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Original Article

Genetic characterization of *Trichomonas gallinae* (Rivolta, 1878) in companion birds in Japan and the genotypical relationship in the Asia region

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Abstract *Background/purpose:* Avian trichomonosis is a parasitic infection that affects a wide range of avian species, including free-ranging and pet birds worldwide, and *Trichomonas gallinae* has been considered as the only causative agent for decades. The sequence of the 5.8S ribosomal RNA with internal transcribed spacer (ITS) regions was widely used for identifying genotypes and determining inter-specific and intra-specific diversity. Moreover, the sequence of Fe-hydrogenase (FeHyd) was proposed as the second genetic marker for providing improved resolution of strain subtyping discrimination. Though the correlation between genetic variability and strain virulence is controversial, FeHyd analyses seemed to be useful to investigate the host or geographic origin of isolates. This study aimed to investigate the genetic characteristics of avian *Trichomonas* spp.
Methods: Forty-seven oral swabs and crop lavage fluids were collected from 9 avian genera, which were diagnosed as *Trichomonas*-positive by microscopy in animal hospitals in Japan,

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were analyzed.
Results: Genetic analysis of clonal isolates revealed the prevalence of the single genotype, ITS-OBT-Tg-1, by ITS region analysis, while two different subtypes, A2 and novel A3, were suggested by FeHyd gene analysis among Japanese companion birds. Phylogenetic analyses of available ITS sequences obtained from the Asia region (China, Iran, Iraq, and Saudi Arabia) were also preformed, revealing endemic ITS-OBT-Tg-1, ITS-OBT-Tg-2, ITS-OBT-Ttl-1, genotype III, and Saudi Arabia's unique lineages. Furthermore, ITS-OBT-Tg-2 predominance in these countries indicates different strains origination from Japan.
Conclusion: This is the first report of the genetic characterization of *T. gallinae* in Japan with discovery of novel subtype A3.

Introduction

Avian trichomonosis, caused by single-celled flagellated protozoa and also known as canker, is a well-known disease that most commonly affects Columbiformes as well as other avian orders, such as Accipitriformes, Anseriformes, Falconiformes, Galliformes, Strigiformes, Passeriformes, and Psittaciformes worldwide.[1,2] This disease usually affects the upper digestive tract of infected birds, forming caseous lesions in the oropharynx, esophagus, and crop, which leads to clinical signs of gagging, regurgitation, vomiting, diarrhea, ptyalism, and ultimately death.[1,3] Moreover, the causative protozoan is able to form diphtheria membranes of wet canker on the internal organs via the blood in pigeons.[2,4,5]

Trichomonas gallinae (Rivolta, 1878) was thought to be the only causative pathogen for avian trichomonosis for decades. Traditionally, *Trichomonas* spp. are identified morphologically by microscopic observation of motile trophozoites in wet mount preparation of crop washes and throat swab from birds. However, recently, new genetic variants and species, including *Trichomonas stableri* Girard et al., 2014 and *Trichomonas gypaetinii* Martínez-Díaz et al., 2015, were characterized morphologically and molecularly.[6,7] Since it is challenging to distinguish these species by morphological means, molecular studies have been performed to investigate genetic polymorphism among these species. The sequence of the 5.8S ribosomal RNA with surrounding internal transcribed spacer (ITS) regions 1 and 2 has been used for identifying genetic heterogeneities and was recognized as a strong molecular tool to determine inter-specific and intra-specific diversity.[8–12] Later, the sequence of Fe-hydrogenase (FeHyd), a housekeeping protein-encoding gene, was proposed as the second genetic marker for providing improved resolution of strain subtyping discrimination.[13–15]

Recently, 12 different (A to L) genotypes of *T. gallinae* were described in the USA[9] and two genotypes (A and B) in Spain[12] based on phylogenetic analyses of the ITS region. Six different genotypes were proposed (ITS-I to VI) in Europe by comparison of ITS sequences of divergence ranging from 84.7% to 97.6%.[10] The abovementioned lineages were integrated into 15 novel genotypes, revealing that ITS-OBT-Tg-1 and ITS-OBT-Tg-2 were the most distributed.[11] Most recently, 15 novel genotypes of *Trichomonas* spp. were discovered from Columbiformes in Australia, exhibiting moderate to high host specificity.[16] On the other hand, FeHyd analyses with an alphanumeric subtyping scheme identified further genotypically distinct subtypes within ITS genotypes, which seemed to be useful to investigate the host or geographic origin of isolates.[13,14] The correlation between genetic variability and strain virulence is controversial. A single clonal strain was said to be responsible for the epidemic trichomonosis in European finches[15,17,18] and this isolate was also obtained from band-tailed pigeon carcass during the trichomonosis outbreak in the USA,[19] indicating that some genotypes had correlation with the presence of clinical signs and lesions. However, the pathogenetic strains were also detected in asymptomatic birds.[12,20,21]

The aforementioned molecular analyses were mainly conducted in Europe and North America, while genotyping of *Trichomonas* species in Asia was reported only from China, Iran, Iraq, and Saudi Arabia.[22–28] Therefore, this study, for the first time, investigated the genetic characteristics of avian *Trichomonas* spp. by analyzing isolates collected from captive-bred parrots, sparrows, and a raptor in Japan.

Methods

Specimens

Between 2019 and 2020, crop lavage fluids or oral swabs were collected from Bengalese finch (*Lonchura striata* var. *domestica*), budgerigar (*Melopsittacus undulatus*), brahminy kite (*Haliastur indus*), cockatiel (*Nymphicus hollandicus*), domestic canary (*Serinus canaria*), Java sparrow (*Lonchura oryzivora*), monk parakeet (*Myiopsitta monachus*), domestic pigeon (*Columba livia*), white northern goshawk (*Accipiter gentilis albidus*), and zebra finch (*Taeniopygia guttata*), which were confirmed to be *Trichomonas*-positive by microscopy in three animal hospitals located in Tokyo and Ishikawa, Japan (Table 1). The samples were inoculated into the commercially available liquid media (*Trichomonas*-RY medium: Nikken Seibutu inc., Japan) immediately after collection and transported at room temperature. The media were incubated under 5% CO_2 at 36.5 °C, and propagation of the protozoa was inspected visually and microscopically every day until needed.

Table 1 List of *T. gallinae* isolates discovered in the present study. Isolates LBG17 and MA1 were collected by oral swabs and the other isolates were all collected from crop lavage fluids.

Isolate ID	Host species	Location	Asymptomatic	ITS genotype	FeHyd subtype
LBG1	*Lonchura oryzivora* (Java sparrow)	Tokyo		**ITS-OBT-Tg-1**	**A2**
LBG2	*Melopsittacus undulatus* (budgerigar)	Tokyo	✓	**ITS-OBT-Tg-1**	NA
LBG4	*Haliastur indus* (brahminy kite)	Tokyo		**ITS-OBT-Tg-1**	**A3 (novel)**
LBG5	*Melopsittacus undulatus* (budgerigar)	Tokyo		**ITS-OBT-Tg-1**	**A2**
LBG6	*Lonchura oryzivora* (Java sparrow)	Tokyo	✓	NA	NA
LBG7	*Melopsittacus undulatus* (budgerigar)	Tokyo	✓	**ITS-OBT-Tg-1**	**A2**
LBG8	*Melopsittacus undulatus* (budgerigar)	Tokyo	✓	**ITS-OBT-Tg-1**	**A2**
LBG9	*Melopsittacus undulatus* (budgerigar)	Tokyo	✓	**ITS-OBT-Tg-1**	**A2**
LBG10	*Melopsittacus undulatus* (budgerigar)	Tokyo	✓	**ITS-OBT-Tg-1**	**A2**
LBG12	*Melopsittacus undulatus* (budgerigar)	Tokyo	✓	**ITS-OBT-Tg-1**	NA
LBG13	*Taeniopygia guttata* (zebra finch)	Tokyo	✓	**ITS-OBT-Tg-1**	**A2**
LBG14	*Melopsittacus undulatus* (budgerigar)	Tokyo	✓	**ITS-OBT-Tg-1**	**A2**
LBG16	*Melopsittacus undulatus* (budgerigar)	Tokyo	✓	**ITS-OBT-Tg-1**	**A2**
LBG17	*Accipiter gentilis albidus* (white northern goshawk)	Tokyo		**ITS-OBT-Tg-1**	**A2**
LBG18	*Columba livia* var. *domestica* (domestic pigeon)	Tokyo		NA	NA
LBG19	*Lonchura oryzivora* (Java sparrow)	Tokyo		NA	NA
LBG20	*Serinus canaria* (domestic canary)	Tokyo		NA	NA
LBG21	*Myiopsitta monachus* (monk parakeet)	Tokyo		NA	NA
LBG22	*Melopsittacus undulatus* (budgerigar)	Tokyo		NA	NA
LBG23	*Melopsittacus undulatus* (budgerigar)	Tokyo		NA	NA
LBG24	*Melopsittacus undulatus* (budgerigar)	Tokyo		NA	**A2**
LBG25	*Lonchura oryzivora* (Java sparrow)	Tokyo		NA	NA
LBG26	*Lonchura oryzivora* (Java sparrow)	Tokyo	✓	NA	NA
LBG27	*Melopsittacus undulatus* (budgerigar)	Tokyo	✓	NA	**A2**
LBG28	*Melopsittacus undulatus* (budgerigar)	Tokyo	✓	NA	NA
LBG29	*Lonchura oryzivora* (Java sparrow)	Tokyo		NA	NA
LBG30	*Nymphicus hollandicus* (cockatiel)	Tokyo	✓	NA	NA
MK1	*Lonchura oryzivora* (Java sparrow)	Tokyo	✓	**ITS-OBT-Tg-1**	NA
MK2	*Serinus canaria* (domestic canary)	Tokyo	✓	NA	NA
MK3	*Serinus canaria* (domestic canary)	Tokyo	✓	**ITS-OBT-Tg-1**	**A3 (novel)**
MK4	*Melopsittacus undulatus* (budgerigar)	Tokyo	✓	**ITS-OBT-Tg-1**	NA
MK5	*Melopsittacus undulatus* (budgerigar)	Tokyo		NA	**A2**
MK6	*Lonchura oryzivora* (Java sparrow)	Tokyo	✓	NA	NA
MK7	*Lonchura oryzivora* (Java sparrow)	Tokyo		**ITS-OBT-Tg-1**	NA
MK9	*Lonchura oryzivora* (Java sparrow)	Tokyo	✓	NA	NA
MK10	*Melopsittacus undulatus* (budgerigar)	Tokyo	✓	**ITS-OBT-Tg-1**	**A2**
MK11	*Melopsittacus undulatus* (budgerigar)	Tokyo	✓	NA	NA
MK12	*Melopsittacus undulatus* (budgerigar)	Tokyo		NA	NA
MK13	*Melopsittacus undulatus* (budgerigar)	Tokyo	✓	**ITS-OBT-Tg-1**	NA
MK14	*Melopsittacus undulatus* (budgerigar)	Tokyo		**ITS-OBT-Tg-1**	NA
MK16	*Melopsittacus undulatus* (budgerigar)	Tokyo		NA	NA
MK17	*Lonchura oryzivora* (Java sparrow)	Tokyo	✓	NA	NA
MK19	*Lonchura striata* var. *domestica* (Bengalese finch)	Tokyo		**ITS-OBT-Tg-1**	**A2**
MK20	*Lonchura oryzivora* (Java sparrow)	Tokyo	✓	NA	**A2**
MK21	*Serinus canaria* (domestic canary)	Tokyo	✓	NA	**A2**
MK22	*Nymphicus hollandicus* (cockatiel)	Tokyo	✓	NA	NA
MA1	*Melopsittacus undulatus* (budgerigar)	Ishikawa	✓	**ITS-OBT-Tg-1**	**A2**

NA: not available.

Molecular analyses

Two hundred microliters of culture medium was used for DNA extraction using the QIAamp DNA MiniKit (QIAGEN, Germany) according to manufacturer's instruction. DNA was stored at 4 °C for immediate use or −20 °C for storage. Partial fragment of the ITS region was amplified using primers, TFR1/TFR2 (TFR), 5′-TGCTTCAGTTCAGCGGG TCTTCC-3′/5′-CGGTAGGTGAACCTGCCGTTGG-3′.[8] The FeHyd gene was amplified using primer pairs, TrichhydFOR/TrichhydREV (TRhyd), 5′-GTTTGGGATGGCCTCAGAAT-3′/5′-AGCC-GAAGATGTTGTCGAAT-3′[15] and newly designed primers, TgHydF/TgHydR (TGhyd), 5′-GAACTYCYGACTGCCACGA-3′/5′-TGCTTGATGCCGAGGAGYTT-3′. Novel primers were designed

as internal primers of TRhyd using Primer 3 (http://frodo.wi.mit.edu/) based on FeHyd sequence alignments of *T. gallinae* (accession nos. JF681141, KC529661, and KP900031), produced an amplicon size of 766 bp, and were used if the FeHyd gene was not amplified with primer pair TRhyd.

PCR was carried out in a 20 μl reaction mixture (TaKaRa Bio Inc., Japan) containing 2.0 μl of 10 × Ex Taq buffer (2.5 mM),1.6 μl of dNTPs mixture (2.5 mM), 0.2 μl of TaKaRa Ex Taq (5 units/μl), 0.2 μl of each primer (50 μM), 14.8 μl of dDW, and 1 μl of template DNA. PCR conditions were as follows: 95 °C for 10 min (TFR) or 94 °C for 15 min (TRhyd and TGhyd), various cycles (TFR: 35, TRhyd and TGhyd: 40) of denaturation at different temperatures (TFR: 95 °C, TRhyd and TGhyd: 94 °C) for different times (TFR: 30 s, TRhyd and TGhyd: 1 min), annealing at various temperatures (TFR: 60 °C, TRhyd: 52 °C, TGhyd: 54 °C) for 30 s, extension at 72 °C for 1 min, followed by final extension at 72 °C for different times (TFR: 7 min, TRhyd and TGhyd: 5 min). PCR products were sent for sequencing by Macrogen Corp., Japan using the abovementioned primers.

All the chromatograms of the sequences obtained from the forward primers and the reverse primers were inspected visually and assembled using Molecular Evolutionary Genetics Analysis software version 7.0.[29] The sequence similarity was determined via the BLASTN program via the nucleotide database provided by the National Center for Biotechnology Information. In this study, the nomenclature of ITS genotypes and FeHyd subtypes was classified as previously described.[10,11,14]

Data treatment and phylogenetic analyses

Two genome datasets were analyzed in this study: datasets of ITS and FeHyd. Sequences of genotype III were used as an out taxon for ITS dataset, and those of *T. stableri* (accession no. KC660123) were used as an out group for the FeHyd dataset. Related sequences of ITS and FeHyd were retrieved from the nucleotide database. Furthermore, available ITS sequences obtained from the Asia region were also used for phylogenetic analysis. MAFFT[30] was used for multiple alignment of ITS sequences, while ClustalW[31] was used for FeHyd sequences. Obvious errors of each alignment were collected with visual inspection.

Phylogenetic analysis for ITS and FeHyd was performed by generating phylogenetic trees using the maximum likelihood (ML) and neighbor joining (NJ) methods. The best-fit model and parameters for the analyses of previous datasets were evaluated based on Akaike Information Criterion for both datasets. The best-fit model for ITS was the Tamura 3-parameter with gamma distribution rate variation among sites for ITS, and the Tamura-Nei model with invariant sites was chosen for the FeHyd dataset. Node support was estimated by bootstrap of 1000 and 2000 replicates for ML and NJ, respectively.

Results

DNA extraction

About three hundred base pair fragments of ITS sequences were obtained from 22 birds: Bengalese finch (n = 1), budgerigar (n = 14), brahminy kite (n = 1), domestic canary (n = 1), Java sparrow (n = 3), white northern goshawk (n = 1), and zebra finch (n = 1). On the other hand, FeHyd sequences were obtained from 20 birds: Bengalese finch (n = 1), budgerigar (n = 12), brahminy kite (n = 1), domestic canary (n = 2), Java sparrow (n = 2), white northern goshawk (n = 1), and zebra finch (n = 1).

Genetic analyses for ITS

All obtained ITS sequences were identical to each other and submitted to GenBank (accession nos. MZ128144 and MZ128145), which had 100% identity to *T. gallinae* sequences (accession no. EU215369), forming a well-supported clade, ITS-OBT-Tg-1 (Fig. 1). Further, strain Tai2 (accession no. KJ721785) and BJ20 (accession no. MH733817), both from China, and strain Tri-IR-22 (accession no. KT869155) and Tri-IR-35 (accession no. KT869157), both from Iran, were also clustered in this clade (Table 2). The remaining sequences from China and Iran were all clustered in the clade ITS-OBT-Tg-2, comprising six sequences obtained from Iraq. Of the 12 sequences reported in Saudi Arabia, strains KSA9, 10, and 11 were identical to the sequences of genotype III, and strain KSA6 was clustered in the genotype ITS-OBT-Ttl. Similar topologies were illustrated with both methods. Phylogenetic relationships among ITS-OBT-Tg-1, ITS-OBT-Tg-2, and a clade comprising KSA3, 5, and 7 failed to obtain a high node support, though they came from a common node, as previously described.[22] Moreover, strain KSA4 also failed to obtain a highly supported phylogenetic position in this study.

Genetic analyses for FeHyd

Eighteen of 996-bp FeHyd sequences, depositing in GenBank under the accession number MZ128142, were identical to subtype A2 (accession nos. KP900030 and JF681141).[15,32] Novel identical sequences (n = 2) from domestic canary and brahminy kite, submitted to GenBank as MZ128143, showed 99.5% identity with subtype A1 (accession nos. JF681136, KP900029) and subtype A2 (accession no. KP900030). This new variant was named A3. In the phylogenetic tree for FeHyd (Fig. 2), similar topologies were illustrated with both methods; novel subtype A3 branched earliest in the subtype A group, while other branches were similar as previously described.[32]

Discussion

The genetic diversity of *Trichomonas* spp. has been widely studied in North America and Europe.[9,11,12,14,15,19] Molecular analyses of endemic strains revealed a single clonal genotype from infected birds, suggesting the correlation between virulence and genotypes.[15,17,18] However, other studies showed that those high-pathogenetic strains were also discovered in asymptomatic birds, making this scenario controversial.[12,20,21] In the Asia region, studies with analysis of ITS region have been conducted in China, Iran, Iraq, and Saudi Arabia[22–28] using mostly domestic pigeons, with low to moderate genetic variations being reported in these countries. By comparison, there have been few reports of

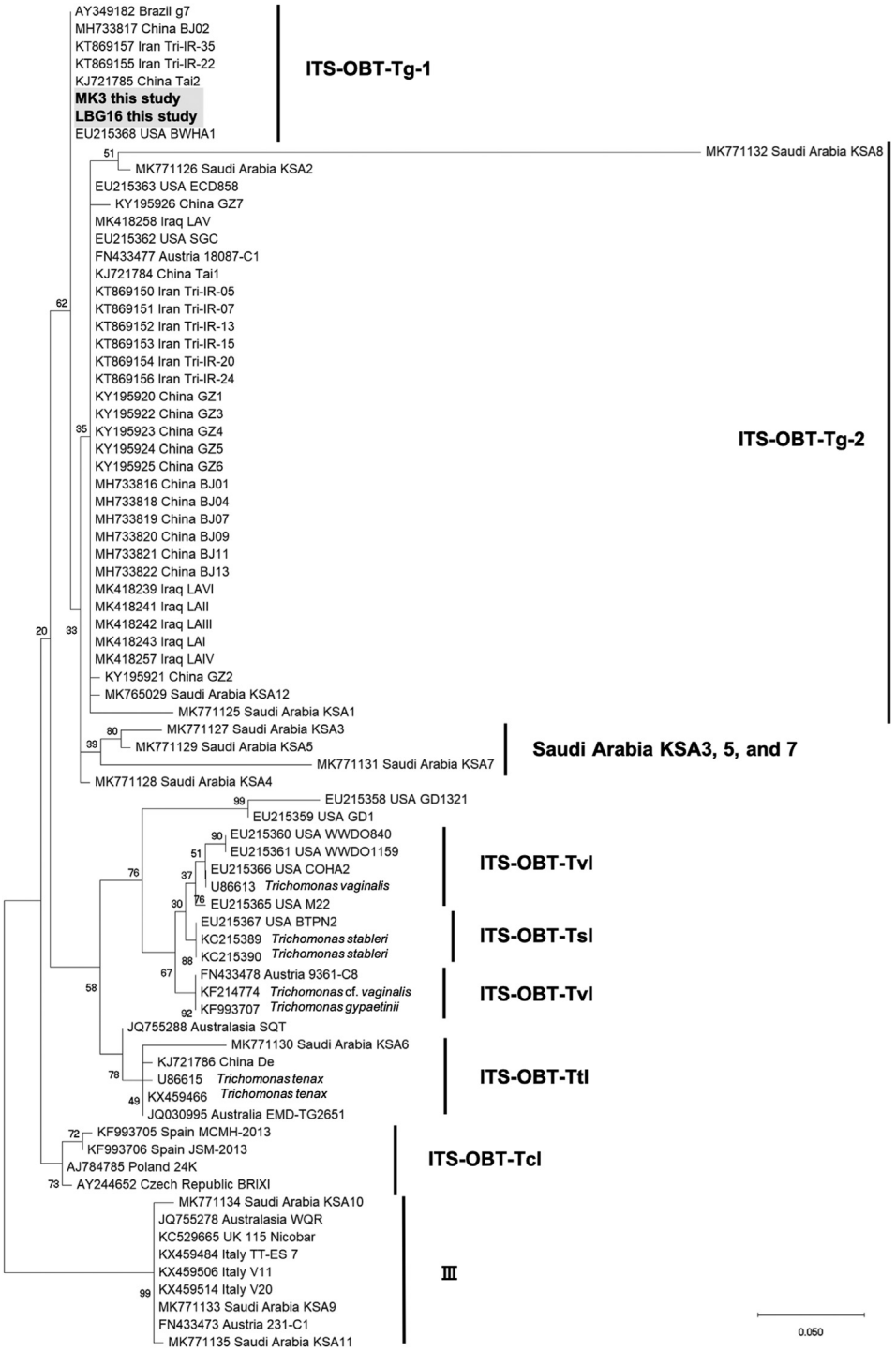

Figure 1. Phylogenetic analysis of ITS region by the maximum likelihood method. Newly obtained sequences in this study are presented in bold face with shaded box and clustered in clade ITS-OBT-Tg-1, while most of the Asian isolates clustered in clade ITS-OBT-Tg-2. Strains obtained from China and Saudi Arabia showed various genotype prevalence.

detection of avian trichomonosis in Japan, and no molecular analyses were performed.[33] In this study, we collected oral swabs and crop lavage fluids from birds, which were confirmed to be *Trichomonas*-positive by microscopy in animal hospitals.

Molecular analyses were performed for those specimens with characterization of the ITS region and the FeHyd gene of *Trichomonas* spp. All the obtained ITS region sequences showed 100% identity to genotype ITS-OBT-Tg-1 and clustered in the same genotype in phylogenetic analyses, revealing that a single clonal strain was endemic in this region. Furthermore, 68.2% (15/22) of the ITS-OBT-Tg-1-positive birds were asymptomatic, indicating that the pathogenicity of these strains might be low. On the other

Table 2 Sequences of Asian strains of *T. gallinae* analyzed in this study.

Isolate	Species	Origin	ITS genotype	Accession no.	Reference
Tai1	*Columba livia* var. *domestica* (domestic pigeon)	China	**ITS-OBT-Tg-2**	KJ721784	[27]
Tai2	*Columba livia* var. *domestica* (domestic pigeon)	China	**ITS-OBT-Tg-1**	KJ721785	[27]
De	*Columba livia* var. *domestica* (domestic pigeon)	China	**ITS-OBT-Ttl-1**	KJ721786	[27]
GZ1	*Columba livia* var. *domestica* (domestic pigeon)	China	**ITS-OBT-Tg-2**	KY195920	[28]
GZ2	*Columba livia* var. *domestica* (domestic pigeon)	China	**ITS-OBT-Tg-2**	KY195921	[28]
GZ3	*Columba livia* var. *domestica* (domestic pigeon)	China	**ITS-OBT-Tg-2**	KY195922	[28]
GZ4	*Columba livia* var. *domestica* (domestic pigeon)	China	**ITS-OBT-Tg-2**	KY195923	[28]
GZ5	*Columba livia* var. *domestica* (domestic pigeon)	China	**ITS-OBT-Tg-2**	KY195924	[28]
GZ6	*Columba livia* var. *domestica* (domestic pigeon)	China	**ITS-OBT-Tg-2**	KY195925	[28]
GZ7	*Columba livia* var. *domestica* (domestic pigeon)	China	**ITS-OBT-Tg-2**	KY195926	[28]
BJ01	*Columba livia* var. *domestica* (domestic pigeon)	China	**ITS-OBT-Tg-2**	MH733816	[26]
BJ02	*Columba livia* var. *domestica* (domestic pigeon)	China	**ITS-OBT-Tg-1**	MH733817	[26]
LAI	*Columba livia* var. *domestica* (domestic pigeon)	Iraq	**ITS-OBT-Tg-2**	MK418243	[25]
LAII	*Columba livia* var. *domestica* (domestic pigeon)	Iraq	**ITS-OBT-Tg-2**	MK418241	[25]
LAIII	*Columba livia* var. *domestica* (domestic pigeon)	Iraq	**ITS-OBT-Tg-2**	MK418242	[25]
LAIV	*Columba livia* var. *domestica* (domestic pigeon)	Iraq	**ITS-OBT-Tg-2**	MK418257	[25]
LAV	*Columba livia* var. *domestica* (domestic pigeon)	Iraq	**ITS-OBT-Tg-2**	MK418258	[25]
LAVI	*Columba livia* var. *domestica* (domestic pigeon)	Iraq	**ITS-OBT-Tg-2**	MK418239	[25]
Tri-IR-05	*Columba livia* (rock pigeon)	Iran	**ITS-OBT-Tg-2**	KT869150	[23]
Tri-IR-07	*Melopsittacus undulatus* (budgerigar)	Iran	**ITS-OBT-Tg-2**	KT869151	[23]
Tri-IR-13	*Serinus canaria* (domestic canary)	Iran	**ITS-OBT-Tg-2**	KT869152	[23]
Tri-IR-15	*Spilopelia senegalensis* (laughing dove)	Iran	**ITS-OBT-Tg-2**	KT869153	[23]
Tri-IR-20	*Falco tinnunculus* (common kestrel)	Iran	**ITS-OBT-Tg-2**	KT869154	[23]
Tri-IR-22	*Melopsittacus undulatus* (budgerigar)	Iran	**ITS-OBT-Tg-1**	KT869155	[23]
Tri-IR-24	*Columba livia* (rock)	Iran	**ITS-OBT-Tg-2**	KT869156	[23]
Tri-IR-35	*Acridotheres tristis* (common mynah)	Iran	**ITS-OBT-Tg-1**	KT869157	[23]
12_Feral_Pigeon	*Columba livia* var. *domestica* (domestic pigeon)	Saudi Arabia	**KSA1**	MK771125	[22]
31_Feral_Pigeon	*Columba livia* var. *domestica* (domestic pigeon)	Saudi Arabia	**ITS-OBT-Tg-2**	MK771126	[22]
35_Feral_Pigeon	*Columba livia* var. *domestica* (domestic pigeon)	Saudi Arabia	**KSA3**	MK771127	[22]
25_Feral_Pigeon	*Columba livia* var. *domestica* (domestic pigeon)	Saudi Arabia	**KSA4**	MK771128	[22]
67_Feral_Pigeon	*Columba livia* var. *domestica* (domestic pigeon)	Saudi Arabia	**KSA5**	MK771129	[22]
29_Feral_Pigeon	*Columba livia* var. *domestica* (domestic pigeon)	Saudi Arabia	**ITS-OBT-Ttl-1**	MK771130	[22]
9_Feral_Pigeon	*Columba livia* var. *domestica* (domestic pigeon)	Saudi Arabia	**KSA7**	MK771131	[22]
28_Feral_Pigeon	*Columba livia* var. *domestica* (domestic pigeon)	Saudi Arabia	**ITS-OBT-Tg-2**	MK771132	[22]
27_Feral_Pigeon	*Columba livia* var. *domestica* (domestic pigeon)	Saudi Arabia	**III**	MK771133	[22]
8	*Columba livia* var. *domestica* (domestic pigeon)	Saudi Arabia	**III**	MK771134	[22]
7_Feral_Pigeon	*Columba livia* var. *domestica* (domestic pigeon)	Saudi Arabia	**III**	MK771135	[22]
68	*Columba livia* var. *domestica* (domestic pigeon)	Saudi Arabia	**KSA12**	MK765029	[22]

hand, based on the phylogram of the FeHyd gene, these strains could be divided into subtype A2 and novel A3; A2 was said to be involved in clinical episodes of North American band-tailed pigeon and Seychelles' turtle dove in the USA,[9,15,19,32] suggesting that the endemic strains might have originated from North America. Novel subtype A3 was detected in one brahminy kite (*H. indus*) with swollen mandible and one domestic canary (*S. canaria*) without symptoms. Hence, as mentioned above, the fact that most of the *Trichomonas*-positive birds were asymptomatic supported the hypothesis that FeHyd sequences are more useful for investigating the origin of isolates than for determining pathogenicity.[32]

We also investigated the available ITS sequences obtained from the Asia region for clarification of the genotypical relationships among those countries. In Iran, ITS region sequences were identified as ITS-OBT-Tg-1 and ITS-OBT-Tg-2,[23] while only genotype ITS-OBT-Tg-2 was discovered in pigeons from Iraq.[25] A third genotype, ITS-OBT-Ttl, was additionally discovered in China.[27] This difference could be due to the sampling population and area. Thirty avian species from eight orders were collected from an avian hospital in Iran; this may have contributed to the variation in the origin of the birds, resulting in two genotypes. Though only pigeons were used in the studies in China and Iraq, pigeons from more than six farms in three distinct areas were used for the genetic identification in China, leading to the discovery of ITS-OBT-Ttl. On the other hand, strains from Saudi Arabia showed a high degree of genetic diversity from those of other regions. Besides ITS-OBT-Tg-1, ITS-OBT-Tg-2, and ITS-OBT-Ttl, genotype III (strains KSA9, 10, and 11) was reported only from Saudi Arabia in the Asia region. Moreover, a unique clade, comprising strains KSA3, 5, and 7, clustered in clade ITS-OBT-Tg-2.[22] Although strain KSA4 failed to obtain highly-supported phylogenetic positions in this study, it seemed

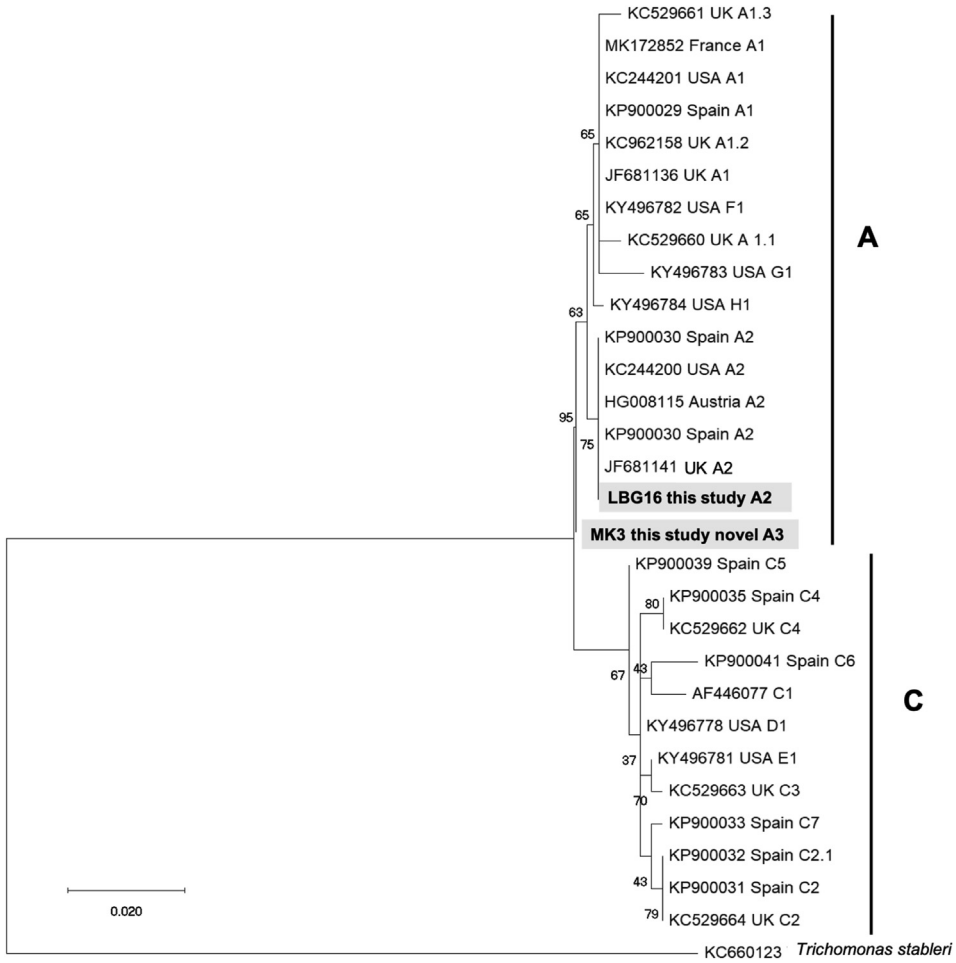

Figure 2. Phylogenetic analysis of FeHyd region by the maximum likelihood method. Newly obtained sequences in this study are presented in bold face with shaded boxes. Strain MK3 branched earliest among subtype A, and was named novel subtype A3. The remaining isolates of this study were subtyped as A2.

to cluster in clade ITS-OBT-Tg-2 with KSA1, 2, 8 and 12. Unfortunately, no FeHyd sequences were available for these Asian isolates. Nevertheless, these strains seemed to have different origins in Japan.

In summary, this is the first molecular characterization of *T. gallinae* in Japan, suggesting that a single genotype ITS-OBT-Tg-1 is the predominant strain in Japanese companion birds, and novel subtype A3 was also discovered by phylogenetic analyses of FeHyd sequences. By comparing ITS sequences, ITS-OBT-Tg-2 was found to be the major genotype in the Asia region, revealing that there are different origins for this protozoan in Japan. However, further studies with a large specimen number and variety of avian species from Japan, including free-ranging birds, should be conducted for molecular analysis to provide precise genetic information of *Trichomonas* species.

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