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Molecular detection and characterization of *Trichomonas gallinae* isolated from pigeon and chicken of Assam, India

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Abstract
Trichomonas gallinae isolates from pigeon and chicken were amplified by polymerase chain reaction using *Fe hydrogenase* gene and characterized by molecular tools to determine the geographical location and the sequences were analyzed phylogenetically. The isolates in the present study were 100% similar to *T. gallinae* isolates of Iran and Austria. Random samples found positive for trichomoniasis in culture and throat swab in live birds were confirmed by PCR. The positive samples showed clear band at 290 bp.

Keywords: *Trichomonas gallinae*, DNA extraction, PCR, pigeon, chicken, phylogenetic analysis

Introduction
Avian trichomoniasis caused by the protozoan *Trichomonas gallinae*, is an acute and often fatal disease that is generally manifested as a caseous lesion within the upper digestive tract of affected birds (Stabler, 1954) [1]. The disease also known as canker, has significant health and economic impact on poultry industry, especially in pigeon and game birds rearing and breeding and is considered as a major factor for regulation and even decline of avian populations (Robinson *et al*., 2010) [2]. In natural settings, the parasite *T. gallinae* primarily infects the columbiformes birds (pigeon and mourning dove) although several other birds including the wild carnivorous ones may be infected (Girard *et al*., 2014) [3]. The site of predilection of the parasite in the host is mainly the upper digestive tract although respiratory system and other visceral organs may also be involved in the pathogenic process (Borji *et al*., 2011) [4]. The parasite is transmitted from infected mother doves and pigeons to their nestlings through crop milk feeding while adult to adult transmission occurs through billing activities during courtship, feed and water contamination. Prey on infected columbiformes may cause infection (commonly known as Frounce) in the raptors. Typical lesions include development of caseous masses (canker) in the upper digestive tract which in severe cases become completely blocked to cause death by starvation. Surviving birds remain as carrier and become a source of infection for others. The disease has been recognized as an emerging and potentially fatal disease of birds (Robinson *et al*., 2010; Girard *et al*., 2014) [2, 3]. The protozoon is mainly diagnosed based on clinical symptoms exhibited and lesions, demonstration of the organisms in wet mount and stained smears and culture of the organisms. Detection of *T. gallinae* by PCR gives confirmatory result. According to Grabensteiner and Hess (2006) [5], the presence of trichomonad's DNA could be detected in oral fluids, tissue taken from the crop, pharynx or from faeces by polymerase chain reaction (PCR) using a variety of primers, most of them targeting ITS1-5.8S rRNA ITS2 and 18S rRNA regions. Chi *et al*. (2013) [6] reported that the *Fe-hydrogenase* primers were designed to specifically amplify the *Fe-hydrogenase* gene of *T. gallinae*, which was used to support the classification of strains. There is not much report on molecular detection and phylogenesis of *Trichomonas gallinae* in pigeons from India. However, Saikia (2016) [7] carried out a molecular study for confirmation of *T. gallinae* isolated from domestic pigeon in Assam using ITS1-5.8S-ITS2 gene which resulted positive DNA fragment at 290 bp. The present paper reports on the molecular analysis and phylogeny of *Trichomonas gallinae* isolates from pigeon and chicken of Assam, India and is probably the first of its kind from North East India.

Materials and Methods

Molecular detection of *Trichomonas gallinae*

For molecular detection, DNA extraction from *T. gallinae* positive cultured samples were done by centrifuging the tube for 5 min at 1,000× g, the supernatant was discarded, and the pellet was re-suspended in 1 ml of phosphate-buffered saline (PBS). The samples were centrifuged again for 5 min at 1,000× g, the pellet was resuspended in five drops of PBS (approximately 100 µl) and kept at -20 °C until DNA extraction.

Isolation of genomic DNA

One ml aliquots from *Trichomonas gallinae* positive cultures were kept for DNA extraction during the first hour of *in vitro* sub cultivation. DNA was extracted randomly from positive samples of cultured throat swabs with the help of GSure™ Fast Tissue Kit (West Bengal) following manufacture's guidelines. The extracted DNA was stored at -20 ºC until further use.

PCR protocol for *Trichomonas gallinae*

The PCR was performed for *T. gallinae* following the method described by Forzan *et al*. (2010) [8] with slight modification. Amplification of *Fe hydrogenase* gene was carried out in a Technee-5000 thermal cycler (Bibby Scientific) using oligonucleotide primers in 25 µl reaction mixture volume containing 5 µl of genomic DNA, 2.5 µl 10x PCR buffer, 1.0 µl $MgCl_2$ (50 mM), 0.5 µl dNTP (10 mM), 1.0 µl (20 pmol/ µl) of each forward and reverse primer, 0.2 µl Taq DNA polymerase (5 IU/ µl). The final volume of 25 µl was made by adding nuclease free water. Oligonucleotide primers used for amplification of *Fe hydrogenase* gene of *Trichomonas gallinae* were Trih-1F (5′-ATGAGTCAACACACGCCATCAG-3′); Trich-1R (5′-CACCTGGACGTCTGTGACCTTC- 3′).

PCR was performed with initial denaturation at 95 ºC for 2 min, followed by 35 cycles each consisting of denaturation for 1min at 94 ºC, annealing for 30 sec at 50 ºC, elongation for 30 sec at 72 ºC and final elongation at 72 ºC for 5 min. For visualization of the PCR product, gel electrophoresis of amplified DNA was done in 1.5% agarose gel for 1 hour at 60 Volts.

Purification and Sequencing

The unpurified PCR amplicons were sent for purification and sequencing by outsourcing (MolBiogen). The sequencing results were analyzed by BLAST tool with the help of freely available software (Bio Edit Mega).

Phylogenetic analysis

Twenty four numbers of nucleotide sequences of hydrogenosomal *Fe hydrogenase* gene belonging to *Trichomonas gallinae* were retrieved from GenBank database (http://www.ncbi.nlm.nih.gov/). Totally, twenty seven numbers of nucleotide sequence (including the three partial nucleotide sequence of *Fe hydrogenase* gene of *T. gallinae* isolated from pigeon and chicken of Assam, viz. T. gal/AAU-1/P, T. gal/AAU-2/C and T. gal/AAU-3/P were considered for phylogenetic analysis of *T. gallinae*. The whole phylogenetic analysis was done in MEGA X software (Kumar *et al*., 2018) [9]. Alignment of multiple sequences was carried out using the Clustal W program implemented within MEGA X. The best-fit substitution model was estimated in MEGA X and selection was done on the basis of Bayesian information criteria (BIC). All positions that contained alignment gaps and missing data were eliminated from the analysis. Phylogenetic tree was constructed using the Maximum Likelihood (ML) method based on Kimura 2-parameter substitution model (Kimura, 1980) [10] and statistical significance of the tree was tested by bootstrap analysis (Felsenstein, 1985) [11] of 1000 pseudo-replicates.

Pairwise sequence distance and identity of *Fe hydrogenase* gene at nucleotide level

The pairwise distance of *Fe hydrogenase* gene of *T. gallinae* was estimated in MEGA X software (Kumar *et al*., 2018) [9]. Sequence Demarcation Tool (SDT) v1.2 (Muhire, 2014) [12] was used to estimate the pairwise identity. For estimation of pairwise sequence distance and identity of partial *Fe hydrogenase* gene at nucleotide level, the same twenty seven sequences used in phylogenetic analysis were taken into consideration.

Results and Discussion

PCR and molecular detection

Random samples found positive for trichomoniasis in culture and throat swab in live birds were confirmed by PCR. The positive samples showed the clear 290 bp band (Fig. 1)

Most of the works on molecular detection and characterization of *T. gallinae* in different countries has been done based on ITS1/5.8S rRNA/ITS gene and *Fe hydrogenase* gene. In line with the present investigation, Saikia (2016) [7] also confirmed *T. gallinae* infection in domestic pigeons of Assam by amplification of ITS1/5.8S rRNA/ITS gene by PCR and obtained positive fragment at 290 bp while Forzan *et al*. (2010)[8] diagnosed 37.5% *T. gallinae* in finches in Canada based on histopathology and PCR by amplification of the ITS1/5.8S r RNA/ITS2 region obtaining 290-bp fragment. According to Grabensteiner and Hess (2006) [5], the majority of primers were originally designed to detect all trichomonads thus agreeing to our present report where DNA was extracted from tissue lesions, throat swab culture. In the same way, Sansano-Maestre *et al*. (2009)[13] examined cultures of oropharyngeal samples obtained from wild and domestic pigeons and raptors to determine the prevalence of *T. gallinae* eastern Spain and amplified a 369 bp band fragment by PCR using 5.8S rRNA gene and the surrounding ITS1 and ITS2 regions.

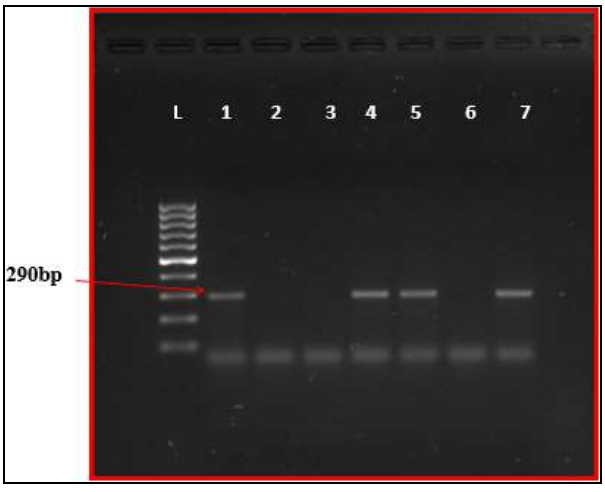

Fig 1: Amplification of *Fe hydrogenase* gene of *Trichomonas gallinae*, PCR product at 290 bp (L-Ladder 100 bp, Lane-1, 4,5 positive sample , 2-3 Negative sample, 6- Negative control, 7-positive control

Sequencing and phylogenetic analysis

In the present study, *Fe hydrogenase* gene was used for molecular characterization and phylogenetic tree. The substitution model Kimura 2-parameter was estimated to be best fit model in MEGA X software on the basis of BIC. The phylogenetic tree constructed by Maximum likelihood method using MEGA X software based on partial *Fe hydrogenase* gene (290 bp) of *T. gallinae* depicted that *T. gallinae* isolated from pigeons (T.gal/AAU-1/P; T.gal/AAU-3/P) and chicken (T.gal/AAU-2/C) of Assam were very closely related to the *T. gallinae* isolates of Iran (Accession No. KX894552, KX894548, KX894545) and Austria (KX514371) as they formed single cluster (Fig. 2). The analysis also revealed that the *T. gallinae* isolated from other geographical locations such as UK, USA, Canada, France, Spain, Malta, Italy and Madagascar were found to form different clades indicating a distance relationship with *T. gallinae* of Assam (Fig. 2).

Pairwise sequence distance and identity of *Fe hydrogenase* gene at nucleotide level

Pairwise distance analysis (at nucleotide level) of partial *Fe hydrogenase* gene belonging to *T. gallinae* isolated from different geographical location of the globe was estimated using MEGA X programme. The overall mean genetic distance among different isolates of *T. gallinae* considered in this study was 4% with a range from 0 to 10% (Table 1). There was no genetic variation in partial *Fe hydrogenase* gene of *T. gallinae* isolated from Assam with those of Iran (KX894552, KX894548, KX894545) and Austria (KX514371) (Table 1). The *T. gallinae* of Assam showed maximum of 10% genetic distance with isolates from Spain (Accession No. KP900029, KP900031, KP900025), UK (JF681136, KC962158, KC529661, KC529664), Madagascar (JF681141), Canada (KJ184169, KJ184171) and France (MK172852, MK172850) while divergence of 9% was seen from Spain (KP900037, KP900032, KP900035), UK (KC529662), Canada (KJ184172), USA (KC249971), Malta (KY675299) and Italy (KY675297), as shown in Fig. 3.

Finally, the results of phylogenetics, pair wise distance and identity analysis based on partial sequence of *Fe hydrogenase* gene depicted that *T. gallinae* of Assam are closely related to *T. gallinae* of Iran and Austria and have the potential to form a new subgroup but a clear picture can be deduced only after considering full-length or marker region of *Fe hydrogenase* gene sequence.

Our present report seems to be the first one in India on molecular characterization and phylogenetic analysis of *T. gallinae* isolate from pigeon and chicken as there are no data available from India in GenBank to compare our findings.

Chi *et al.* (2013)[6] reported that the *Fe hydrogenase* primers were designed to specifically amplify the *Fe hydrogenase* gene of *T. gallinae*, which was used to support the classification of strains, which recently demonstrated the potential for detecting fine-scale variations amongst *T. gallinae* strains. Lawson *et al.* (2011) [14] characterized the *T. gallinae* genotypic heterogeneity within the affected wild British avifauna, green finches and chaffinches by analyzing individual isolates from affected species using hydrogenosomal *Fe-hydrogenase* gene as a single marker locus for fine-typing which showed no evidence of heterogeneity amongst the parasites infecting British passerines, indicating that a clonal strain of *T. gallinae* was the causative agent of the emerging infectious disease. However, Girard *et al.* (2014) [3] on their study on free-ranging Pacific Coast band-tailed pigeons (*Patagioenas fasciata monilis*) in California found them to be infected with trichomonads that were genetically and morphologically distinct from *T. gallinae* and phylogenetic analysis of the ITS1/5.8S/ITS2, rpb1, and *Fe hydrogenase* regions revealed that the protozoan shared an ancestor with *T. vaginalis* of human in spite similar clinical and pathologic features with *T. gallinae*.

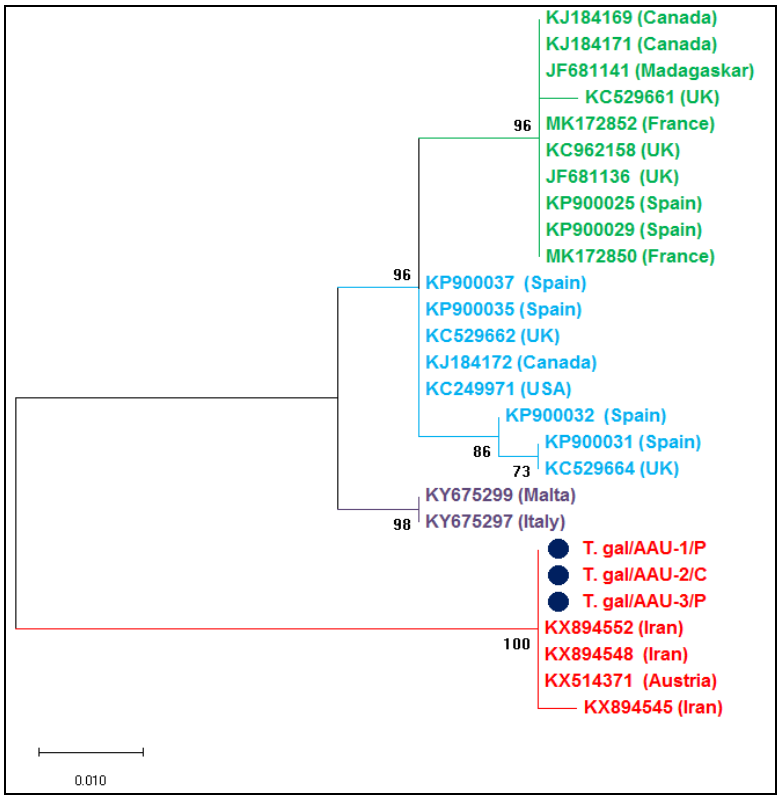

Fig 2: Phylogenetic tree constructed for *T. gallinae* from the *Fe hydrogenase* gene using Clustal Wof MEGA 10.0.5

Table 1: *Fe hydrogenase* sequence distance analysis of *T. gallinae* compared with other isolates

		Standard Error																										
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
Mean Genetic distance	1. T._gal/AAU-1/P		0.00	0.00	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.00	0.00	0.00	0.00	0.02	0.02	0.02	0.02	0.02
	2. T._gal/AAU-2/C	0.00		0.00	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.00	0.00	0.00	0.00	0.02	0.02	0.02	0.02	0.02
		0.00	0.00		0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.00	0.00	0.00	0.00	0.02	0.02	0.02	0.02	0.02
	4. KP900037__(Spain)	0.09	0.09	0.09		0.01	0.01	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.00	0.01	0.01	0.00	0.02	0.02	0.02	0.02	0.00	0.01	0.01	0.01	0.01
	5. KP900029_(Spain)	0.10	0.10	0.10	0.01		0.01	0.01	0.01	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.00	0.00	0.01	0.02	0.02	0.02	0.02	0.01	0.01	0.01	0.00	0.00
	6. KP900032__(Spain)	0.09	0.09	0.09	0.01	0.02		0.01	0.00	0.01	0.01	0.01	0.01	0.01	0.00	0.01	0.01	0.01	0.01	0.02	0.02	0.02	0.02	0.01	0.01	0.01	0.01	0.01
	7. KP900035_(Spain)	0.09	0.09	0.09	0.00	0.01	0.01		0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.00	0.01	0.01	0.00	0.02	0.02	0.02	0.02	0.00	0.01	0.01	0.01	0.01
	8. KP900031_(Spain)	0.10	0.10	0.10	0.01	0.02	0.00	0.01		0.01	0.01	0.01	0.01	0.01	0.00	0.01	0.01	0.01	0.01	0.02	0.02	0.02	0.02	0.01	0.01	0.01	0.01	0.01
	9. KP900025_(Spain)	0.10	0.10	0.10	0.01	0.00	0.02	0.01	0.02		0.00	0.00	0.00	0.00	0.01	0.01	0.00	0.00	0.01	0.02	0.02	0.02	0.02	0.01	0.01	0.01	0.00	0.00
	10. JF681136__(UK)	0.10	0.10	0.10	0.01	0.00	0.02	0.01	0.02	0.00		0.00	0.00	0.00	0.01	0.01	0.00	0.00	0.01	0.02	0.02	0.02	0.02	0.01	0.01	0.01	0.00	0.00
	11. KC962158_(UK)	0.10	0.10	0.10	0.01	0.00	0.02	0.01	0.02	0.00	0.00		0.00	0.00	0.01	0.01	0.00	0.00	0.01	0.02	0.02	0.02	0.02	0.01	0.01	0.01	0.00	0.00
	12. KC529661_(UK)	0.10	0.10	0.10	0.02	0.00	0.02	0.02	0.03	0.00	0.00	0.00		0.00	0.01	0.01	0.00	0.00	0.01	0.02	0.02	0.02	0.02	0.01	0.01	0.01	0.00	0.00
	13. JF681141_(Madagaskar)	0.10	0.10	0.10	0.01	0.00	0.02	0.01	0.02	0.00	0.00	0.00	0.00		0.01	0.01	0.00	0.00	0.01	0.02	0.02	0.02	0.02	0.01	0.01	0.01	0.00	0.00
	14. KC529664_(UK)	0.10	0.10	0.10	0.01	0.02	0.00	0.01	0.00	0.02	0.02	0.02	0.03	0.02		0.01	0.01	0.01	0.01	0.02	0.02	0.02	0.02	0.01	0.01	0.01	0.01	0.01
	15. KC529662_(UK)	0.09	0.09	0.09	0.00	0.01	0.01	0.00	0.01	0.01	0.01	0.01	0.02	0.01	0.01		0.01	0.01	0.00	0.02	0.02	0.02	0.02	0.00	0.01	0.01	0.01	0.01
	16. KJ184169_(Canada)	0.10	0.10	0.10	0.01	0.00	0.02	0.01	0.02	0.00	0.00	0.00	0.00	0.00	0.02	0.01		0.00	0.01	0.02	0.02	0.02	0.02	0.01	0.01	0.01	0.00	0.00
	17. KJ184171_(Canada)	0.10	0.10	0.10	0.01	0.00	0.02	0.01	0.02	0.00	0.00	0.00	0.00	0.00	0.02	0.01	0.00		0.01	0.02	0.02	0.02	0.02	0.01	0.01	0.01	0.00	0.00
	18. KJ184172_(Canada)	0.09	0.09	0.09	0.00	0.01	0.01	0.00	0.01	0.01	0.01	0.01	0.02	0.01	0.01	0.00	0.01	0.01		0.02	0.02	0.02	0.02	0.00	0.01	0.01	0.01	0.01
	19. KX894552_(Iran)	0.00	0.00	0.00	0.09	0.10	0.09	0.09	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.09	0.10	0.10	0.09		0.00	0.00	0.00	0.02	0.02	0.02	0.02	0.02
	20. KX894548__(Iran)	0.00	0.00	0.00	0.09	0.10	0.09	0.09	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.09	0.10	0.10	0.09	0.00		0.00	0.00	0.02	0.02	0.02	0.02	0.02
	21. KX514371__(Austria)	0.00	0.00	0.00	0.09	0.10	0.09	0.09	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.09	0.10	0.10	0.09	0.00	0.00		0.00	0.02	0.02	0.02	0.02	0.02
	22. KX894545_(Iran)	0.00	0.00	0.00	0.09	0.10	0.10	0.09	0.10	0.10	0.10	0.10	0.11	0.10	0.10	0.09	0.10	0.10	0.09	0.00	0.00	0.00		0.02	0.02	0.02	0.02	0.02
	23. KC249971_(USA)	0.09	0.09	0.09	0.00	0.01	0.01	0.00	0.01	0.01	0.01	0.01	0.02	0.01	0.01	0.00	0.01	0.01	0.00	0.09	0.09	0.09	0.09		0.01	0.01	0.01	0.01
	24. KY675299_(Malta)	0.09	0.09	0.09	0.02	0.03	0.02	0.02	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.02	0.03	0.03	0.02	0.09	0.09	0.09	0.09	0.02		0.00	0.01	0.01
	25. KY675297_(Italy)	0.09	0.09	0.09	0.02	0.03	0.02	0.02	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.02	0.03	0.03	0.02	0.09	0.09	0.09	0.09	0.02	0.00		0.01	0.01
	26. MK172852_(France)	0.10	0.10	0.10	0.01	0.00	0.02	0.01	0.02	0.00	0.00	0.00	0.00	0.00	0.02	0.01	0.00	0.00	0.01	0.10	0.10	0.10	0.10	0.01	0.03	0.03		0.00
	27. MK172850_(France)	0.10	0.10	0.10	0.01	0.00	0.02	0.01	0.02	0.00	0.00	0.00	0.00	0.00	0.02	0.01	0.00	0.00	0.01	0.10	0.10	0.10	0.10	0.01	0.03	0.03	0.00	

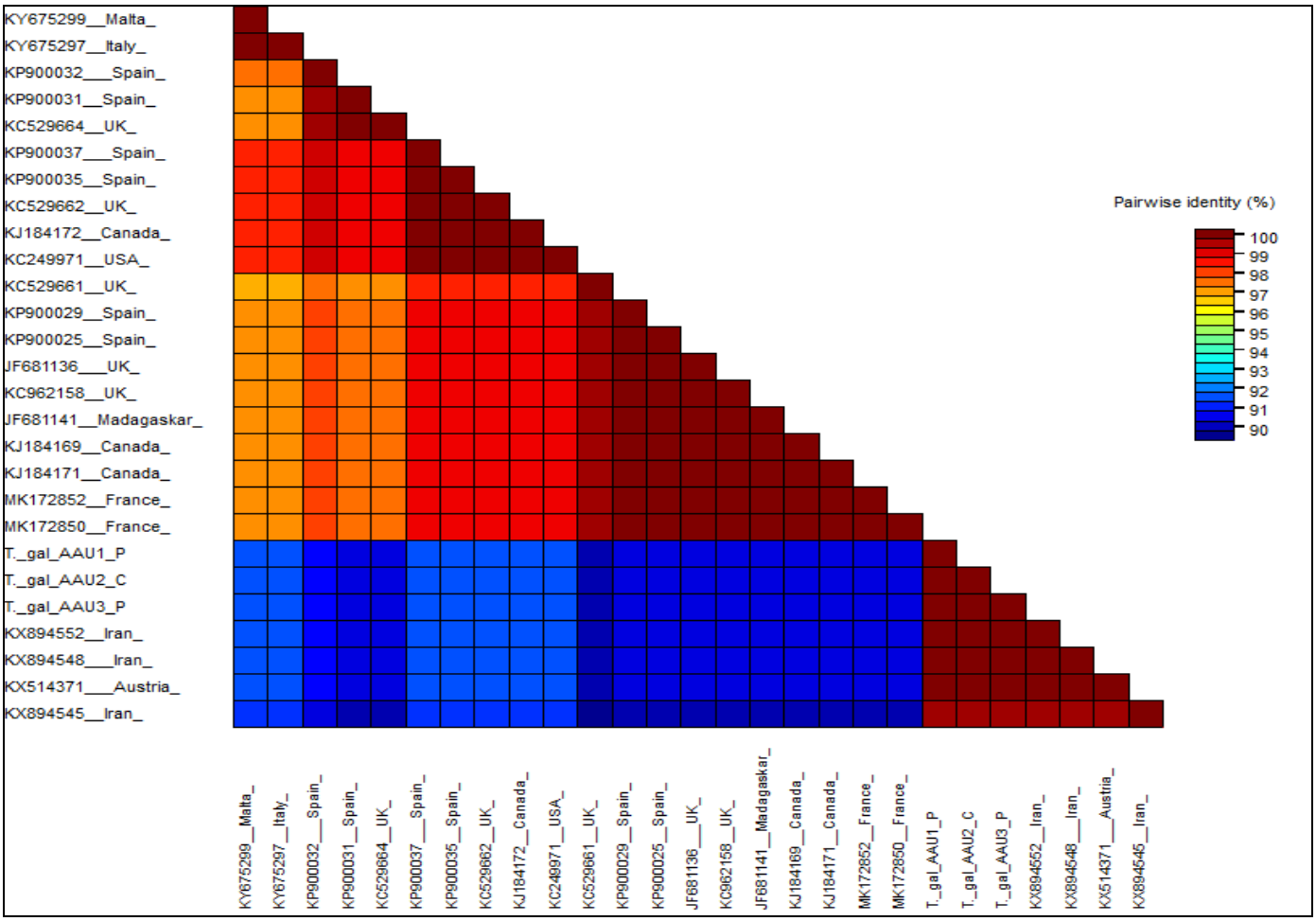

Fig 3: Pair wise identity matrix

Conclusion

From the present work it can be concluded that molecular analysis and phylogeny of *Trichomonas gallinae* isolates from pigeon and chicken of Assam is probably the first report from this part of the country as there are no data available from India in GenBank on phylogeny.

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References

1. Stabler RM, Kihara JT. Infection and death in the pigeon resulting from the oral implantation of single individuals of *Trichomonas gallinae*. Journal of Parasitology. 1954;40:706.
2. Robinson RA, Lawson B, Toms MP, Peck KM, Kirkwood JK, Chantrey J. Emerging infectious disease leads to rapid population declines of common British birds. PLoS ONE. 2010;5:122-125.
3. Girard YA, Rogers KH, Woods LW, Chouicha N, Miller WA, Johnson CK. Dual pathogen etiology of avian trichomonosis in a declining band-tailed pigeon population. Infection, Genetics and Evolution. 2014;24:146-56.
4. Borji H, Razmi GH, Movassaghi AH. Prevalence and pathological lesion of *Trichomonas gallinae* in pigeons of Iran. Journal of Parasitic Diseases. 2011;35(2):186-189.
5. Grabensteiner E, Hess M. PCR for the identification and differentiation of *Histomonas meleagridis*, *Tetratrichomonas gallinarum* and *Blastocystis* spp. Veterinary Parasitology. 2006;142:223-230.
6. Chi JF, Lawson B, Durrant C, Beckmann K, Alrefaei AF, Kirkbride K, *et al*. The finch epidemic strain of *Trichomonas gallinae* is predominant in British non-passerines. Parasitology. 2013;140:1234-1245.
7. Saikia M. Studies on protozoa infection of domestic pigeon (*Columba livia domestica*) in Assam. M.V.Sc. Thesis, Assam Agricultural University, Khanapara, Guwahati-781022; c2016.
8. Forzan MJ, Vanderstichel R, Melekhovets YF, McBurney S. Trichomoniasis in finches from the Canadian Maritime provinces-An emerging diseases. Canada Veterinary Journal. 2010;51:391-396.
9. Kumar S, Stecher G, Li M, Knyaz C, Tamura K. MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. Molecular Biology and Evolution. 2018;35:1547-1549.
10. Kimura M. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. Journal of Molecular Evolution. 1980;16:111-120.
11. Felsenstein J. Confidence limits on phylogenies: An approach using the bootstrap. Evolution. 1985;39:783-791.
12. Muhire BM, Varsani A, Martin DP. SDT: a virus classification tool based on pairwise sequence alignment and identity calculation. PloS One. 2014;9:e108-277. https://doi.org/10.1371/journal.pone.0108277.
13. Sansano-Maestre J, Garijo-Toledo MM, Gomez-Munoz MT. Prevalence and genotyping of *Trichomonas gallinae* in pigeons and birds of prey. Avian Pathology. 2009;38:201-207.
14. Lawson B, Cunningham AA, Chantrey J, Hughes LA, John SK, Bunbury N, *et al*. Clonal strain of *Trichomonas gallinae* is the aetiologic agent of an emerging avian epidemic disease. Infection, Genetics and Evolution. 2011;11:1638-1645.