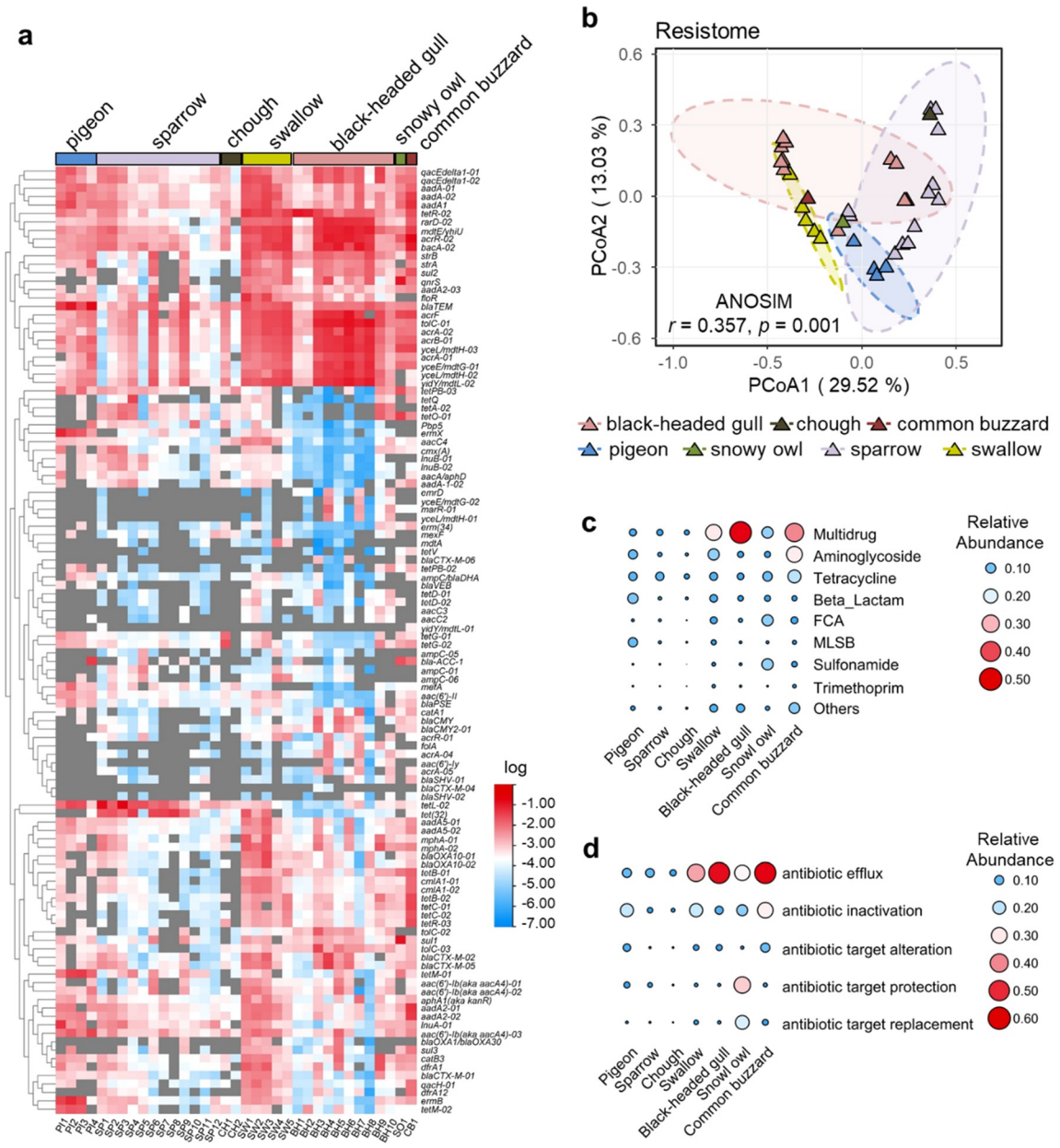

Figure 1. Characteristics of antibiotic resistance genes (ARGs) in wild bird feces. (a) Heatmap shows the abundance ARGs (ARG copies/16S rRNA copies) in each bird feces sample. (b) Principal coordinate analysis (PCoA) plots depict Bray−Curtis distances between bird feces samples. Fecal resistome from different bird species clusters separately (analysis of similarity, ANOSIM, r = 0.357, p = 0.001). Comparisons of ARG abundance among different bird species based on classification of (c) the antibiotics to which they conferred resistance and (d) the mechanism of resistance. FCA: fluoroquinolone, quinolone, florfenicol, chloramphenicol, and amphenicol, MLSB: macrolide-lincosamide-streptogramin B. Pigeon: PI1−PI4, sparrow: SP1−SP12, chough: CH1−CH2, swallow: SW1−SW5, black-headed gull: BH1−BH10, snowy owl: SO1, and common buzzard: CB1.

Analysis of Residual Antibiotics. A total of 33 of the 35 bird fecal samples collected were used to determine antibiotic residues in this study (i.e., two black-headed gull fecal samples collected from Tianjin, China, were not tested due to insufficient sample mass). A total of 19 antibiotic compounds were quantified by high-performance liquid chromatography combined with tandem mass spectrometry (HPLC-MS/MS) as previously described,[61,62] including two β-lactams (cefalexin and ampicillin), four quinolones (enrofloxacin, lomefloxacin, ofloxacin, and ciprofloxacin), six sulfonamides (sulfadiazine, sulfachlorpyridazine, sulfadimethoxine, sulfamethoxazole, sulfadimidine, and trimethoprim), four tetracyclines (chlortetracycline, doxycycline, oxytetracycline, and tetracycline), and three macrolides (roxithromycin, tylosin, and erythromycin). Details of antibiotic extraction procedures, limit of quantification (LOQ), relative standard deviation (RSD), and validation of analytical methods are further described in Text S3.

Targeted Gene Sequencing and Analysis. A partial fragment of bla_{TEM} (12F to 478R) was amplified using barcode primers, and the amplicons were sequenced using the Illumina HiSeq 2500 platform as detailed in Text S4. Sequencing and library construction were performed by Beijing Biomarker Technologies Co., Ltd. Raw tags were obtained by merging paired-end reads using FLASH (v1.2.7)[63] followed by filtering

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and clustering. The merged tags were aligned with the primers, while the FASTX-toolkit was used to discard tags with more than six mismatches.[64,65] Tags with an average quality score <20 in a 50 bp sliding window were truncated using Trimmomatic,[66] and tags shorter than 350 bp were removed. We identified possible chimeras by employing UCHIME.[67] Finally, an average of 163,891 clean sequences were obtained from each sample, and the average length was 426 bp. The denoised sequences were clustered using VSEARCH, and tags with similarity ≥99% were regarded as a bla_{TEM} variants.

Data Analysis. The mean and standard deviation of ARGs and MGEs were calculated using Microsoft Excel 2017. SPSS version 22.0 was used to determine the normally distribution of the data by the Shapiro−Wilk test. Differences of resistome and microbiome between different bird species and between bird and soil and water were analyzed by the analysis of similarities (ANOSIM) test using RStudio with the vegan package. The Pearson correlation coefficients between log(tetracycline concentration) and log(tetracycline ARG abundance) were calculated using SPSS version 22.0. The Spearman correlation coefficients between ARGs and MGEs were calculated using RStudio with the Hmisc package, and the p-value was adjusted with the fdrci package. Network analysis based on Spearman's correlation coefficients ($r > 0.6$, $p < 0.05$) between bacterial genera and genes was visualized using Gephi 0.9.2. Principal coordinates analysis (PCoA), the Procrustes test, and the Mantel test of ARGs and microbial communities were performed using RStudio with the vegan package and visualized using the ggplot2 package.

RESULTS AND DISCUSSION

Characteristics of Wild Bird Fecal Resistomes. A qPCR analysis with 292 primer sets was performed to investigate the abundance and diversity of ARGs and MGEs in fecal microbiomes from seven wild bird species: pigeon, sparrow, chough, swallow, black-headed gull, snowy owl, and common buzzard. A total of 112 ARGs and eight MGEs (six transposase genes, a class 1 integron-integrase gene− *IntI1* class1, a "clinical" class 1 integron-integrase gene−clinical *IntI1*[59]) were detected in bird feces. The number of detected ARGs in each fecal sample ranged from 54 to 108 (Figure 1a). We designated the 33 ARGs found in more than 90% of the bird fecal samples as core ARGs; these included genes conferring resistance to aminoglycoside (*aac(6′)-Ib*, *aac(6′)-II*, *aacA/aphD*, *aacC4*, *aadA*, *aadA1*, *aadA2*, *aadA5*, *aphA1*, *strB*), β-lactam (bla_{TEM}, bla_{CTX-M}, bla_{PSE}), FCA (fluoroquinolone, quinolone, florfenicol, chloramphenicol, amphenicol) (*catB3*), MLSB (Macrolide-Lincosamide-Streptogramin B) (*lnuA*, *lnuB*, *mphA*, *ermB*), tetracycline (*tetB*, *tetG*, *tetL*, *tetR*, *tetM*), and multidrug resistance (*acrA*, *acrB*, *acrF*, *acrR*, *mdtE/yhiU*, *rarD*, *tolC*, *yceE/mdtG*, *yceL/mdtH*, *yidY/mdtL*).

ARG profiles varied among different bird species. A principal coordinates analysis (PCoA) showed that parts of the bird resistome clustered based on the host bird species (Bray−Curtis distance, ANOSIM, $r = 0.357$, $p = 0.001$) (Figure 1b), which agrees with previous observations that fecal ARG profiles differ between bird species.[42,68] Genes coding for antibiotic efflux pumps predominated in feces from black-headed gull, swallow, common buzzard, chough and sparrow, while snowy owl fecal ARGs were dominated by those coding for antibiotic target protection, and pigeon feces ARGs were dominated by those coding for antibiotic inactivation (Figure 1c). The most abundant β-lactam resistance gene in wild bird feces was bla_{TEM}, which was 10-fold more abundant in pigeon [$(8.77 \pm 5.79) \times 10^{-2}$ copies/16S rRNA copy] than in all other bird samples (averaging 8.09×10^{-3} copies/16S rRNA copy). Sparrows excreted the highest abundance of *tetL*-02 (approximately 2-fold higher than that for pigeons, 37-fold higher than for choughs, 47-fold higher than for swallows, and over 100-fold higher than for black-headed gulls) and *tet*(32) (68-fold higher than for pigeons, 67-fold higher than for choughs, 2-fold higher than for swallows, and 327-fold higher than for black-headed gulls).

We observed divergence of resistance profiles among sparrow feces collected from different regions (Figure S1, Bray−Curtis distance, ANOSIM, $r = 0.738$, $p = 0.001$). Sparrow feces from Weinan had lower abundances of nearly all ARG types than those from Tianjin and Shijiazhuang cities (Figure S2). A regional difference of bird fecal resistomes was also observed in black-headed gulls (Figure S3, Bray−Curtis distance, ANOSIM, $r = 0.992$, $p = 0.002$). Gulls from Kunming (BH3−BH8) excreted predominantly multidrug, aminoglycoside and β-lactam resistance genes, while those from Qingdao (BH1 and BH2) excreted predominantly tetracycline resistance genes. The regional differences of the fecal resistome of black-headed gulls might be related to their different dietary intake; i.e., gulls from Kunming mostly forage on bird feed provided by the local government, while those from Qingdao and Tianjin mostly pray on fish and other aquatic organisms.

Antibiotics and MGEs Contribute to a Rich and Diverse Wild Bird Fecal Resistome. Wildlife can ingest pollutants that exert selective pressure for the maintenance and enrichment of ARGs (e.g., veterinary pharmaceuticals[69,70] and heavy metals[71]). Here, 12 of the 19 antibiotic compounds tested were detected in bird feces. Quinolones were detected in all bird feces samples (17.9 ± 7.2 ng/kg) (Figure 2a). Quinolones were previously found at high levels (54.5 ± 6.6 μg/L) in other wild birds, such as in the blood of nestling golden eagles[69] and vultures.[70] The prevalence of quinolones in wild birds might be due to their common presence in soil[72] and surface water.[73] Tetracycline and oxytetracycline were detected in over 80% of the samples and were more abundant in sparrow feces with a maximum oxytetracycline concentration reaching 5.5 μg/g dry feces. Notably, tetracyclines were present at significantly higher concentrations in sparrow feces from Tianjin (SP6-SP9) and Shijiazhuang (SP1-SP5), which may partly explain the higher abundance of tetracycline resistance genes in sparrow feces than in those from the Weinan region. Furthermore, a significant positive correlation was observed for sparrow feces between the total concentration of tetracyclines and the abundance of total tetracycline resistance genes (Figure 2b, Pearson correlation coefficient $r = 0.717$, $p = 0.009$). These data corroborate that residual antibiotics (from dietary intake or produced by bacteria and fungi) may contribute some selective pressure for ARGs.[58] Dietary intake of residual antibiotics (and possible resistant bacteria harboring ARGs) may be more obvious for synanthropic birds such as sparrows and swallows that have frequent opportunities to interact with human communities.

MGEs were also analyzed to assess their potential role in ARG dissemination through HGT. Eight MGEs including six transposases genes (*tnpA-01*, *tnpA-02*, *tnpA-03*, *tnpA-04*, *tnpA-05*, *tnpA-07*), *IntI1* class1, and clinical *IntI1*, were broadly detected in wild bird feces. The abundance of total MGEs was $(8.55 \pm 9.83) \times 10^{-2}$ copies/16S rRNA copy (Figure 1a), with *tnpA-05* (a marker gene of the insertion sequence *IS6* group)

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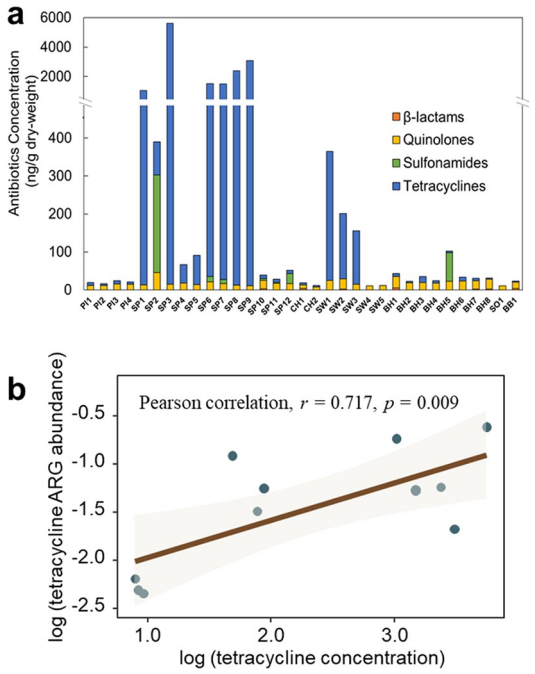

Figure 2. Antibiotics partly contribute to the wild bird fecal resistome. (a) Concentrations of antibiotics in the bird feces, including β-lactams, quinolones, sulfonamides, and tetracyclines. (b) Positive correlations between the log (tetracycline ARG abundances) and log (tetracyclines concentration) (Pearson, $r = 0.717$, $p = 0.009$) in the feces of sparrow.

as the most abundant transposase gene [$(5.76 \pm 6.92) \times 10^{-2}$ copies/16S rRNA copy]. *IntI1* class1 (which may disseminate ARGs as mobile gene cassettes[59]) were detected in 97% of the bird fecal samples, with the highest abundance reaching 0.33 copies/16S rRNA copy in the feces of migratory snowy owl. Moreover, clinical *IntI1*, which is known to correlate with anthropogenic activities,[59] were found in 80% of the bird fecal samples (except chough). Such high abundance of MGEs is conducive to facilitating the transfer of bird fecal ARGs to the indigenous microbial community in the receiving environments.

The abundance of total MGEs was positively correlated with the abundance of total ARGs in wild bird feces (Spearman, $r = 0.685$, $p < 0.0001$) (Figure 3a). Specifically, *tnpA*-05 was positively correlated with 24 ARG subtypes (Spearman, $r > 0.6$, $p < 0.05$, Figure 3b). The *IntI1* class1 was positively correlated with 18 ARG subtypes. In addition, 16 ARG subtypes were positively correlated with clinical *IntI1*. MGEs are known to contribute to the worldwide spread of *mcr*-1 and *NDM*-1 across different habitats, including clinical[74−76] and environmental settings,[77−79] farm animals,[5,80,81] and wildlife.[42,82]

Fecal Resistome Was Influenced by the Corresponding Microbiome. The bacterial community structure has been previously proposed to determine the structure of the resistome of another system, soil.[83] Here, wild bird feces microbial communities were generally dominated by *Proteobacteria*, *Firmicutes*, *Actinobacteria*, and *Tenericutes* phyla (Figure 4a), as documented by previous research.[52] Among these phyla, *Proteobacteria* and *Firmicutes* were the most dominant phyla for all bird species considered in this study, accounting for 77.9 ± 14.5% of the total bacterial community. *Tenericutes* was particularly higher in swallows (25.6 ± 13.1%). *Actinobacteria* was more abundant in choughs (28.8 ± 4.3%) and pigeons (14.8 ± 7.3%). At the genus level, 522 bacteria genera in the tested wild bird feces were classified (Figure 4b).

Dominant genera of bird fecal bacteria generally varied among bird species. Sparrow fecal bacteria were dominated by the genus *Catellicoccus* (20.4%), *Pantoea* (8.7%), *Lactobacillus*

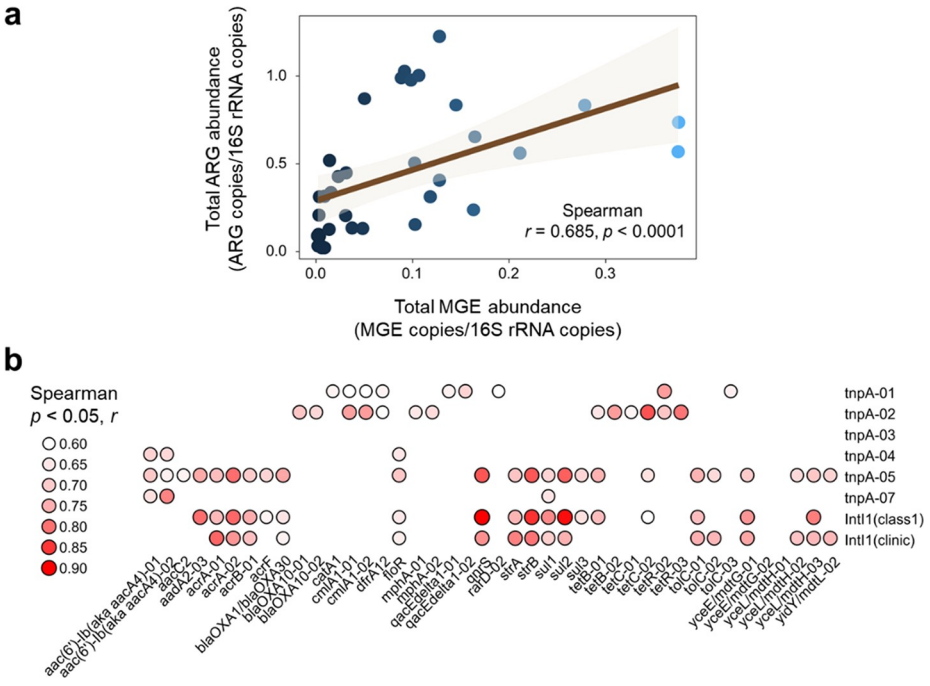

Figure 3. A richer bird feces resistome was correlated with higher abundance of MGEs that facilitate horizontal gene transfer. (a) Positive correlations between the total abundances of MGEs and ARGs (Spearman, $r = 0.685$, $p < 0.0001$) in the feces of wild birds. (b) Positive correlations between certain ARG subtypes and MGE subtypes (Spearman correlation, $p < 0.05$, $r > 0.6$, circles filled with white, light pink, to dark red represent r values ranging from 0.6 to 0.9) in wild birds.

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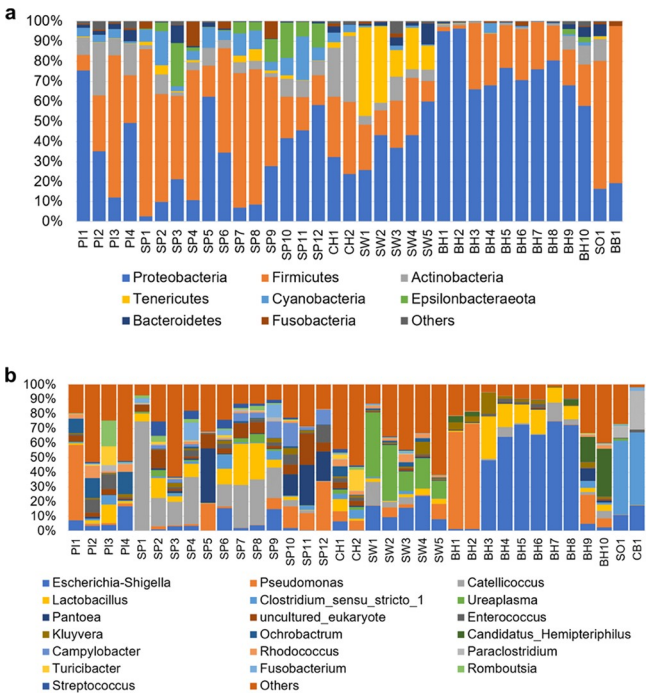

Figure 4. Microbial community composition of wild bird feces at the (a)phylum level and (b) genus level.

(8.7%), and *Pseudomonas* (7.7%), while swallow fecal bacteria were dominated by *Ureaplasma* (25.6%), *Escherichia-Shigella* (14.8%), *Catellicoccus* (5.0%), *Pseudomonas* (4.1%), and *Lactococcus* (2.9%). Common buzzard and snowy owl fecal microbiomes were similar and were both dominated by *Clostridium_sensu_stricto_1* (50.5% and 49.5%). Black-headed gulls from different regions harbored different fecal microbiomes. While *Escherichia-Shigella* and *Lactobacillus* genera were predominant in feces from black-headed gulls in Yunnan (BH3-BH8), the *Pseudomonas* genus was predominant gull feces from Qingdao (BH1 and BH2). Previous studies have demonstrated that diet contributes to alterations in bird gut microbiota[84] and have an even greater impact on the gut microbiome than the host's phylogeny.[85] Besides host phylogeny and diet, other characteristics that may influence the fecal microbiomes include bird age, sex, reproductive cycle, and environmental factors (e.g., temperature and pollution).[86−88]

A principal coordinates analysis shows that parts of the bird fecal microbiome clustered with the corresponding bird species (Bray−Curtis distance, ANOSIM, r = 0.424, p = 0.001, Figure 5a). Procrustes analysis and Mantel tests revealed that the bacterial community structures (at the genus level) of wild bird feces and fecal ARGs pass a goodness-of-fit test (Figure 5b, Protest $M2$ = 0.555, p < 0.001, 999 permutations; Mantel test r = 0.530, p = 0.001, 999 permutations) based on the Bray−

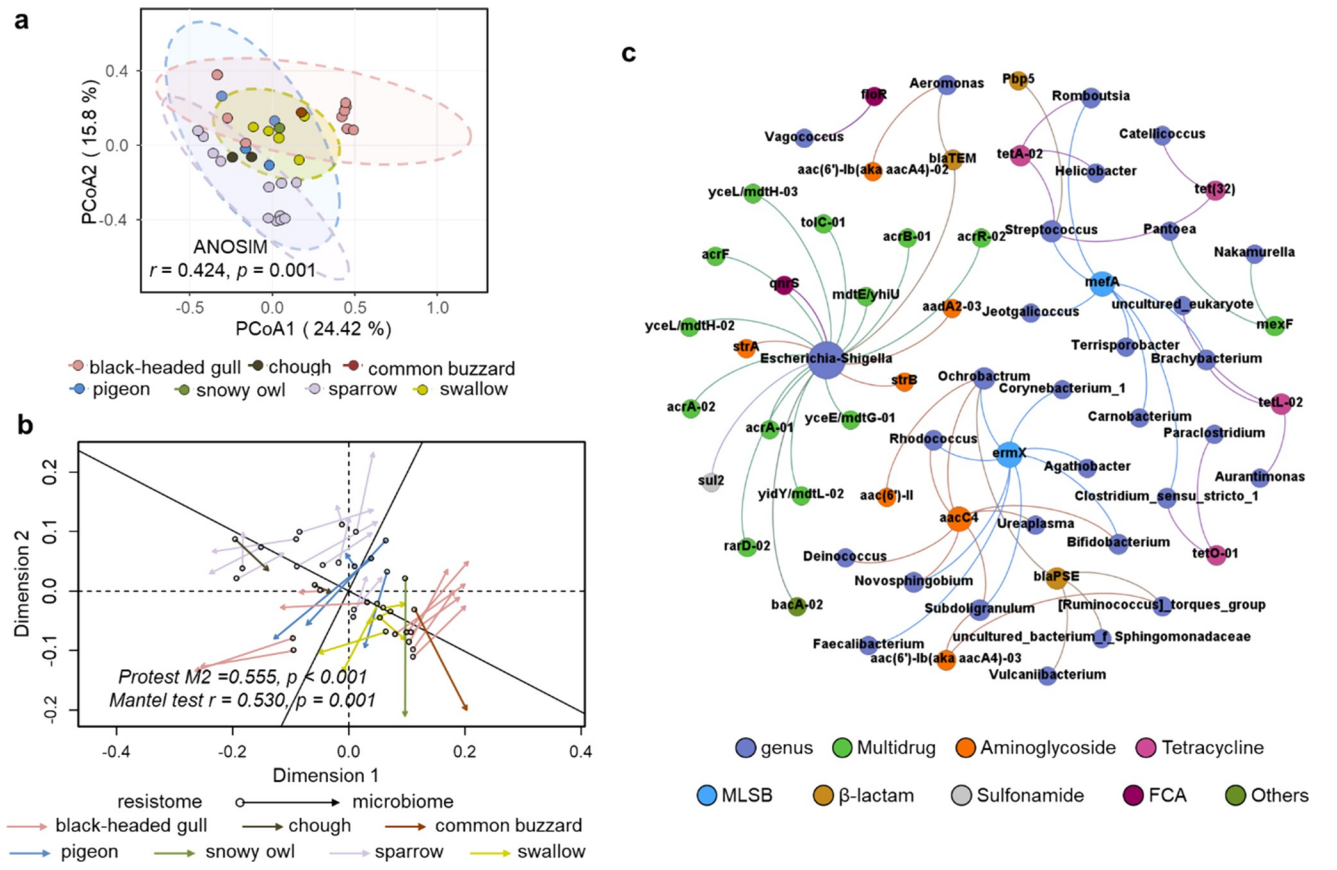

Figure 5. Associations between bird fecal microbiomes and resistomes. (a) Principal coordinate analysis (PCoA) plots depict Bray−Curtis distances between bird feces samples. Microbiomes from different bird species cluster separately (analysis of similarity, ANOSIM, r = 0.424, p = 0.001). (b) Procrustes analysis depict significant correlation between ARG abundance (Bray−Curtis) and bacterial composition (Bray−Curtis) for wild bird feces (Protest $M2$ = 0.555, p < 0.001, 999 permutations; Mantel test r = 0.530, p = 0.001, 999 permutations). (c) Network analysis revealing co-occurrence patterns of ARGs and bacterial genera. Nodes are colored based on the ARG type and bacterial genera. The node size is proportional to the node degree, and the edges present the spearman positive correlation between nodes (r > 0.6, p < 0.05).

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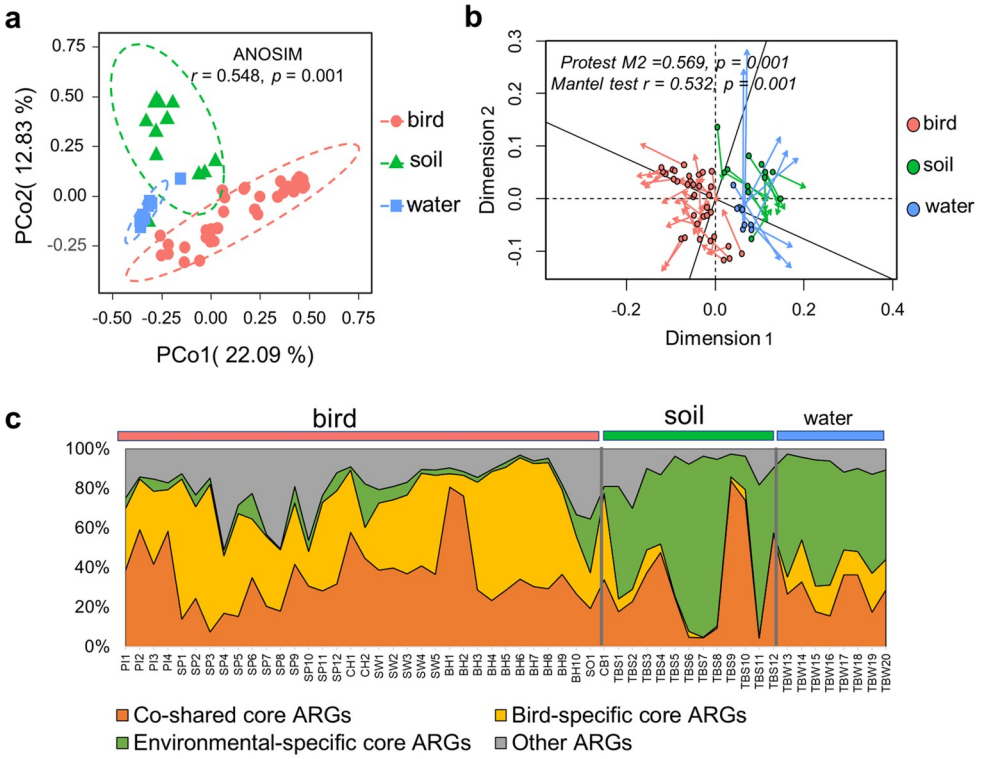

Figure 6. Shared and specific core resistome between wild bird feces and habitat samples. (a) Principal coordinate analysis (PCoA) plots depict Bray−Curtis distances. The resistome of bird feces (n = 35), soil (n = 12), and water (n = 8) cluster separately (analysis of similarity, ANOSIM, r = 0.548, p = 0.001). (b) Procrustes analysis depict significant correlation between ARG abundance (Bray−Curtis) and bacterial composition (Bray−Curtis) for soil, water and wild bird feces samples (Protest $M2$ = 0.569, p = 0.001, 999 permutations; Mantel test r = 0.532, p = 0.001, 999 permutations). (c) Relative abundance of habitat-specific core ARGs, bird-specific core ARG abundances, shared core ARGs, and other ARGs in each sample.

Curtis dissimilarity metric, which infers a modest correlation between bird resistome profiles and their microbial community structure and corroborates a recent report that the fecal resistome of migratory birds is associated with their fecal microbiome structure.[42] The correlation between fecal resistome and microbiome for black-headed gull was stronger (Protest $M2$ = 0.256, p = 0.001, 999 permutations; Mantel test r = 0.876, p = 0.001, 999 permutations, Figure S4a,b) than that for sparrow (Protest $M2$ = 0.608, p = 0.016, 999 permutations; Mantel test r = 0.528, p = 0.003, 999 permutations, Figure S4c,d). These results indicate that the fecal resistome of black-headed gulls could be mainly explained by the microbiome structure. In contrast, the sparrows' resistome was not fully explained by their microbiome and was apparently significantly influenced by relatively high levels of residual antibiotics (i.e., 8 to 5602 ng/g of tetracyclines in sparrow feces).

A spearman correlation-based network analysis (r > 0.6, p < 0.05) was used to investigate the co-occurrence pattern of ARGs and bacterial genera (Figure 5c). *Escherichia-Shigella* correlated with multiple ARGs, including multidrug resistance genes *acrA*, *acrB*, *acrF*, *tolC*, *yceE/mdtG*, *yceL/mdtH*, *yidY/mdtL*, *mdtE/yhiU*, etc. These efflux pump ARGs (such as AcrAB-TolC and its homologues, and *mdtE*, *mdtG*, *mdtH*) are commonly found in the chromosome of Gram-negative bacteria.[89] The enrichment of *Escherichia-Shigella* may contribute to the high abundance of multidrug resistance genes in the feces of black-headed gulls, common buzzards and swallows (Figure 4b). *Tet*(32) was correlated with *Catellicoccus* and *Streptococcus*, which were relatively abundant in sparrow feces. Tetracycline resistance genes such as *tetK*, *tetL*, *tetM*, *tetO*, *tetQ*, and *tetT* are frequently reported in *Streptococcus* isolates,[90] as are MLSB, β-lactam, and fluoroquinolone resistance genes.[90] Here, *Streptococcus* was also correlated with *mefA*, *tetA*-02, and *Pbp*5. Overall, our analysis suggests that both microbial community structure and HGT mediated by MGEs are key contributors to the maintenance and dissemination of the wild bird fecal resistome (Figure S5).

High Interconnectivity between the Wild Birds Fecal Resistome and That of Their Habitat. *Principal Coordinates and Procrustes Analyses Infer Significant ARGs Interconnectivity.* The resistomes of soil samples from the habitats of terrestrial birds (pigeon, sparrow, swallow, and chough) and water samples from the habitats of waterfowl (black-headed gull) were investigated to assess the interconnectivity of ARGs between wild birds and their habitats. A PCoA analysis showed that the fecal ARG profiles were different from those of the birds' habitats (Bray−Curtis distance, ANOSIM, r = 0.548, p = 0.001, Figure 6a). This is likely due, in part, to differences in bacterial community structure between bird feces and their habitats (Figure 6b, Protest $M2$ = 0.569, p = 0.001, 999 permutations; Mantel test r = 0.532, p = 0.001, 999 permutations). Nevertheless, 16 ARG subtypes with presence frequency equal or higher than 90% in both bird fecal and habitat samples were identified as coshared core ARGs, including *aac(6′)-Ib*, *aac(6′)-II*, *aacC4*, *aadA*, *aadA1*, *aadA2*, *aphA1*, *strB*, *bla*$_{TEM}$, *ermB*, *mphA*, *acrF*, *yceL/mdtH*, *catB3*, *tetG*, and *tetR*. These ARGs occupied a large proportion of the total ARG abundance in both bird feces (35.0 ± 15.9%) and environmental samples (water and soil) (29.9 ± 21.4%) (Figure 6c), corroborating the high

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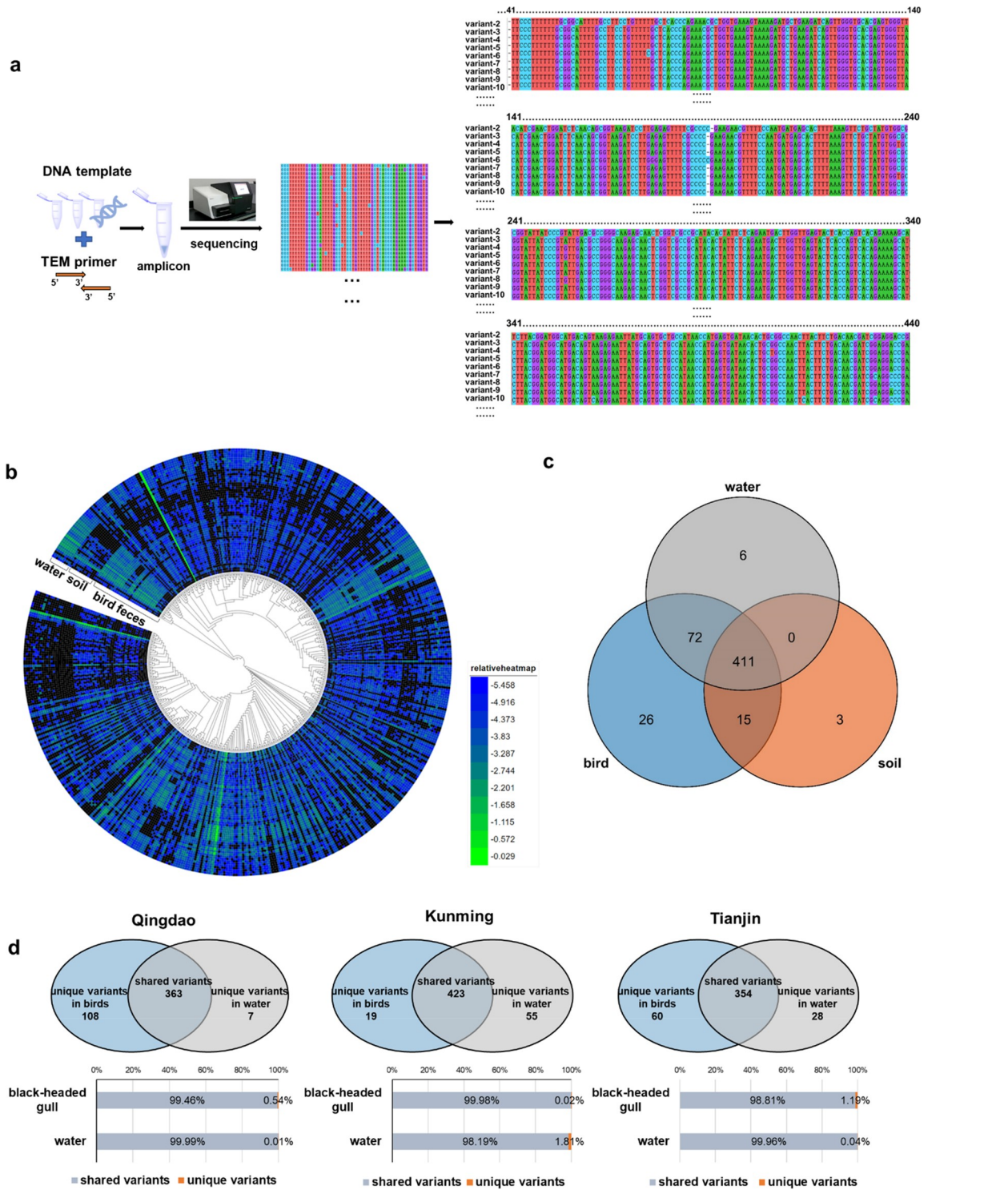

Figure 7. *Bla*$_{TEM}$ polymorphisms derived from high-through sequencing indicate resistome interconnectivity between wild birds and their habitats. (a) Flowchart of high-similarity *bla*$_{TEM}$ variants (>99% identity) acquired through high-throughput sequencing. (b) Phylogenetic tree of *bla*$_{TEM}$ variants (using the Maximum Likelihood method and Tamura-Nei model) and their relative abundances in bird feces, soil, and water samples depicted by a heatmap. (c) Co-occurrence of *bla*$_{TEM}$ variants between bird feces, soil, and water environment. (d) Shared and unique *bla*$_{TEM}$ variants and their abundances in black-headed gull feces and the surrounding water samples from Qingdao, Kunming, and Tianjin cities.

interconnectivity between the fecal bird resistome and that of their habitat.

High Interconnectivity between the Fecal Wild Bird Resistome and Their Habitat Microbiome Is Also Inferred by Bla$_{TEM}$ *Polymorphism analysis.* The polymorphisms of *bla*$_{TEM}$, which is a shared core ARG, were investigated by high-throughput sequencing to assess ARGs interconnectivity between bird feces and their habitats and the associated dissemination potential. We obtained 533 *bla*$_{TEM}$ variants with the largest sequence divergence between clusters that had as many as 20 site mutations, and the sequence identity between these variants varied from 93% to 100%. The phylogenetic tree

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of these variants and other class A β-lactamases are shown in Figures 7b and S6. These results reflect the wide ecological niches and increasing evolutionary adaptations associated with bla_{TEM} genes. We found that 411 bla_{TEM} variants were shared by birds, soil, and water environments (Figure 7c), suggesting the common transfer of these bla_{TEM} variants among wild birds and their habitat microbiomes. The bla_{TEM} sequences were dominated by five bla_{TEM} variants (variant-2, -5, -4, -7, and -8) that had higher relative abundances than 1%; together, they added up to 86.6% of the total abundance on average in the samples. Variant-2 was the most abundant, accounting for 52 ± 31% of the total bla_{TEM} sequences in each sample. These sequence divergences of clusters were preserved with high-resolution molecular signatures to assess the transferability of those detected ARGs.

Among the birds considered, black-headed gulls have a very large global population (9,932,500)[91] and migrate over long distances,[92] potentially contributing to ARG dissemination across international boundaries. A total of 363, 423, and 354 bla_{TEM} variants were coshared by gull fecal and water samples from Qingdao, Kunming, and Tianjin, accounting for more than 96% of the bla_{TEM} abundance in both bird feces and water samples. This suggests extensive translocation of ARGs between wild birds and their habitats. Some bird activities such as nesting, digging soil, and ingestion could facilitate ARG translocation between the bird gut microbiome and bacteria in the birds' habitats, and ARGs genetically linked with MGEs may disseminate further via HGT. Overall, these findings represent converging evidence of ARG transfer across ecological boundaries and underscore the need to assess and mitigate potential dissemination of high-risk ARB that can be harbored by both humans and animals.

Environmental Implications. This study provides a comprehensive analysis on the wild bird fecal resistome and microbiome and infers that bacterial community structure and MGEs play important roles in shaping these resistomes. A considerable proportion of coshared ARGs between the birds and their habitats suggests high interconnectivity of wild birds' and environmental resistomes, which was corroborated by co-occurrence of nearly identical bla_{TEM} nucleic acid sequences that prevailed in both bird feces and their habitats. Wild birds could serve as mobile sources of ARGs, and long-distance migratory species could expand local environmental ARG transmission to a global scale. Overall, this study sheds light on the potential role of the wild bird resistome in mediating ARG global dissemination and highlights the need for more attention to the propagation of ARGs across the interfaces between wildlife, their living environments, and humans.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.2c01633.

Detailed description of the sampling protocols, 16S rRNA sequencing, antibiotic quantification, and targeted gene sequencing (Text S1−S4 and Figures S1−S6); information on the collected samples (Tables S1 and S2); details of the primers used (Table S3) (PDF)

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Notes

The authors declare no competing financial interest.

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