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Molecular characterization of the nematode *Heterakis gallinarum* (Ascaridida: Heterakidae) infecting domestic chickens (*Gallus gallus domesticus*) in Tunisia

Nabil AMOR[1,*], Sarra FARJALLAH[2], Osama Badri MOHAMMED[1], Abdulaziz ALAGAILI[1], Lilia BAHRI-SFAR[2]

[1]KSU Mammals Research Chair, Department of Zoology, King Saud University, Riyadh, Saudi Arabia

[2]Research Unit of Integrative Biology and Evolutionary and Functional Ecology of Aquatic Systems, Faculty of Science of Tunis, University of Tunis El Manar, Tunis, Tunisia

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Abstract: *Heterakis gallinarum* is one of the most recurrently diagnosed nematodes within the gastrointestinal tract of galliform birds. In the present study, we investigated the genetic diversity of 96 *H. gallinarum* collected from free-range chickens from different localities in Tunisia. We assessed phylogeny and genetic variability using the internal transcribed spacers (ITS) of the nuclear ribosomal DNA and the mitochondrial cytochrome c oxidase subunit I gene. Haplotype and nucleotide diversities indicate that *H. gallinarum* is a species with extremely low genetic diversity. Based on the networks and phylogenetic trees, there was strong support for the absence of significant geographical structuring among the *H. gallinarum* populations in different localities in Tunisia. Mismatch distributions and neutrality tests of both genetic markers suggest that at least one expansion event occurred in the population demographic history of *H. gallinarum*. Our data showed a lack of population structure using the pairwise fixation index (FST) and an extensive gene flow. It indicated that the most likely major contributor to the low genetic diversity and gene flow is the movement of the parasitized birds; in other words, the frequency of the poultry trade considerably affects patterns of worm gene flow.

Key words: *Heterakis gallinarum*, genetic diversity, Tunisia, internal transcribed spacers, cytochrome oxidase gene

1. Introduction

The caecal poultry nematode *Heterakis gallinarum* (Schrank, 1788) has a large host and geographical range, and it is frequently described in avian helminth studies (1). In fact, it is one of the most recurrently identified nematodes within the gastrointestinal tract of galliformes (2).

Heterakis gallinarum is a vector of the protozoan *Histomonas meleagridis*, a causative agent of histomoniasis (blackhead disease). Histomoniasis induces severe pathological lesions in the gut and liver, leading to an important increase of host mortality and exerting serious economic impacts on the poultry industry (3,4).

In Tunisia, the domestic chicken *Gallus gallus domesticus* is one of the most valuable sources of protein since poultry production constitutes more than half of the entire protein production (5). Despite this economic importance, studies of helminth infections damaging Tunisian poultry are limited to parasitological surveys (5).

Different genetic markers, such as internal transcribed spacers (ITS) and the subunit I of the mitochondrial cytochrome oxidase gene (COXI), proved their abilities to infer phylogenetic relationships among parasites species (6,7). Despite the low level of the intraspecific polymorphism, ITS sequences show high rates of interspecific variability (8–10). Currently, COXI is one of the most common and easily used mt-DNA genes in phylogeny. Thus, COXI applications have allowed the revision of phylogenetic relationships between parasite species, as well as the barcoding of several taxa (11).

In Tunisia, there is a lack of molecular study on *H. gallinarum* populations, and sequence information is still at a pioneering level. In fact, we found only a few COXI and ITS sequences from China, published recently in a study by Gu et al. (9). Consequently, in this study, we aimed to evaluate the occurrence of *H. gallinarum* isolated from domestic chickens (*Gallus gallus domesticus*) reared in different Tunisian localities and to characterize this nematode and its population structure using two common molecular markers, namely the ITS and COXI genes.

2. Materials and methods

2.1. Sample collection and morphological identification

Between May 2016 and January 2017, the caecal content of 25 domestic chickens *Gallus gallus domesticus* (Linnaeus,

* Correspondence: nabil.amor@gmail.com

1758) from five Tunisian localities (five chickens/locality) were examined. The localities and number of adult *H. gallinarum* collected for molecular study were as follows: Beja, located in the north of Tunisia (36°44′30.3″N; 9°11′22.1″E): N = 20; Sousse (35°49′48.2″N; 10°35′41.8″E): N = 19; Kairouan (35°41′01.8″N; 10°05′13.7″E): N = 19; in the center in Gafsa (34°23′46.9″N; 8°47′57.7″E): N = 19; and Gabes (33°53′21.0″N; 10°05′28.7″E): N = 19 in the south. Worms were identified morphologically according to existing keys and descriptions (12) and stored in 75% ethanol until DNA extraction.

2.2. DNA extraction, PCR amplification and sequencing

We extracted genomic DNA using a Wizard Genomic DNA Purification kit (Promega, Madison, WI, USA). The polymerase chain reaction (PCR) was performed in a 25-µL reaction mix containing 1 µL of template (the genomic DNA of each sample was used as a template), 1 U of MyTaq DNA polymerase (Bioline, Meridian Life Science, Memphis, TN, USA), 5-µL 5X MyTaq buffer (including dNTPs and $MgCl_2$), 10 pmol of each primer, and ddH_2O. Negative controls were always included in PCR to assess possible contamination.

The DNA region (960 bp) comprising *ITS*-1, 5.8S rDNA and *ITS*-2 (ITS) was amplified using primers NC5/ NC2 (13). Two primers (9), COXI-R/ COXI-F, were used to amplify the COX1 partial gene (1325 bp). The thermal profile included an initial denaturation at 94 °C for 5 min, 35 cycles of 45 s at 94 °C, 45 s at 50 °C (COXI), or 62 °C (ITS), 45 s at 72 °C, followed by a final extension at 72 °C for 10 min and a final cooling at 4 °C. All the PCR products were sequenced using forward primers in a Macrogen sequencing facility (Macrogen Inc., Seoul, Korea).

2.3. Data analysis

We performed multiple alignments with SeaView V 3.2 (14). We translated COX1 sequences into amino acids to check for possible amplification of pseudo genes. We added to the dataset several sequences from China (KP308308-63) (9). Concerning the ITS, we found only 3 haplotypes covering only the *ITS*1 and *ITS*2 regions (JQ995320 from USA and AJ876757 from China, AJ007453). Finally, we used *H. beramporia* (KU529972) and *Ascaridia columbae* (JX624729, KC905082) as outgroups. We estimated haplotype (Hd) and nucleotide diversities (π) (15), number of polymorphic sites (S), and the mismatch distribution using DnaSP v 5.10 (16).

In order to calculate the pairwise fixation index (FST), the gene flow (*Nm*), the neutrality indexes Tajima's D (17), Fu's Fs (18), and estimate the molecular variability among and within Tunisian populations, Arlequin 3.5 (19) was used.

The best-fitting models of molecular evolution applied to both datasets in the phylogenetic reconstructions and the partition scheme of COXI were inferred with PartitionFinder 1.1.0 (20) and jModeltest 2.1 (21). For the ITS dataset, we tested 11 schemes with jModeltest. We obtained a starting tree using the BIOINJ method fixed for parameter estimation under the Akaike Information Criterion. Under the assumption that each codon position evolves at different rates, we partitioned the COXI dataset by codon position, and the appropriate model was selected using the Bayesians Information Criterion implemented in PartitionFinder.

To infer the relationships among haplotypes, we conducted neighbor joining (NJ), Bayesian, and maximum likelihood analyses by using SeaView V 3.2, MrBayes 3.2 (22), and RAxML (23), respectively. We evaluated Bootstrap support in the NJ and ML trees by 1000 pseudoreplicates. We carried out two independent runs for the Bayesian analyses of 5 million generations each, sampling trees every 500 generations. We generated a consensus topology using 50% of the resulting trees. We analyzed the evolutionary relationships between haplotypes for the two markers with a median-joining network (24) constructed with Network 5.0 (Fluxus Technology Ltd.).

3. Results

In all the inspected localities (5 chickens/locality) between May 2016 and January 2017, the prevalence of *H. gallinarum* was 100%. We did not observe any clinical signs in the parasitized chickens. Total genomic DNA was isolated from 96 adult individuals (Beja: N = 20, Sousse: N = 19, Kairoun: N = 19, Gafsa: N = 19, and Gabes: N = 19).

3.1. Genetic diversity

We deposited all haplotypes generated for both markers into the GenBank under the accession numbers MF066712–MF066726. ITS dataset analysis showed 5 parsimony informative sites. All sites were singleton variable defining 6 haplotypes (H1–H6). None of the haplotypes was unique or found in only one locality. H1 is the major haplotype, including 74 *H. gallinarum* (77%). Nucleotide diversity in the 96 samples was very low (0.00046). Among the studied localities, the nucleotide diversity ranged from 0.0004 (Gafsa and Gabes) to 0.00072 (Kairouan). Haplotype diversity (Hd) was also very low (0.399), and the values of Hd within the 5 different geographical populations ranged from 0.35 (Beja) to 0.58 (Kairouan). Haplotype and nucleotide diversities between all samples were very low (Hd = 0.39, π = 0.00046) (Table 1).

The analysis of COXI sequences revealed 9 haplotypes differentiated by 10 variable sites. Only 3 sites were parsimony informative. Six unique haplotypes were found in only one locality and a major one included 83 sequences (86%).

Nucleotide diversity and Haplotype diversity were also very low for COXI in all the studied localities (Table 1). Kairouan exhibited the lowest Hd and Beja the highest

value. π ranged from 0.00014 in Sousse to 0.0005 in Beja. K ranged from 0.182 (Sousse) to 0.657 (Beja). Overall haplotype diversity and nucleotide diversity was 0.252 and 0.00025, respectively.

3.2. Population structure and demographic analysis

Both makers showed the absence of genetic structure among *H. gallinarum* populations (FST_{ITS} = –0.02, FST_{COXI} = –0.001). The inferred $Nm_{(ITS)}$ value was 23.3 between Beja and Sousse, and it reached infinite in the remaining cases. The $Nm_{(COXI)}$ values range was 60.3 (between Gabes and Kairouan), 674.75 (between Gafsa and Kairouan) to infinite, thereby indicating an extensive gene flow. AMOVA analyses confirmed this result when sample sites were assembled as three groups (north, center, and south). Actually, several AMOVA analyses at different hierarchical levels were performed and 97.28% and 94.69% of the total variations corresponded to the variation within populations for ITS and COXI, respectively. Pairwise, FST for both markers were small or zero with significant P-values. In Table 2, we set negative FST values to zero.

Mismatch distributions analyses of both genetic markers showed clear unimodal curves (Figures 1a and 1b). Tajima's D and Fu's FS test of neutrality were both significantly negative (D_{ITS}= –1.576, D_{COXI}= –1.513, FS_{ITS}= –3.103, and FS_{COXI}= –1. 372). Mismatch distributions analyses and neutrality indices suggested that a population expansion in *H. gallinarum* occurred. It is possible that demographic expansions of the parasite occurred after introducing particular individuals into the endemic areas by anthropogenic movements of the birds.

3.3. Phylogeography and phylogenetic analysis

We investigated the relationships between haplotypes by building median-joining networks. Both haplotype datasets displayed star-like networks. H1 (ITS) and H2 (COXI), the most common haplotypes, were situated in the star's center and were linked to the remaining haplotypes by short branches (Figures 2a and 2b). Only H1 (ITS) and H2 (COXI) were found in all the sampling localities. All ITS haplotypes were directly derived from H1. The COXI network shows the same result for 6 haplotypes, but H4 and H9 were connected to H2, respectively, by 3 and 2 mutations. These median-joining network results support a population expansion from an ancestral haplotype.

PartitionFinder v1.1.0 suggested a nonpartitioned codon scheme for the COXI dataset with GTR + G and GTR + G + I substitution models as the best fit for these data for the Bayesian inferences (BI) and the maximum likelihood (ML) approaches, respectively. Using jModeltest, we selected HKY + G for ITS alignment. Node support values within the text consist of NJ bootstrap values, ML bootstrap values, and Bayesian posterior probabilities (NJ/ML/PP).

The phylogenetic reconstructions based on ITS data (Figure 3a) show the same topology and strongly support

Table 1. Summary of ITS and COXI genetic diversity of the five populations of *H. gallinarum* collected from chickens in Tunisia. Hap nb: haplotype number; Hd: haplotype diversity; π: nucleotide diversity; K: average number of nucleotide differences.

	ITS				COXI			
Locality (n)	Hap nb	Hd	π	K	Nb hap	Hd	π	K
Beja (20)	5	0.3524	0.00040	0.381	5	0.4238	0.00050	0.657
Gafsa (19)	4	0.3632	0.00041	0.389	3	0.1947	0.00015	0.200
Gabes (19)	6	0.3538	0.00040	0.385	4	0.2764	0.00022	0.291
Sousse (19)	3	0.4394	0.00049	0.470	2	0.1818	0.00014	0.182
Kairouan (19)	6	0.5882	0.00072	0.691	2	0.1176	0.00022	0.291

Table 2. Pairwise FST values for ITS (above diagonal) and COXI (below diagonal) datasets.

Locality	Beja	Gafsa	Gabes	Sousse	Kairouan
Beja	-	0	0	0.021	0
Gafsa	0	-	0	0	0
Gabes	0	0	-	0.005	0
Sousse	0	0	0.00062	-	0
Kairouan	0	0.00074	0.00821	0.691	-

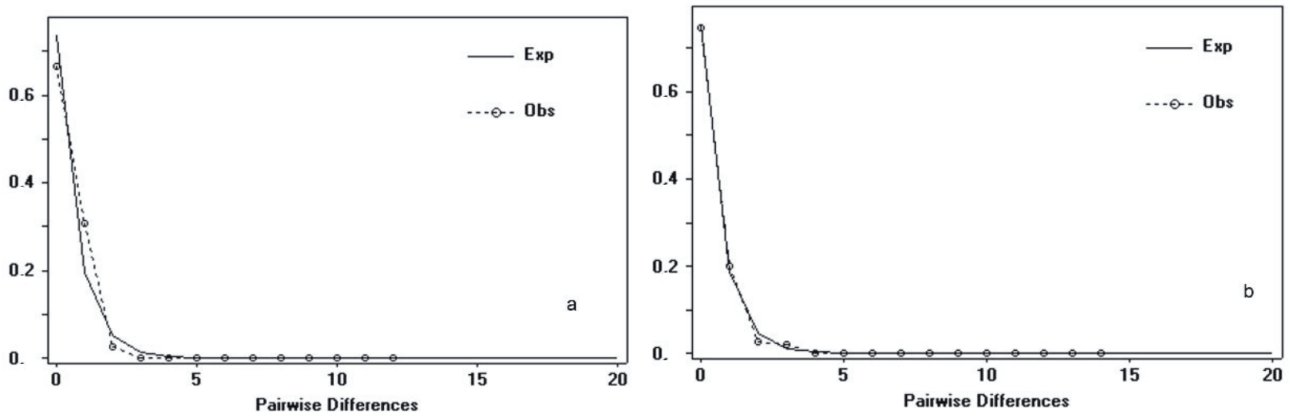

Figure 1. Mismatch distribution to test the expansion of 96 *H. gallinarum* isolates. The number of nucleotide differences between pairs of sequences is indicated along the x-axis and their frequency along the y-axis; a) ITS and b) COXI.

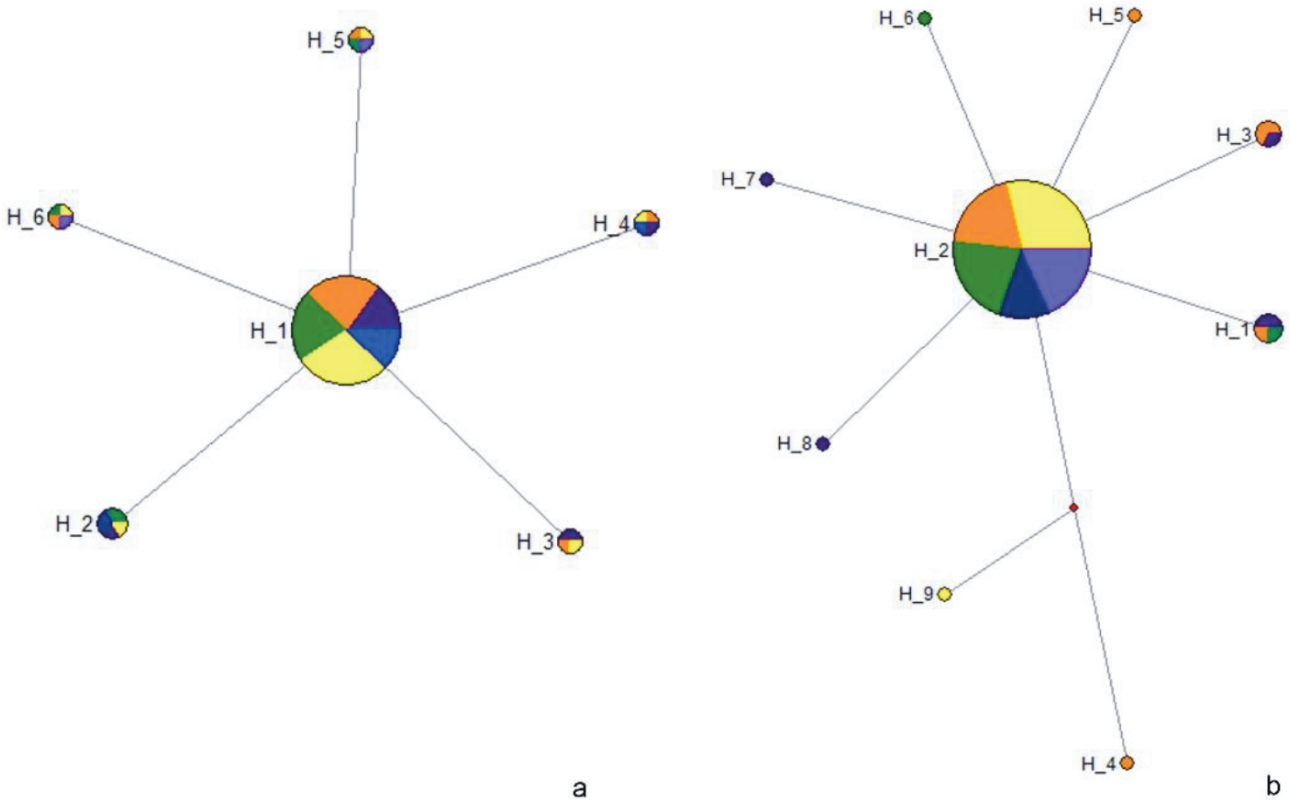

Figure 2. Haplotype network obtained from COXI: a) ITS and b) sequences. The area of each circle is proportional to the haplotype frequency and each branch represents one mutation.

the monophyly of *H. gallinarum* (100/100/1). All nodes within *Heterakis* clade present low bootstrap support. The USA haplotype appears as a sister group to the most common Tunisian *Heterakis* H1, while Chinese and Australian haplotypes form a sister group to the Tunisian and USA clade.

The phylogenetic trees resulting from ML and NJ and Bayesian analyses on COXI exhibited similar topologies (Figure 3b). These phylogenetic analyses were mostly congruent with ITS results and strongly supported the monophyly of *H. gallinarum* (100/100/1). Within this lineage, *Heterakis* from Tunisia were nested together as

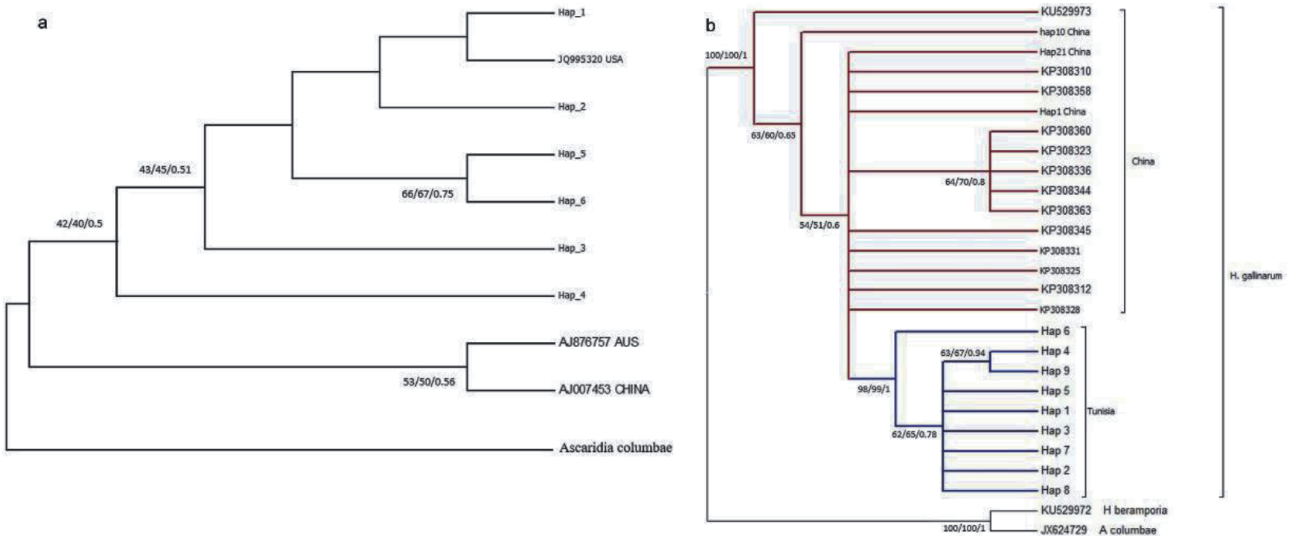

Figure 3. Consensus trees constructed with NJ, ML, and BI methods showing phylogenetic relationships among *H. gallinarum*: a) Phylogenies inferred from COXI sequences and b) Phylogenies inferred from ITS sequences (ITS1–5.8S–ITS2). Numbers shown at nodes of branches are the posterior probability (BI) and bootstrap values (NJ and ML), respectively. *H. beramporia* (COXI) and *Ascaridia columbae* (COXI and ITS) were used as the outgroup.

one strongly-supported clade (98/99/1) and are sister to the remaining haplotypes mainly originating from China (bootstrap value = 98). These results agree with the median-joining network of the Tunisian haplotypes of *H. gallinarum*.

4. Discussion

Molecular techniques have provided a better understanding of the phylogeny, population structure, and epidemiology of nematodes (13). *Heterakis gallinarum* appears as one of the main nematodes affecting poultry production in Tunisia; however, no molecular studies are currently available on this issue. In our study, we assessed the genetic variability of *H. gallinarum* from Tunisia. Previous studies showed the ability of COXI and ITS to resolve taxonomic questions within various parasitic nematodes (13,25). These markers have been widely used due to their high interspecific levels of variability and to the availability of common universal primers.

In both datasets, namely ITS rDNA and COXI mtDNA, the levels of haplotype and nucleotide diversities indicate that *H. gallinarum* is a species with extremely low genetic diversity. The π values did not exceed 0.0005 within and among the studied populations. The Hd value was slightly higher in the ITS dataset than in COXI. The observed low π values are in accordance with the pattern of genetic diversity previously reported across Chinese populations of *H. gallinarum* based on the same markers (9,10). However, the authors in these studies detected higher Hd values. This difference can be due to the higher geographic distances between the studied Chinese populations. The observed low level of genetic diversity is in agreement with patterns of polymorphism reported in several nematode species. Based on ITS-2 rDNA sequence polymorphism, Posedi et al. (26) noticed low genetic variability (0.3%) within *Cylicocyclus elongatus* and *C. insigne* populations. Furthermore, Bazh (3) reported high identity between ITS sequences of chicken nematodes from Egypt, namely *Heterakis gallinae* and *H. gallinarum*, confirming the conspecific status of these samples. Using ITS-1 rDNA sequences, Lin et al. (27) revealed no genetic polymorphism among *Baylisascaris schroederi* isolates. For the mtCytb gene, Zhao et al. (25) detected an important gene flow and low *p* distances between Chinese populations of *B. schroederi*. In our study, the observed genetic pattern within *H. gallinarum* is generally characteristic of several invertebrate species, including highly dense populations with important fecundity rates (28). The observed low variability can also indicate high matrilineal inheritance in *H. gallinarum* populations or the occurrence of an episode of genetic drift and recent sample size expansion.

The haplotype networks analysis displayed obvious star-like patterns, with the most common haplotypes being H1 (ITS) and H2 (COXI) in the star's center. All ITS and COXI haplotypes were directly derived from H1 and H2 haplotypes, respectively. Darriba et al. (21) considered such haplotypes as the most ancient or ancestral ones within a certain species. Often, ancestral haplotypes include high individual frequency and may cover most of the species' geographic ranges. Both networks and

phylogenetic analyses revealed the absence of significant geographical structure among *H. gallinarum* in Tunisian populations, as reported previously (3,8,9). FST values confirmed a lack of significant differentiation among the *H. gallinarum* populations (FST_{ITS} = –0.02, FST_{COXI} = –0.001). This suggests a high genetic exchange based on several molecular markers and is consistent with previous reports (8–10,25,27). AMOVA confirmed a lack of genetic structures between the 3 studied regions. The observed gene flow between *H. gallinarum* populations can be explained by the wide geographical range of the most common haplotypes H1 (ITS) and H2 (COXI).

In theory, a pattern of gene flow within parasite populations is generally modulated by two main factors: the parasites' own life history and their hosts' evolutionary dynamics. As in previous studies dealing with population genetics of parasitic roundworms (9,10,27), our results confirm that dispersion patterns of parasitized birds considerably affect parasite gene flow. Furthermore, direct migration within nematodes species is extremely limited and a major part of the ITS pattern of dispersion should be associated to their definitive host (29,30). Consequently, the model of population parasite structure will be a direct function of the host pattern of dispersion. In addition, the values of Tajima's D, Fu's Fs, and mismatch distributions analyses of both genetic markers suggest that *H. gallinarum* populations experienced an expansion event during their demographic history. It is likely that anthropogenic activities might accelerate *H. gallinarum* expansions by introducing new individuals into endemic areas.

In conclusion, this study provides the first data on the genetic variability of *H. gallinarum* isolates from different localities in Tunisia. Our data showed a lack of genetic structure using the FST indices and an important gene flow between *H. gallinarum* Tunisian populations. It suggests that the definitive host movement and the frequency of the poultry trade within this region are the main contributors controlling patterns of parasite gene flow. In addition, these results reveal that *H. gallinarum* populations experienced at least one expansion event during their demographic history.

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