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Molecular characterization of the Fe-hydrogenase gene marker in *Trichomonas gallinae* isolated from birds in Riyadh, Saudi Arabia

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ABSTRACT

Trichomonas gallinae causes avian oropharyngeal trichomonosis. This pathogen affects a large number of bird species and may cause substantial economic losses to the poultry industry. Al-Azizia poultry market in Riyadh, Saudi Arabia is among the largest poultry markets in the Arabian Gulf. Birds traded in this market may be exposed to a variety of *T. gallinae* strains. Genetic diversity of *T. gallinae* among birds in the market was examined using Fe-hydrogenase gene sequences. These sequences were amplified by PCR for twenty-nine isolates of *T. gallinae* from four different avian species, including 21 feral pigeons, one common mynah, three chickens, and four turkeys. Sequence analysis showed ten variant gene sequences. Nine sequences comprise a new subtype, including A(KSAF1), C(KSAF1) and C(KSAF3) with 34.48% (n = 10), 6.90% (n = 2), 6.90% (n = 2) of the isolates, respectively. Analyses also showed an additional five new sequences (KSAF1.1., KSAF2, KSAF13, KSAF14, KSAF15), representing 17.24% of the isolates. Subtype II (KSAF) was found in four feral pigeons (13.80%). To our knowledge, this report is the first to describe genotypes of *T. gallinae* from pigeons in Saudi Arabia using Fe-hydrogenase gene sequences for subtyping. Subtype analysis infers the presence of multiple genotypes of *T. gallinae* in Saudi avian populations.

1. Introduction

Infectious diseases and parasites are both regulate populations, producing marked effects on host abundance and evolution [1]. Parasites are an important component of ecosystems at various levels, and understanding the influences that underlie parasite diversity is vital to identifying ecological principles that govern biodiversity. The relationship between parasitic diseases and birds differ significantly in their health consequences and ecological complexity [2]. Disease can also be a factor in species decline to threatened, endangered or extinct status [3].

Trichomonosis is a well-known disease caused by a flagellated protozoan parasite, *Trichomonas gallinae*. The disease is widespread among avian species, commonly affecting Columbiformes [4,5], Falconiformes and Strigiformes [6–8], Psittacines [9], and Galliformes [10]. In addition, *T. gallinae* is also predominantly found in the rock dove (*Columba livia*), making it one of the primary host for this parasite [4]. The major factor contributing to the dispersion of trichomonosis is the worldwide distribution of *C. livia*, which is a clinical carrier and reservoir of *T. gallinae* [11,12]. However, trichomonosis less frequently infects *Gallus gallus* [13].

The disease predominantly spreads when one or both infected parents feed their squabs, and when adult birds share the same food and water sources [5,14]. Further, transmission can occur to birds of prey via feeding on infected birds, such as pigeons or doves [15].

Trichomonosis causes swelling of the neck that may be visible externally, and the disease may progress over several days or even weeks. Birds often become emaciated due to the formation of large

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necrotic and inflammatory lesions in the mouth and crop. These lesions may prevent swallowing food and water. Oral lesions may also impede normal breathing by obstructing the upper digestive and respiratory tracts. Frequently, mortality is secondary to starvation due to severe blockage of the esophagi [4,16].

Molecular characterization has identified different strains of *T. gallinae*. The most common gene used to sequence *T. gallinae* is 5.8S ribosomal RNA (rRNA; ITS ribotype) [17–20]. Internal transcribed spacer (ITS) region sequences are non-coding and evolve rapidly, which makes them suitable for construction of phylogenetic trees of closely related organisms [17].

Another subtype marker used to differentiate *T. gallinae* isolates is the gene encoding Fe-hydrogenase [21,22]. This gene can be targeted as a single marker locus for genotyping, specifically for amitochondrial protists, and for detecting fine-scale differences in sequence variation within isolates [21]. In the present study, we selected the Fe-hydrogenase gene as a single-marker subtyping tool for trichomonad parasites. This selection takes advantages of the genes ability to provide additional resolution for discrimination of *T. gallinae* strains [22].

Hundreds of different birds including chickens, ducks, pigeons, doves, turkeys, parrots, falcons and pet birds are offered for sale daily at the poultry market in Riyadh, Saudi Arabia. Some, such as chickens, ducks, pigeons, doves, and turkeys, come from local poultry farms; however, others, such as parrots and pet birds, are imported from outside the country [13]. The diverse geographical origins of these birds are likely to expose poultry to a variety of *T. gallinae* strains [13]. Endemic wild bird species may be threatened by this source of parasites. Moreover, this source may inflict economic losses on poultry farmers in the Kingdom of Saudi Arabia.

Strains of *T. gallinae* in the poultry market in Riyadh have been studied using the ITS region [13]; this study found 15 ribotypes, of which 12 were novel, and three were previously described as ribotypes A, C, and II. In the present study, we used the Fe-hydrogenase gene to subtype *T. gallinae* isolates from different bird species (feral pigeon, *Columba livia*; common mynah, *Acridotheres tristis*; chicken, *Gallus Gallus domesticus*; and turkey, *Meleagris gallopavo*) in Riyadh, Saudi Arabia.

2. Materials and methods

2.1. Sources of isolates

Twenty-nine isolates of *Trichomonas gallinae* were obtained by oral swab from four different bird species (feral pigeon, common mynah, chicken, and turkey) collected from the Al-Azizia poultry market Riyadh, Saudi Arabia. All samples were collected between March and December 2018. Birds were visually observed for gross presentation of oropharyngeal trichomonosis lesions.

2.2. Bird sampling and DNA extraction

DNA was extracted from all isolates of *T. gallinae* in this study following the protocol as previously described by Albeshr and Alrefaei (13), and stored at −20 °C. Procedures for sample collection were performed in strict accordance with recommendations by the Research Ethics Committee of the King Saud University, Riyadh, Saudi Arabia (Ethic Reference KSU-SE-19-77).

2.3. PCR amplification of the Fe-hydrogenase gene

The Fe-hydrogenase gene was amplified from isolated DNA by PCR using standard molecular biology protocols. Primers used for amplification were: Forward primer TrichhydFOR (5'-GTTTGGGATGGCCTCAGAAT-3′) and reverse primer TrichhydREV (5'-AGCCGAAGATGTTGTCGAAT-3′) [21,22]. PCR amplification used a 25 μL reaction volume containing 8.5 μL of Green Master Mix (2×; Thermo Fisher Scientific, Waltham, MA), 3 μL each of forward (TrichhydFOR) and reverse (TrichhydREV) primer (Eurofins Genomics, Anzinger, Germany), 8.5 μL of nuclease-free water, and 1 μL of DNA. Each reaction was run with a negative control containing no DNA and a positive control containing *T. gallinae* DNA.

PCR was performed with the following cycling conditions: initial denaturation at 94 °C for 15 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 52 °C for 30 s, and extension at 72 °C for 1 min, and a final extension at 72 °C for 5 min.

PCR products were separated with a 1% agarose gel stained with ethidium bromide and analyzed under UV light. The expected product size was approximately 1 kb. Positive PCR products were sent to Macrogen Inc. (Seoul, Republic of Korea) for sequencing.

2.4. Sequence analysis and phylogenetic tree

The molecular and phylogenetic analyses of Fe-hydrogenase sequences were performed using MEGA software version 7 [23] and CLUSTALX 2.1 [24]. All sequence data were aligned using forward and reverse complements of the reverse primer. Available *Trichomonas gallinae* and *Trichomonas vaginalis* sequences in the NCBI GenBank database (https://www.ncbi.nlm.nih.gov/genbank/) were used to compare amplified Fe-hydrogenase sequences. Phylogenetic trees of datasets obtained from the Fe-hydrogenase gene were constructed separately using neighbor-joining (NJ) and Tamura-Nei models to illustrate taxonomic relationships by nucleotide analysis [23,24]. We used Felsenstein's bootstrap test (1000 iterations) to cluster associated taxa clustered [25]. A total of 756 positions were assessed in the final dataset. Patterns of synonymous and non-synonymous substitution rates were evaluated to assess selective pressure on alleles between isolates using DnaSP v6 [26]. Allelic diversity was evaluated with a Median Joining algorithm in Network v10.1 (fluxus-engineering.com; [27]).

3. Results

3.1. Sequencing analysis of the Fe-hydrogenase gene

Twenty-nine *T. gallinae* isolates were genetically characterized using Fe-hydrogenase gene amplification. The PCR amplified fragment of about 756 bp was consistent with previous studies [21,22]. *T. gallinae* DNA was extracted amplified from four different avian orders: Columbiformes, Anseriformes, Galliformes and Passeriformes. Species included 21 (72.4%) feral pigeons, *C. livia*; four (13.8%) turkeys *M. gallopavo*, three (10.4%) chickens *G. gallus domesticus* and one (3.4%) Common mynah, *A. tristis*. Twelve (41.38%) of twenty-nine birds showed characteristic of lesions; the remaining birds (58.62%) had no visible lesions (Table 1).

The Fe-hydrogenase gene was amplified from DNA extracted from *T. gallinae* in all samples and successfully sequenced. We discovered ten variant sequences; nine were of a novel subtype. These sequences were registered with the GenBank database (accession no. MT367178, MT367179 and MT367187) (Fig. 1; Table 1). Subtype A(KSAF1) accounted for 34.48% of isolates ($n = 10$) (one common mynah, two feral pigeons, three chickens, and four turkeys). This subtype was similar to the clonal UK finch epidemic strain (A1) (GenBank: JF681136) [21]. Subtype C(KSAF1) accounted for 20.68% of isolates ($n = 6$) (all feral pigeons), and with similarity to subtype C1 isolated from rock pigeon (GenBank: AF446077) [21]. Subtype C(KSAF2) accounted for 6.90% of isolates ($n = 2$) (both feral pigeons), and with similarity to subtype C4 isolated from feral pigeon (GenBank: KC529662) [28]. Subtype C (KSAF3) accounted for 6.90% of isolates ($n = 2$) (both feral pigeons), with similarity to subtype C1 isolated from feral pigeon (GenBank: AF446077) [28]. Finally, five subtypes were novel (KSAF1.1., KSAF2, KSAF13, KSAF14, KSAF15), accounting for 17.24% of isolates (all feral pigeons). Subtype II (KSAF) was found in four feral pigeons (13.80%) and was identical to isolated obtained from Eurasian collared dove (GenBank: KP900034) [29]; this finding is the first for this genotype

Table 1
Overview of isolate ID, bird species, clinical signs, characteristic lesions, and culture using ITS ribotype and Fe-Hyd subtype of *T. gallinae* in birds from Riyadh, Saudi Arabia.

	Case ID	Host species	Origin	Clinical signs[a]	Lesions	Lesion location	Culture extract	ITS ribotype	Fe-Hyd subtype
1	R2	Feral pigeon	Domestic	−	−	−	+	−	KSAF15
2	R17	Feral pigeon	Domestic	−	−	−	+	C	C(KSAF1)
3	R18	Feral pigeon	Domestic	−	−	−	+	C	C(KSAF1)
4	R19	Feral pigeon	Domestic	−	+	Mouth	+	KSA (II)	II (KSAF)
5	R21	Feral pigeon	Domestic	−	−	−	+	−	II (KSAF)
6	R22	Feral pigeon	Domestic	−	−	−	+	−	A(KSAF1)
7	R23	Feral pigeon	Domestic	−	−	−	+	−	C(KSAF2)
8	R24	Feral pigeon	Domestic	−	−	−	+	C	C(KSAF2)
9	R27	Feral pigeon	Domestic	−	+	Mouth	+	−	C(KSAF1)
10	R30	Feral pigeon	Domestic	−	+	Mouth	+	C	C(KSAF1)
11	R31	Feral pigeon	Domestic	−	+	Mouth	+	KSA2	KSAF2
12	R36	Chicken	Domestic	+	+	Mouth	+	−	A(KSAF1)
13	R39	Feral pigeon	Wild	−	−	−	+	−	KSAF13
14	R40	Feral pigeon	Domestic	−	+	Mouth	+	−	II (KSAF)
15	R42	Feral pigeon	Domestic	−	+	Mouth	+	−	II (KSAF)
16	R43	Feral pigeon	Domestic	−	+	Mouth	+	−	KSAF14
17	R49	Chicken	Domestic	+	+	Mouth	+	A	A(KSAF1)
18	R51	Chicken	Domestic	−	−	−	+	A	A(KSAF1)
19	R54	Feral pigeon	Domestic	−	−	−	+	−	A(KSAF1)
20	R57	Turkey	Domestic	−	−	−	+	A	A(KSAF1)
21	R58	Common mynah	Wild	−	−	−	+	A	A(KSAF1)
22	R59	Turkey	Domestic	−	−	−	+	A	A(KSAF1)
23	R60	Turkey	Domestic	−	−	−	+	A	A(KSAF1)
24	R61	Turkey	Domestic	−	−	−	+	A	A(KSAF1)
25	R62	Feral pigeon	Wild	−	−	−	+	C	C(KSAF3)
26	R63	Feral pigeon	Wild	−	−	−	+	−	C(KSAF3)
27	R69	Feral pigeon	Domestic	+	+	Mouth	+	C	C(KSAF1)
28	R71	Feral pigeon	Domestic	+	+	Mouth	+	A	A(KSAF2)
29	R72	Feral pigeon	Domestic	+	+	Mouth	+	C	C(KSAF1)

[a] Clinical signs in birds are general illness, excessive salivation, dysphagia, lethargy, and difficulties in swallowing food and water.

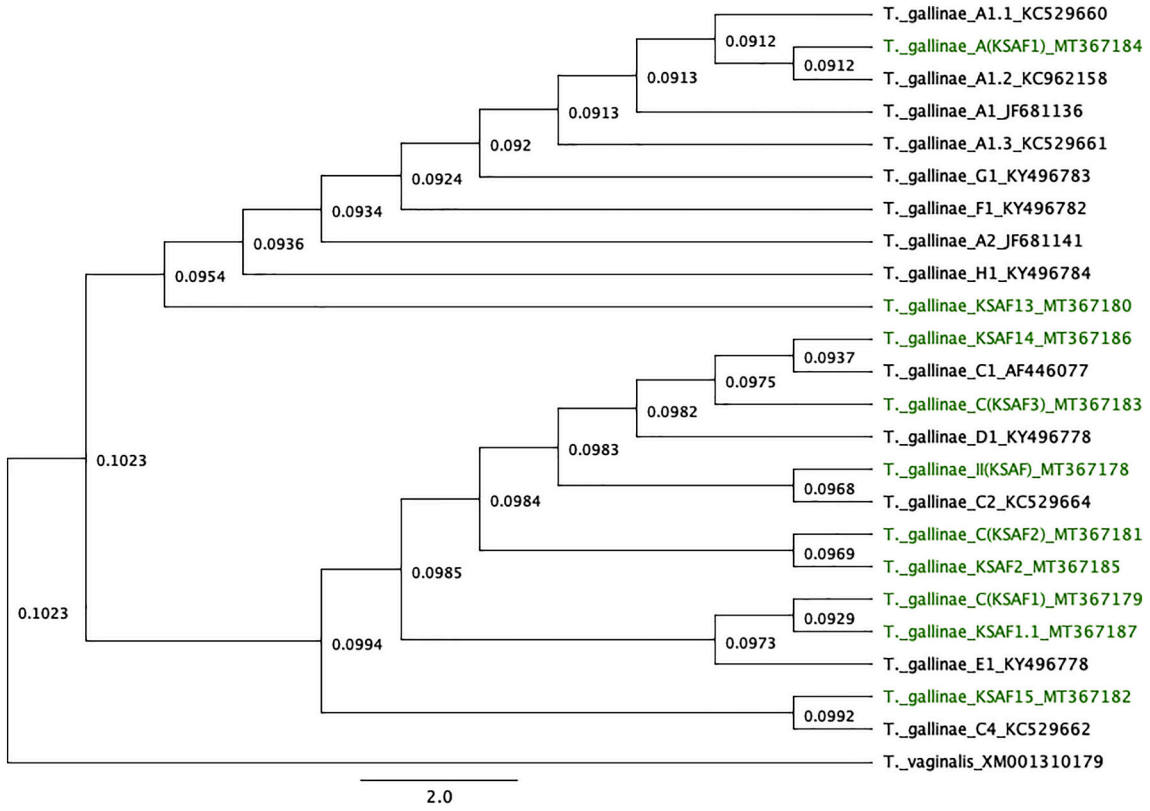

Fig. 1. Phylogenetic tree based on ITS region relationships of *T. gallinae* isolates using NJ. References to GenBank accession numbers are: JF681136, JF681141, AF446077 (Lawson et al. 2011); KC529660, KC962158, KC529661, KC529664, KC529662 (Chi et al. 2013); KY496783, KY96782, KY496784, KY496778, KY496781 (Alrefaei et al. 2019). *T. vaginalis* (GenBank accession XM_001310179 (Carlton et al. 2007) was included in the present study as an outgroup.
New subtypes identified in this study are shown in green. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

from pigeons in Saudi Arabia.

3.2. Allelic diversity and genetic differentiation of the Fe-hydrogenase gene

A phylogenetic network analysis of 29 Fe-hydrogenase sequences, plus 12 previously published corresponding sequences, showed distinct sub-clustering into two groups of alleles (Fig. 2). Fe-hydrogenase alleles sequenced from local feral pigeons mostly clustered into a single subgroup with other pigeons, with the exception of two isolates (R22 and R54) that displayed alleles identical to alleles identified in chickens, turkeys, and the common myna. This finding could indicate the presence of some admixture of alleles within the Saudi bird population or a reflection of convergent evolution.

Population genetic diversity and differentiation at the Fe-hydrogenase locus was assessed using nucleotide diversity (π), and the ratio of non-synonymous (Ka) to synonymous (Ks) nucleotide substitution rates (Ka/Ks) (Fig. 3A). This analysis showed low mutation rates across all four bird species (π: 0.000–0.024), and an absence of signals for positive selective pressure (Ka/Ks: 0.000–0.213). The Fe-hydrogenase locus appears to be under negative or purifying selection, at least for the species examined. A parallel analysis for the complete set of available Fe-hydrogenase sequences for *T. gallinae* deposited to date was generated for comparative purposes (Fig. 3B). A somewhat higher degree of divergence at the nucleotide level (π: 0.000–0.228) was found, but the same low selective pressure was indicated by the large majority

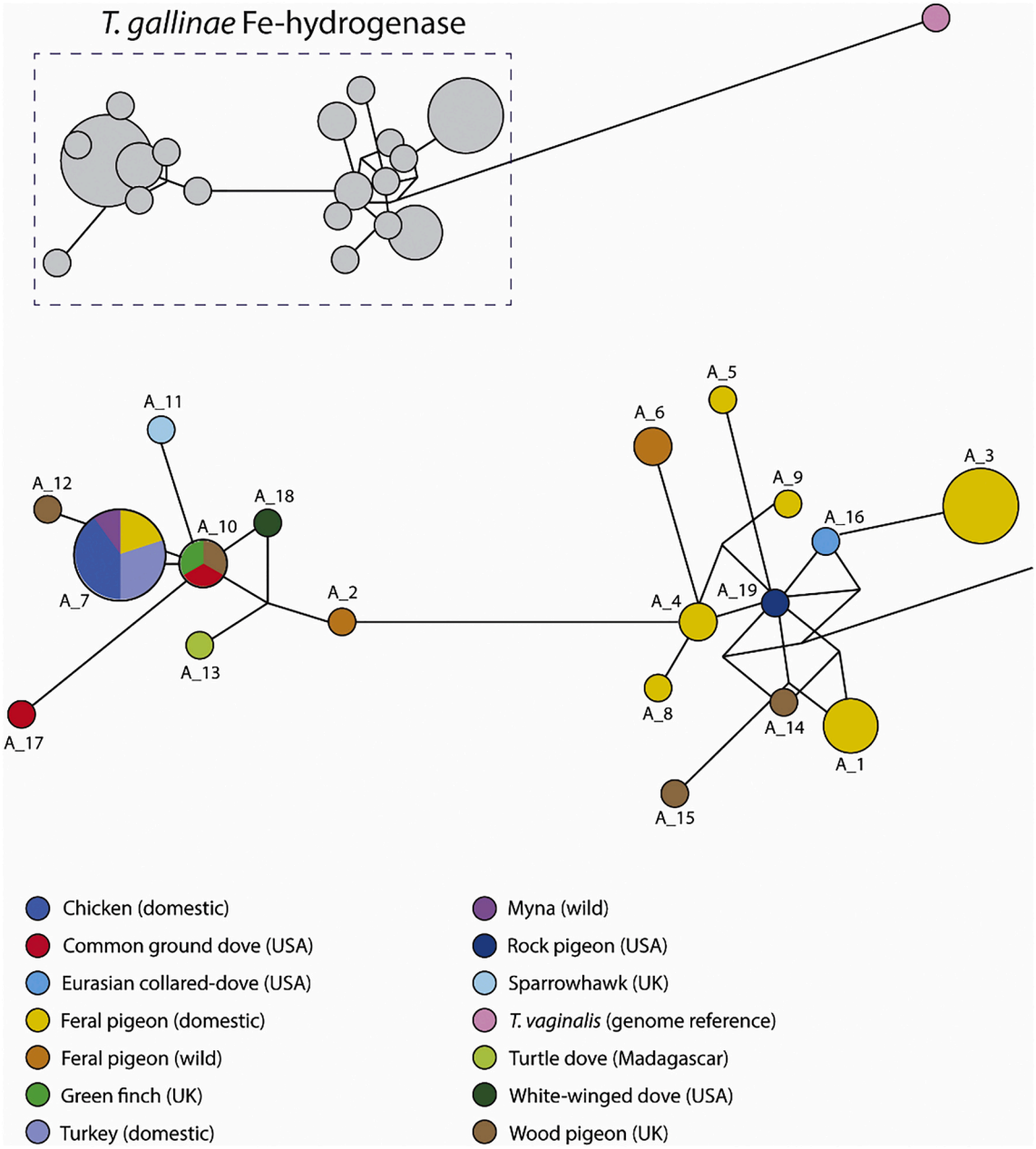

Fig. 2. Phylogenetic network of Fe-hydrogenase consensus sequences (521 bp) from 41 *T. gallinae* isolates from birds and a single *T. vaginalis* outgroup (genome reference). Genetic variants are grouped into identical allele types based on nucleotide composition, and circle sizes are proportional to the number of isolates. **A_1**: MT367178 (R19, R21, R40, R42), **A_2**: MT367180 (R39), **A_3**: MT367179 (R17, R18, R27, R30, R69, R72) and MT367187 (R71), **A_4**: MT367181 (R23, R24), **A_5**: MT367182 (R2), **A_6**: MT367183 (R62, R63), **A_7**: MT367184 (R22, R36, R49, R51, R54, R57, R58, R59, R60, R61), **A_8**: MT367185 (R31), **A_9**: MT367186 (R43), **A_10**: JF681136, KC529661 and KY496782, **A_11**: KC529660, **A_12**: KC962158, **A_13**: JF681141, **A_14**: KC529664, **A_15**: KC529662, **A_16**: KY496781, **A_17**: KY496783, **A_18**: KY496784, **A_19**: KY496778.

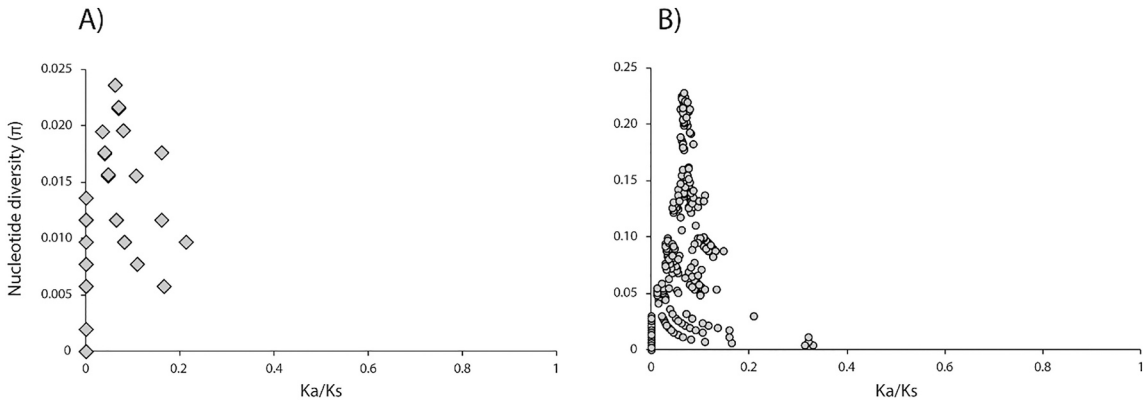

Fig. 3. Pairwise analyses of nucleotide diversity (π) and Ka/Ks for *T. gallinae* Fe-hydrogenase nucleotide sequences. Panel A illustrates the average number of pairwise nucleotide differences versus the ratio of non-synonymous (Ka) to synonymous (Ks) nucleotide substitution rates. Analysis is based on 521 bp alignment of Fe-hydrogenase sequence data from 29 novel *T. gallinae* isolates. Panel B describes pairwise results for all available Fe-hydrogenase sequence data for *T. gallinae* covering the 521 bp region deposited into NCBI's BLASTn database ($N = 87$) (https://blast.ncbi.nlm.nih.gov/; NCBI Resource Coordinators, 2018).

of Ka/Ks values <0.2, and none >0.4. This finding substantiates evidence for negative selective pressure acting at the Fe-hydrogenase locus on a global scale.

Only Fe-hydrogenase sequences from local feral pigeons were sufficient to further explore patterns of selective evolution at the population level. Tajima's D statistic was slightly negative but non-significant (Tajima's D: −0.25416, $P > 0.10$), indicating a neutral process for accumulation of DNA sequence mutations, and that the *T. gallinae* population is likely evolving following a standard coalescent model at this locus.

4. Discussion

Trichomonosis is an avian disease that can affect the upper digestive tract of many species of birds worldwide. The disease was previously restricted mainly to pigeons and their avian predators. In the present study, we used the Fe-hydrogenase gene to investigate the genetic diversity of *T. gallinae* in various avian species for the first time in Riyadh, Saudi Arabia. As a result, molecular and genetic analysis of isolated *T. gallinae* from 29 birds revealed new subtypes within infected bird species. Multiple subtypes of *T. gallinae* were found in four different species of the birds, and nine novel subtypes of *T. gallinae* were documented in the present study.

Sequences identified were grouped into ten branches by phylogenetic analysis (Fig. 1). We detected nine new subtype sequences and all were uploaded into GenBank of NCBI with accession nos. MT367179, MT367180, MT367181, MT367182, MT367183, MT367184, MT367185, MT367186, MT367187. One subtype (MT367178) was clustered with KP900034 [29]. These results contrast with heterogeneity reported in other studies using the same gene in among different species in the USA, UK, some countries in Europe, and others [21,22,28–32].

The sequence for the isolate A(KSAF1) (MT367184) showed only 99.87% identity to the consensus subtype A1 sequence (JF681136) [21]. Also, the sequence for C(KSAF1) MT367179 demonstrated 99.34% identity to the sequence C1obtained for a *T. gallinae* isolate from a rock pigeon, *Columba livia* (AF446077) [21].

The Fe-hydrogenase gene sequence analysis showed considerable variation among the isolates in this study, but we cannot determine which are associated with pathognomonic lesions. Albeshr and Alrefaei (13), reported that ribotype C is associated with oral lesions and other visible clinical sign of trichomonosis in pigeons and chickens. Also, previous studies indicate that the pathogenicity of British finch epidemic genotype A1 is robust compared with genotype C4 [21,28]. We used the Fe-hydrogenase gene, which has the sensitivity to confidently discriminate strains within existing genotypes, and provided evidence that subtype C4 is different from the Saudi C(KSAF1) strain. Our analysis shows that two subtypes of *T. gallinae,* A(KSAF1) and C(KSAF1), are widespread among Saudi birds. Limited numbers of samples in our study restrict our ability to link subtypes with lesions. Consequently, additional studies are needed to clarify relationships between pathogenicity and genotype of *T. gallinae*.

The present results suggest that the resolution of the ITS region is insufficient for the study of pathogenicity of strains, and work with other loci, such as performed in this study, is therefore necessary to identify *T. gallinae* strains most important for infection of birds. Chi et al. (2013) [28] supported the use of the Fe-hydrogenase gene for genotyping *T. gallinae*, because it allows detection of neutrally evolving fine-scale variation in amitochondrial protists [21,33]. Alrefaei et al. (2019) [22] reported that the subtype of Fe-hydrogenase genes offers additional resolution for discrimination of *T. gallinae* strains, and provides higher resolution genotyping than ITS ribotypes alone.

Finally, more analysis with additional samples is encouraged along with application of different molecular approaches. For instance, MLST is a sequence-based genotyping method that relies on the identification of single nucleotide polymorphisms (SNPs) in sequences of housekeeping genes. This technique may provide unambiguous data with high reproducibility and is proven to be a valuable technique for tracing the global spread of clones, particularly in long-term analyses [34].

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