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**Prevalence and genotyping of *Trichomonas gallinae* in pigeons and birds of prey**

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## Abstract

Avian trichomonosis is a world-wide parasitic disease caused by the protozoan *Trichomonas gallinae*. Although several degrees of pathogenicity have been described on the basis of the clinical signs in birds, there are few reports concerning the genetic characterization of the parasite and its relationship with pathogenicity. The parasite usually appears apathogenic but is occasionally responsible for outbreaks of the disease in avian populations, particularly affecting nestlings of ornithophagous raptors. For three years, cultures of oropharyngeal samples from 612 wild and domestic pigeons (*Columba livia*) and 102 birds of prey from 15 different species were made in an attempt to determine the prevalence of *T. gallinae* in the Valencian Community (eastern Spain). To establish the genotype of the isolates, 5.8S rRNA and the surrounding internal transcribed spacer regions (ITS) were amplified by PCR and sequenced. After Restriction map analysis, sequencing and PCR-restriction fragment length polymorphism (RFLP) using *Hae*III showed two genotypes (A and B) in isolates from both groups of birds were found, although genotype prevalence differed in each group, genotype A being more prevalent in columbiforms and genotype B in raptors. In addition, genotype B was present in every bird that displayed macroscopic lesions.

## 1 Introduction

2

3 Avian trichomonosis is a parasitic disease caused by the protozoan *Trichomonas gallinae*. Parasites  
4 live mainly in the bird's anterior digestive tract, where they can cause granulomatous lesions that  
5 occlude the oesophagic lumen, leading to the death of birds as a result of severe starvation. However,  
6 it is well known that virulence of the strains varies and some can reach parenchymatous organs and  
7 generate necrotic foci (Pérez-Mesa and Stabler, 1960; Narcisi *et al.*, 1991).

8 Columbidae are known as the parasite's main host, particularly the domestic pigeon (*Columba*  
9 *livia*), which has been considered responsible for the worldwide spread of *T. gallinae* (Harmon *et al.*,  
10 1987). Despite the parasite's preference for pigeons and doves, *T. gallinae* can affect a wide range of  
11 bird families, and infections in bustards (Silvanose *et al.*, 1998), psittacine birds (Baker, 1996;  
12 McKeon *et al.*, 1997), fowl (McDougald, 2000) and passerine birds (Cousquer, 2005) have been  
13 reported. Birds of prey (diurnal and nocturnal) are also frequently infected by the flagellated  
14 protozoan, especially those species that usually feed on doves or even bird carcasses (Work and Hale,  
15 1996; Boal *et al.*, 1998; Erwin *et al.*, 2000; Krone *et al.*, 2005).

16 Data on prevalence found in Columbidae by different authors throughout the world vary  
17 greatly, ranging from 5.6% cited by Schulz *et al.* (2005) in mourning doves (*Zenaida macroura*), to  
18 95%, detected by Conti and Forrester (1981) in white-winged doves (*Zenaida asiatica*) in the USA. In  
19 Spain, Höfle *et al.* (2004) reported an outbreak of trichomonosis in the south that affected 15% of  
20 wintering woodpigeons (*Columba palumbus*), while Villanúa *et al.* (2006) established a prevalence of  
21 34.2% in the same species in different parts of the Peninsula. The parasite has been reported  
22 throughout the year, but most outbreaks seem to occur in spring, summer and autumn (Cole, 1999;  
23 Gerhold *et al.*, 2007).

24

Differences in prevalence between captive and wild birds have been demonstrated, proving to be higher in the first. In Australia, McKeon *et al.* (1997) observed a prevalence of 49% in captive pigeons, compared with 46% in wild birds. More remarkable differences were described in Saudi Arabia by Bailey *et al.* (2000), who detected a prevalence of 35% in wild pigeons, and 68% in captive birds.

Due to the ability of the parasite to infect numerous avian species, its role in the conservation of threatened species has recently been reviewed (Höfle *et al.*, 2004; Swinnerton *et al.*, 2005; Bunbury *et al.*, 2007; Hegemann *et al.*, 2007). This seems to be particularly important in the case of birds of prey that, due to loss of habitat, are forced to nest near urban areas, and whose traditional prey is mainly replaced by urban pigeons. A noticeable variation was found in the prevalence of the parasite in Cooper's hawk (*Accipiter cooperi*) in Arizona, since the nestlings of couples breeding far from urban areas displayed a prevalence of only 9% of *T. gallinae*, while those in urban areas had a value of 85%. According to the authors, this was due to the increased consumption of urban columbiforms in urban areas (Boal *et al.*, 1998). This observation was further corroborated by Estes and Mannan (2003), who determined that 57% of the urban Cooper's hawks diet consisted of columbiforms compared with 4% in rural areas. This was also observed in goshawk (*Accipiter gentilis*) nestlings close to urban areas in Europe, e.g. 100% in Poland by Wieliczko *et al.* (2003) and 65% in Germany by Krone *et al.* (2005). In the Iberian Peninsula, studies of *T. gallinae* prevalence have focused on Bonelli's eagle (*Hieraaetus fasciatus*), a vulnerable species that can be found in the Peninsula and that accounts for between 75% and 93% of the total European population (Real & Mañosa, 1997). In southern Portugal *T. gallinae* was diagnosed in 50% of the Bonelli's eagle chicks analysed by Höfle *et al.* (2000). In northeast Spain, Real *et al.* (2000) found the parasite to be present in this species of raptor in 36% of the chicks studied and a death rate of 3% was attributed to his disease. In both cases a high percentage of pigeons was observed in the eagle's diet.

1           Several authors have shown that there are variations in the virulence of different parasite  
2 strains, but only a few attempts to characterise them genetically have been made, including some  
3 recent studies on *Histomonas meleagridis* (Harold *et al.*, 2006). Studies carried out on the gene 5.8S  
4 rRNA and the surrounding ITS (Internal Transcribed Spacer regions) have proved to be very useful  
5 for the taxonomy of the Trichomonadidae family (Felleisen, 1997; Hayes *et al.*, 2003; Walker *et al.*,  
6 2003; Kleina *et al.*, 2004; Gerhold *et al.*, 2008a). Gaspar DaSilva *et al.* (2007) used this sequence to  
7 characterise the strains of *T. gallinae* found in a wild population of pink pigeons from Mauritius. The  
8 authors discovered that every strain isolated belonged to the genotype sequenced by Kleina *et al.*  
9 (2004). Later, the same authors used the randomly amplification of polymorphic DNA (RAPD)  
10 technique and described seven groups which proved to be species-specific and geographic-specific  
11 strains. To our knowledge, genetic characterization of *T. gallinae* from birds of prey has not been  
12 reported to date except for three sequences obtained from Cooper's hawks and one from a Broad-  
13 winged hawk published by Gerhold *et al.* (2008a).

14           The objective of this study is to determine the prevalence of *T. gallinae* in different species of  
15 birds of prey and its main host – pigeons – and to determine the genotype of the strains from both  
16 groups using PCR-restriction fragment length polymorphism (RFLP) of the 5.8S rRNA gene and the  
17 surrounding ITS1 and ITS2 regions. Besides, the relationship between strain genotype and virulence  
18 is investigated.

19

20   **Material and methods**

21

22   **Area of study and birds.** The area studied was the Valencian Community, a region on the east of the  
23 Iberian Peninsula (40° 47'N, 1° 32'W). It is characterized by a Mediterranean climate, with mild  
24 winters and hot summers, and mountainous terrain. For a period of three years (January 2005 -April  
25 2008) a total of 612 domestic pigeons (*Columba livia*) and 102 birds of prey from 15 species were

1 sampled. In the case of pigeons, 439 samples were taken from wild birds while 173 were obtained  
2 from barrier pigeon lofts. Wild birds were captured in the course of a pest control program undertaken  
3 by the local authorities using cage traps in public parks located in urban areas. After sampling, the  
4 birds were humanely euthanized. Pigeons from lofts were captured in situ and released after sampling.  
5 Raptor samples were taken from 48 nestlings and 54 adult birds. Sixteen samples from 15 to 25-day-  
6 old Bonelli's eagle chicks and 23 from 15-day-old European kestrel (*Falco tinnunculus*) chicks were  
7 taken directly from the nest. The other samples taken from chicks and adult birds were obtained from  
8 local Wildlife Recovery Centres (Table 1).

9  
10 **Sampling and parasite culture.** Oral swabs were taken from the oral cavity, placed in  
11 Tryptone/Yeast extract/Maltose (TYM) medium supplemented with 10% fetal calf serum (Sigma  
12 Chemical Co., St. Louis, MO) and incubated at 37 °C (Diamond, 1957). Cultures were observed over  
13 three consecutive days to check the growth of parasites. Positive samples were frozen at -80°C in  
14 TYM medium containing 5% of dimethyl sulfoxide. Necropsies were carried out on euthanized or  
15 dead birds to determine if macroscopic lesions were present.

16  
17 **Isolation of genomic DNA.** DNA was obtained from 1 ml of medium with parasites in an  
18 exponential growing phase, using a commercial DNA extraction kit (DNeasy Tissue extraction kit,  
19 QIAGEN, Valencia, CA, USA). Parasites were recovered by centrifugation at 350 x g 5 min.  
20 Supernatant was discarded and the sediment was washed three times with PBS. After the washes, the  
21 pellet was processed in accordance with the manufacturer's instructions. Final products were eluted in  
22 100 µl of elution buffer and stored at 4 °C until used.

23  
24 **PCR amplification of the ITS1/5.8S rRNA/ITS2.** PCR reactions were carried out following the  
25 method used by Felleisen (1997) and using TFR 1 (5'-TGCTTCAGCTCAGCGGGTCTTCC3') and



TFR 2 (5'-CGGTAGGTGAACCTGCCGTTGG-3') oligonucleotides. A final volume of 50 µl was used in each reaction containing: 5 µl of 10x buffer, 1.5 mM MgCl<sub>2</sub>, 2 mM dNTP, 2 µM of each primer, 2.5 IU of AmpliTaq Gold polymerase (PE Applied Biosystems, Foster City, CA, USA) and 5 µl of DNA sample. PCR was performed using the following temperature profile: initial denaturation at 94 °C for 9 min, followed by 40 cycles of denaturation at 94 °C for 30 sec., annealing at 66 °C for 30 sec., extension at 72 °C for 30 sec. and a final extension step at 72 °C for 15 min. Ten µl of the final product were loaded into 2% agarose gel stained with ethidium bromide and observed under UV light.

**Sequence analysis of the ITS1/5.8S rRNA/ITS2 region.** Twenty-five PCR products obtained from pigeons and nine obtained from birds of prey were randomly selected and sequenced in the facilities of "Sistemas Genómicos, S.A." (Paterna, Valencia, Spain) using ABI PRISM BigDye Terminator Cycle Sequencing kit. The PCR products had previously been purified using a commercial kit (Minielute Purification kit, QIAGEN, Valencia, CA, USA). Sequencing reactions were carried out in an automatic sequencer 3730xl DNA Analyzer. Chromatograms of each sample in both directions were manually checked and edited using BioEdit 7.0.9.0. software available at <http://www.mbio.ncsu.edu/BioEdit/bioedit.html> (Hall, 1999) and assembled using Lasergene analysis software SeqMan 7.0 (DNASTAR, Madison, USA). A Multiple alignment was performed using the Clustal W method of the MegAlign 7.0 program (DNASTAR, Madison, USA). This multiple alignment contained seven representative sequences obtained in this study and 24 sequences of ITS1/5.8S rRNA/ITS2 region from related species selected from GenBank database. Mega 4.0 software available at <http://www.megasoftware.net> (Tamura *et al.*, 2007) was used to generate the phylogenetic tree using the neighbor-joining method with a bootstrap value of 1000 replicates.

**Restriction Fragment Length Polymorphism (RFLP).** Final PCR products of each sample were digested using *HaeIII* enzyme (New England Biolabs Inc. Beverly MA, USA). This enzyme was selected after generating a restriction map with BioEdit 7.0.9.0. software using *T. gallinae* sequences obtained in this study. *HaeIII* displayed a cutting site at position 243 only in one type of sequences, but not in the other. Ten µl of each sample were digested using 10 IU of the enzyme and 10 µl of specific buffer, obtaining a reaction volume of 25 µl. Reactions were carried out at 37 °C for one hour and digested products were run in 3% agarose gel stained with ethidium bromide and observed under UV light.

**Statistical analysis.** Independent variables studied for pigeons were “period of the year”, divided into period 1 (March-April-May), period 2 (June-July-August), period 3 (September-October-November) and period 4 (December-January-February), and “aptitude” of the birds: birds from barrier lofts or birds trapped in the wild. Association between variables was studied using binary logistic regression analysis. The Chi-square test was used to check differences between age groups in raptors. Association was considered significant when p values were lower than 0.05. SPSS software version 13.0. was used for the statistical analysis.

**GenBank submission.** The sequences obtained in this study were deposited in GenBank with accession numbers EU881911 to EU881917.

## Results

**Prevalence of *T. gallinae* in pigeons.** At the end of the study 274 out of the 612 samples analysed were positive to culture in TYM medium, indicating a global prevalence of 44.8% in pigeons. Taking into account the prevalence results according to the “aptitude” variable, a significant difference was

found between the prevalence in barrier lofts (52.7%) and the values found in wild birds (41%) (OR= 1.712, 95%, CI=1.20, 2.44,  $p < 0.05$ ). In spite of the high percentage of parasitized birds, only one bird with macroscopic lesions in the oral cavity was found (0.2%). Annual variations were observed in prevalence, which ranged from 35.3% in period 3 to 57.1% in period 4, showing significant differences between periods (Table 2).

**Prevalence of *T. gallinae* in birds of prey.** At the end of the study *T. gallinae* was found in 6 out of the 15 species analysed. The parasite was isolated in 20 out of 102 samples, giving an overall prevalence of 19.6%. The endangered Bonelli's eagle was the species that displayed the highest prevalence value (52.4%) (Table 1). With regard to the presence of lesions, *T. gallinae* was responsible for oral and pharyngeal granuloma in 5 out of 20 infected birds (25%). Four of the birds, 3 European kestrels and a barn owl (*Tyto alba*), displayed serious lesions and emaciation, dying shortly after they were examined. These are the first cases of trichomonosis, to the authors' knowledge, described in both species in the wild in Spain. Although the nestlings analyzed displayed higher percentages of parasitization (27.1%) than adult birds (13%), age did not appear to be a significant risk factor for the birds infected by the parasite ( $\chi^2 = 3.23$ ,  $p > 0.05$ ). However, in the endangered Bonelli's eagle only chicks sampled directly on the nest were positive to *T. gallinae* infection.

**ITS1/5.8S rRNA/ITS2 sequences.** One hundred and sixteen samples from pigeons and 14 samples from birds of prey grew sufficiently for DNA extraction. PCR was carried out, and all of them produced a 369 pb band fragment, as previously described by Felleisen (1997) for *T. gallinae*. From all these samples, 34 were randomly sequenced, 25 from pigeons and 9 from birds of prey. After analysing the sequences, only two genotypes, A and B, were found in both pigeons and birds of prey. Genotypes differed in nucleotides at positions 64, 106, 236, 243 and 344, locating the restriction site

for *HaeIII* at position 243. The phylogenetic tree generated with the selected sequences clearly separated the two genotypes from the rest of species of related protozoa analysed (Figure 1). One representative sequence of each genotype obtained from different species was sent to GenBank: EU881911 from *C. livia*, EU881913 from *T. alba*, EU881915 from *H. fasciatus* and EU881916 from *F. tinnunculus* were affiliated with genotype B whereas sequences EU881912 from *C. livia*, EU881914 from *H. fasciatus* and EU881917 from *H. pennatus* belonged to genotype A. The rest of the sequences obtained in this study (23 from pigeons and 4 from raptors) were identical to either genotype A or B (not shown in figure 1). Differences were observed in the prevalence of each sequenced genotype in both groups of birds studied. In pigeons, genotype A appeared more frequently, representing 75% of the sequenced samples. On the contrary, genotype B was isolated in most of the samples from birds of prey (66.7%).

**PCR-RFLP analysis.** All PCR positive samples were processed for digestion with *HaeIII* enzyme. After the reaction, genotype B displayed two bands in agarose of approximately 243 and 126 pb, while genotype A was not digested, displaying a band of 369 pb. A third, minor pattern appeared after digestion, producing bands of 369, 243 and 126 pb. This was interpreted as co-infection with both genotypes in the same bird (mixed pattern). In most of the infected pigeons (76.1%) genotype A was identified, while genotype B was isolated in 20.5% of samples. The mixed pattern was observed in 3.4% of cases. In birds of prey genotype B was again found to be the more prevalent after digestion (64.3%) while genotype A was identified in 35.7% of samples. None of the samples from birds of prey belonged to the mixed pattern. It is remarkable, though, that all the samples isolated from birds with lesions belonged to genotype B.

## 1 Discussion

2 The prevalence of *T. gallinae* found in pigeons (44.8%) was similar to the values found by Villanúa *et*  
3 *al.* in 2006 (34.2%) in wood pigeons in southern Spain. In other parts of the world, previous studies  
4 showed great variation in the prevalence of the parasite in columbiforms between, and even within  
5 countries (Conti and Forrester, 1981; McKeon *et al.*, 1997; Schulz *et al.*, 2005). Variations in season,  
6 age or bird species could explain these differences in prevalence between the studies. Seasonal  
7 variation was observed in this as well as in other studies. Although outbreaks can occur at any time of  
8 the year, most authors agree that spring and summer are ideal for transmission, since they coincide  
9 with the highest reproduction rate of pigeons (Vogel *et al.*, 1994; Gerhold *et al.*, 2007). The  
10 prevalence in the barrier lofts was 52.7%, significantly higher than that found in wild birds (41%).  
11 These rates are comparable to those observed by other authors (McKeon *et al.*, 1997; Bailey *et al.*,  
12 2000). These variations could be due to the closer proximity of pigeons in the barrier loft, since they  
13 have food and water *ad libitum* and do not need to leave the building.

14  
15 In birds of prey the overall prevalence of trichomonosis in this study was 19.6%, although we must  
16 take into account the very heterogeneous sample. Of the species sampled, Bonelli's eagle had the  
17 highest level of parasitisation (52.4%). Other studies carried out with the same species showed similar  
18 prevalence values, between 36% and 50% (Real *et al.*, 2000; Höfle, *et al.*, 2000). Similar studies have  
19 revealed higher prevalence values in nestlings in different species of raptors (Boal *et al.*, 1998; Real *et*  
20 *al.*, 2000; Krone *et al.*, 2005). For the Bonelli's eagle we found higher values (52.4%) than Höfle *et*  
21 *al.* (2000) in Portugal (50%) and Real *et al.* (2000) in northern Spain, who detected a prevalence of  
22 36% in nestlings older than the birds we analyzed. This difference could be explained by the fact that,  
23 as the birds get older, the parents bring less prey to feed their young, reducing the risk of infection  
24 from parasitized prey (Estes and Mannan, 2003). Another possible factor would be the immunity  
25 developed by older nestlings (Tizard, 2002).

Only 0.72% of infected pigeons displayed lesions at the time of sampling. Similar results were observed by Bunbury *et al.* (2008), who detected a small number of birds with clinical symptoms (1.9%) in the pink pigeon from Mauritius. This could be explained by the fact that it is usually difficult to find very sick birds because they tend to hide or attract predators. In addition, the carcasses of birds that have died from disease are rapidly eradicated by other carrion feeders, which would make them impossible to detect (Peterson *et al.*, 2000). The percentage of birds of prey showing clinical signs was 25%, but we must remember that most of the birds were sampled in Wildlife Recovery Centres, where the probability of finding sick birds is higher.

According to other studies, Ribosomal RNA genes are attractive markers for phylogenetic studies. The level of divergence observed in ITS regions is a useful marker to study relationships of closely related species, including Trichomonadidae protozoa (Felleisen, 1997). A difficulty arises from the existence of polymorphisms among repeat units even in a single individual (Vogler and DeSalle, 1994). Some authors have used a cloning approach to solve this situation in protozoa from birds, but after cloning and sequencing PCR products, a high homogeneity was observed among the *Histomonas meleagridis* isolates obtained from the same flocks (Harold *et al.*, 2006). According to some authors, homogenization of ITS repeats is likely to occur after a few generations in individuals lacking sexual reproduction (Vogler and DeSalle, 1994). This situation could explain the fact that in our case, clean sequences were obtained and only two patterns were observed when using a direct sequencing approach. Only one sequence showed a heterogeneous (mixed) pattern, further confirmed by *Hae*III digestion. This direct sequencing approach has also been successfully used by other authors working on *T. gallinae* obtaining a more variable scenario and seems to be a useful tool (Gerhold *et al.*, 2008a).

1 In our study, sequences of related protozoa have been included in the phylogenetic analysis, including  
2 those of *T. gallinae* and *Trichomonas* sp. published in GenBank (Felleisen, 1997; Kleina *et al.*, 2004;  
3 Gaspar DaSilva *et al.*, 2007; Gerhold *et al.*, 2008a). In the phylogenetic tree *T. gallinae* splits into two  
4 very distinct branches: genotype A, which includes the sequences published by Felleisen (1997) and  
5 Gerhold *et al.* (2008a), and genotype B, which includes sequences published by Kleina *et al.* (2004),  
6 DaSilva *et al.* (2007) and Gerhold *et al.* (2008a). Both groups differ from the other species, including  
7 *Trichomonas* sp. from the oral cavity of birds (Gerhold *et al.*, 2008a), and the closest species, such as  
8 *T. vaginalis* and *T. tenax*. We have observed differences in the prevalence of genotypes between  
9 pigeons and raptor species, since genotype A was more prevalent in the samples of *T. gallinae*  
10 obtained from pigeons (76.1%) than from raptors (20.5%), whereas genotype B was more prevalent in  
11 raptors (64.3%) than in pigeons (35.7%). These differences in prevalence could be a consequence of  
12 the parasite's adaptation to its host.

13  
14 It is also remarkable that all the birds that displayed lesions were infected with genotype B. Also, one  
15 pigeon showing a mixed pattern had lesions. Taking altogether, the results obtained here indicate a  
16 relationship between genotype B and clinical disease. The same situation is observed when analyzing  
17 data published by other authors: sequences EU215368, EU215369 (Gerhold *et al.*, 2008a) grouped  
18 together with genotype B in the phylogenetic tree (Figure 1) and correspond with virulent isolates (see  
19 isolate information in Gerhold *et al.*, 2008b). A similar situation has been described in *Histomonas*  
20 *meleagridis* using the same target (Harold *et al.*, 2006): the authors described two genotypes more  
21 frequently related to clinical disease whereas a third genotype was not.

22  
23 In this paper we have described for the first time the co-existence of both genotypes of *T. gallinae* in  
24 the same avian population (pigeons and birds of prey). The existence of these two genotypes supports  
25 the need for genetic characterization of the isolates (Krone *et al.*, 2005; Gaspar DaSilva *et al.*, 2007;

Hegemann *et al.*, 2007; Gerhold *et al.*, 2008a) in order to establish more consistent differences between isolates, including host adaptation and virulence of different strains.

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Table 1. Prevalence of *T. gallinae* in birds of prey.

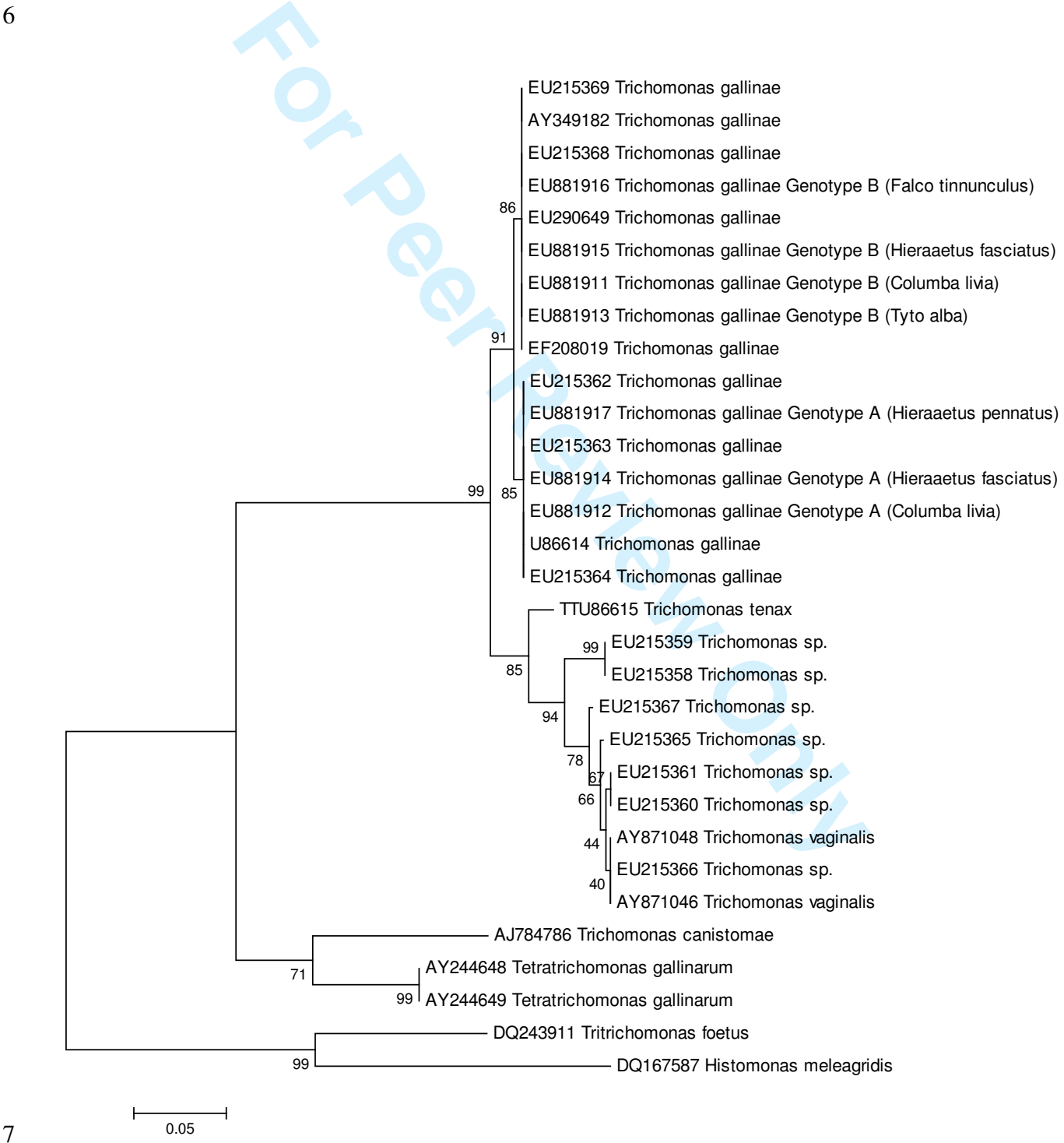
Species sampled in the study		Number of samples tested	Prevalence (%)
Bonelli's eagle	<i>Hieraaetus fasciatus</i>	21	52.4
Booted eagle	<i>Hieraaetus pennatus</i>	20	15
European kestrel	<i>Falco tinnunculus</i>	29	10.3
Peregrine falcon	<i>Falco peregrinus</i>	8	12.5
Barn owl	<i>Tyto alba</i>	7	14.3
Common buzzard	<i>Buteo buteo</i>	4	0
Tawny owl	<i>Strix aluco</i>	3	0
Long-eared owl	<i>Asio otus</i>	2	0
Golden eagle	<i>Aquila crysaetos</i>	2	50
Goshawk	<i>Accipiter gentilis</i>	1	0
Sparrow hawk	<i>Accipiter nisus</i>	1	0
Merlin	<i>Falco columbarius</i>	1	0
Eurasina hobby	<i>Falco subbuteo</i>	1	0
Griffon's vulture	<i>Gyps fulvus</i>	1	0
Short-eared owl	<i>Asio flammeus</i>	1	0

Table 2. Odds Ratio (OR) values and 95% Confidence Interval (CI) obtained after binary logistic regression analysis of the variables included in the study associated with *T. gallinae* infection in pigeons. \*  $p < 0.05$ .

Variable		n	Prevalence (%)	OR and 95% CI (Lower, Upper)
Period	Period 1 (March -May)	57	49.1	1.77 (1, 3.13)*
	Period 2 (June-August)	113	54	2.15 (1.39, 3.33)*
	Period 3 (September-November)	309	35.3	Constant
	Period 4 (December-February)	133	57.1	2.44 (1.61, 3.7)*
Aptitude	Wild	439	41	Constant
	Barrier loft	173	52.7	1.71 (1.2, 2.44)*



1 Figure 1. Phylogenetic analysis of *Trichomonas gallinae* and related protozoa published in GenBank  
2 using ITS1/5.8S rRNA/ITS2 sequence and neighbor-joining method. Branch reliability was assessed  
3 using bootstrap of 1000 replicates and showed as the frequency of 100. Hosts of origin from  
4 sequences obtained in this study are indicated in parentheses. Scale bar, 0.05 substitutions (corrected)  
5 per base pair.



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Golden eagle	<i>Aquila crysaetos</i>	2	50
Goshawk	<i>Accipiter gentilis</i>	1	0
Sparrow hawk	<i>Accipiter nisus</i>	1	0
Merlin	<i>Falco columbarius</i>	1	0
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