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Giardia duodenalis in colony stray cats from Italy

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Abstract

Giardia duodenalis is the most common intestinal protozoan in humans and animals worldwide, including eight morphologically identical assemblages, infecting pets, livestock, wildlife and human beings. Assemblages A and B are those with the higher zoonotic potential, and they have been detected in several mammals other than humans; the others (C to H) show a higher host specificity. Cats can harbour both the specific Assemblage F and the zoonotic ones A and B. Several studies have been carried out on *G. duodenalis* genotypes in cats; however, the role of this species in the epidemiology of giardiasis is still poorly understood. In this scenario, the present study carried out the detection and genetic characterization at sub-assemblage level of *G. duodenalis* from colony stray cats in central Italy. In the period 2018–2019, 133 cat faecal samples were analysed for the presence of *G. duodenalis* cysts by a direct immunofluorescence assay. Positive samples were subsequently subjected to molecular analyses for assemblage/sub-assemblage identification. Forty-seven samples (35.3%) were positive for *G. duodenalis* cysts by immunofluorescence. *G. duodenalis* DNA was amplified at SSU-rDNA locus from 39 isolates: 37 were positive for zoonotic Assemblage A and 2 showed a mixed infection (A + B). Positive results for the β-giardin gene were achieved for 25 isolates. Sequence analysis revealed 16 isolates belonging to Sub-assemblage AII and 8 to Sub-assemblage AIII. One isolate resulted as ambiguous AI/AIII. Large sequence variability at the sub-assemblage level was detected, with several double peaks and mutations, making complex a proper isolate allocation. When compared with previous studies, the 35.3% prevalence of *G. duodenalis* in cats reported in the present article was surprisingly high. Moreover, all positive cats resulted to be infected with zoonotic assemblages/sub-assemblages, thus indicating stray cats as a possible source of human giardiasis and highlighting the sanitary relevance of cat colonies in the study area.

KEYWORDS

cat, *Giardia duodenalis*, molecular characterization, parasite, zoonosis

1 | INTRODUCTION

Flagellate protozoa of the genus *Giardia* are among the most prevalent and widespread enteric parasites in vertebrate species worldwide (Monis et al., 2009). Its taxonomy, once based on morphology, nowadays mainly relies on genetic evidence. At present, eight species within the genus are retained as valid: *Giardia agilis*, *Giardia ardeae*, *Giardia psittaci*, *Giardia microti*, *Giardia muris*, *Giardia duodenalis* (synonyms *Giardia intestinalis* and *Giardia lamblia*) and the two recently described *Giardia peramelis* and *Giardia cricetidarum* (Hillman et al., 2016; Lyu et al., 2018). Among these species, *G. duodenalis* has a paramount importance of being the only one detected in humans, as well as in many species of wild and domestic mammals.

Giardia duodenalis includes at least eight genotypes or assemblages morphologically identical. Assemblages A and B are those with the higher zoonotic potential, and they have been detected in several mammals, including humans; the others show a higher host specificity, and they have been recorded from canids (C and D), livestock (E), cats (F), rodents (G) and marine mammals (H) (Cacciò & Ryan, 2008; Capewell et al., 2020; Hill et al., 2000). In addition, intragenetic variation occurs within zoonotic assemblages, and different sub-assemblages (e.g. AI/AII/AIII and BIII/BIV) with high levels of heterogeneity have been recognized (Capewell et al., 2020; Ryan & Cacciò, 2013).

Despite domestic animals, especially pets, may have a role in the zoonotic transmission due to their close contact with people (Dixon, 2020), few studies have been carried out on *G. duodenalis* genotypes in cats, and their role in the epidemiology of human giardiasis is still poorly known. To date, *G. duodenalis* has been reported in cats from breeding colonies, animal shelters, pet shops and private owners (Hill et al., 2000; McGlade et al., 2003; Sommer et al., 2018), with prevalence values up to 25%. In Italy, most studies are based on low numbers of animals, mainly privately owned (Paoletti et al., 2011), although data on colony stray cats are sporadic (Papini et al., 2007).

Cats can harbour both the cat-specific Assemblage F and the zoonotic ones A and B (Saleh et al., 2019), with one or the others prevailing depending on the study. Regarding the possible role of cats as source of human giardiasis, some authors do not consider this host species relevant (Fayer et al., 2006), and some others even claim a more relevant role of cats than dogs (Pallant et al., 2015). Although cats can harbour both specific and zoonotic assemblages, assemblage or sub-assemblage determination is not usually performed in the diagnostic routine (Pallant et al., 2015). When dealing with *G. duodenalis* genotyping, small subunit ribosomal RNA (SSU-rDNA) and β-giardin gene (*bg*) are ordinarily used, respectively, at assemblage and sub-assemblage levels. In particular, the *bg* region seems to be the most efficient to reveal isolates diversity from feline faecal samples (Sursal et al., 2020).

In this scenario, in a broader study on zoonotic pathogens of colony stray cats from central Italy, the detection and genetic characterization at the sub-assemblage level of *G. duodenalis* were carried out, the results of which are shown later.

Impacts

- *Giardia duodenalis* was detected in stray cats from central Italy with an unexpected prevalence.
- All *G. duodenalis* isolates from stray cats from central Italy belonged to zoonotic assemblages/sub-assemblages.
- The role of cat as a possible source of human giardiasis is highlighted.

2 | MATERIAL AND METHODS

2.1 | Sampling and parasite detection

Individual faecal samples were collected during neutering surgery of stray cats belonging to 25 feline colonies located in the province of Rome, central Italy (Figure 1). In the period March 2018–January 2019, samples were individually analysed for the presence of *G. duodenalis* cysts using a commercial direct immunofluorescence kit (Merifluor® Meridian Diagnostic). Positive samples were subsequently tested by molecular analyses for *G. duodenalis* assemblage and sub-assemblage identification. The protocol and procedures employed were reviewed and approved by the Ethics Committee of the Istituto Zooprofilattico Sperimentale del Lazio e della Toscana 'M. Aleandri'.

2.2 | Assemblage and sub-assemblage identification

DNA extraction was performed using a QIAmp DNA stool mini kit (QIAGEN) following manufacturer's instructions. All samples were investigated at two target genes: primarily, a nested PCR was conducted to amplify a 130-bp fragment of small subunit ribosomal RNA (SSU-rDNA) (Read et al., 2002), followed by a 753-bp fragment of β-giardin gene amplification, as described by Cacciò et al. (2002). Amplicons were ultimately purified using an mi-PCR Purification Kit (Metabion International AG) and sent to an external laboratory for sequencing (Bio-Fab Research).

The resulting chromatograms were manually checked using Finch TV 1.4 software (Geospiza, Inc.), in order to identify possible double peaks for mixed infections or single-nucleotide polymorphisms (SNPs). Consensus sequences were compared with those previously published on GenBank database. SSU-rDNA identities at the assemblage level were verified using the Basic Local Alignment Search Tool (BLAST). Subsequently, β-giardin sequence analysis was conducted in MEGA X (Kumar et al., 2018) to identify isolates at the sub-assemblage level. The sequences were trimmed to the shortest length with high quality in all samples and aligned with representative sequences retrieved from GenBank: EU014385, FJ560591, JQ247029 (AI sub-assemblage); AY072723, KM190678, KY612248 (AII sub-assemblage); MT542767, FJ560590, FN386481, AY072724 (AIII sub-assemblage); HM165226 (Assemblage B); DQ116616

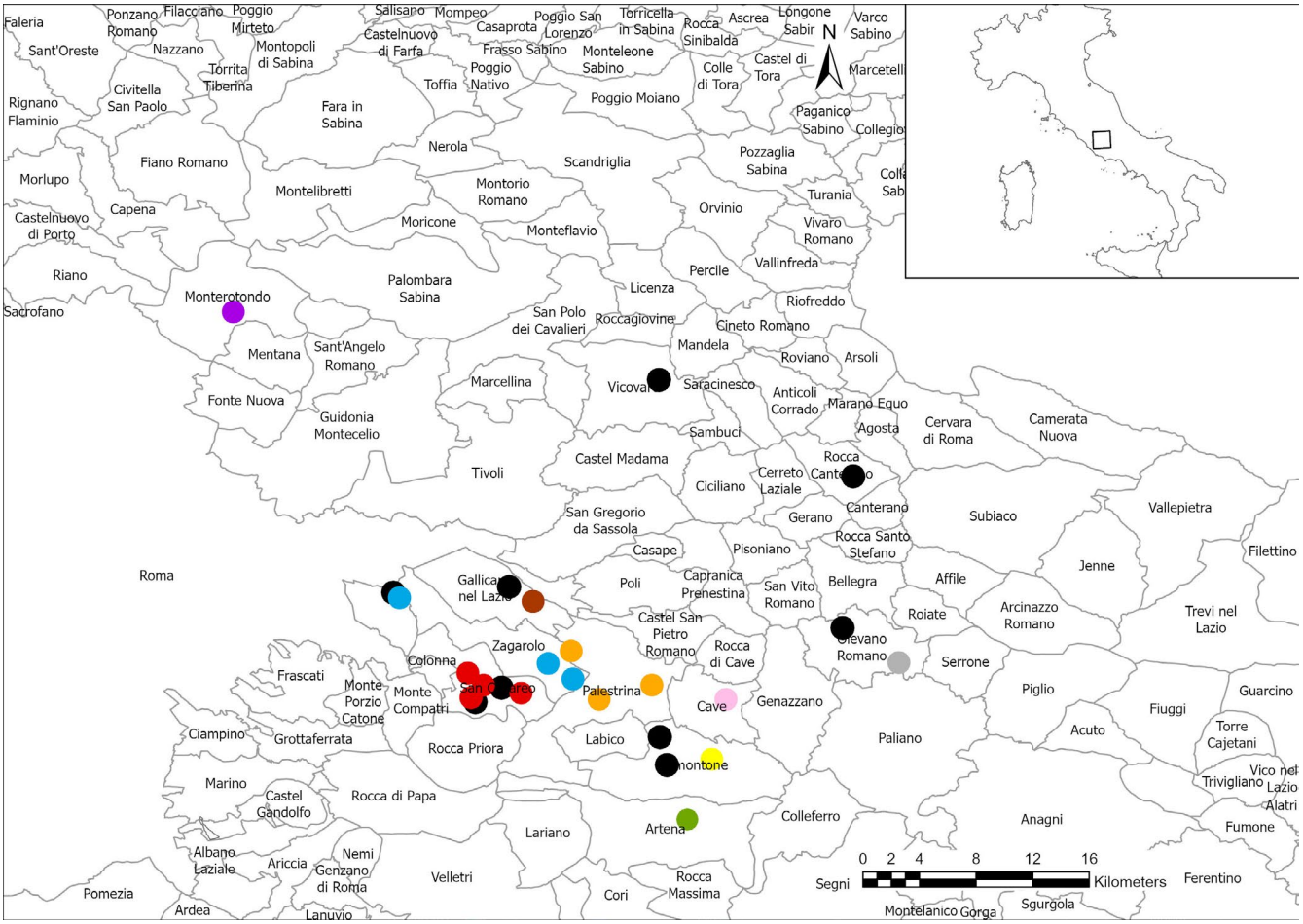

FIGURE 1 Map of the study area with municipalities and the localization of the stray cat colonies indicated by dots. Colour dots indicate colonies with at least one positive cat, with each colour referring to a single municipality. Black dots identify colonies where the parasite was never reported

(Assemblage E); AY647264 (Assemblage F). Phylogenetic analysis was performed using the maximum likelihood method (ML), using Tamura–Nei as the best model (Tamura & Nei, 1993). The sequence AY258618 (*Giardia muris*) was used as the outgroup. Only sequences without ambiguous positions (double peak presence) were considered.

Representative β-giardin sequences obtained in the present study were deposited in GenBank under accession numbers MW969798–MW969808. To visualize the spatial distribution of *Giardia* isolates among cat colonies, haplotype and polymorphic site analyses were conducted by DnaSP v.6 software (Rozas et al., 2017), and PoPART (population analysis with reticulate trees) genetic software (Leigh & Bryant, 2015) was used for the median-Joining network calculation (Bandelt et al., 1999).

2.3 | Statistical analysis

For each sampled animal, the following data were recorded: immunofluorescence outcome (*G. duodenalis* negative, positive), gender (male, female), age (as continuous variable and categorical ≤1, >1 year), body condition score—BCS (normal, underweight, overweight), as an indicator of the animal general health status, and colony identification number. Statistical significances of association between individual variables and positivity were evaluated using the chi-square test and Fisher's exact test for bivariate analysis. The influence of colony population size on prevalence was analysed by using two methods: (a) evaluating the difference among percentages by the chi-square test (WinEpi online software), dividing the colonies according to population size in colonies with ≤13 cats and colonies with >13 cats (cut-off based on the mean number of cats in studied colonies), (b) studying the association between the population size (categorized as before) and positivity of the colony, assuming as positive a colony with at least one positive cat. The analyses were performed using software Stata 12.0 (StataCorp).

3 | RESULTS

A total of 133 cats have been tested. Forty-seven samples from 16 colonies were positive for *G. duodenalis* cysts to immunofluorescence (prevalence 35.3%). The distribution of gender was 63%

female and 37% male. The mean age of sampled cats was 1.8 (*SD* ± 1.4) years; animals ≤1 year of age were 59 (44.4%), and those >1 were 74 (55.6%). Most of the animals had a normal BCS (96%). No significant association was found between the analysed variables at individual level and the positivity to *G. duodenalis* (*p*-value > .05).

The number of cats per colony ranged from 2 to 30 animals (mean 12.9; median 13). Regarding population size, the difference of prevalence between colonies was not significant (*p*-value = .1354), and there was no association between size and positivity of the single colony (*p*-value = .390). Descriptive analyses of the continuous variable distribution between positive and negative colonies showed slight differences in the mean number of cats per colony (13.4 in positive colonies and 12 in negative ones) and in the mean age (1.7 years in positive colonies and 2.4 years in the negative ones). A synthesis of the aforementioned reported results is illustrated in Table 1.

Among the 47 microscopically positive samples, 39 *G. duodenalis* isolates were successfully amplified at SSU-rDNA: 37 were positive for the zoonotic Assemblage A and two showed a mixed infection of the two zoonotic Assemblages A and B, presenting double peaks in the diagnostic positions. Positive results for β-giardin gene amplification were achieved for 25 out of 39 isolates, confirming, where possible, SSU-rDNA assignments. Sequence analysis revealed high genetic heterogeneity within the isolates, with the presence of several SNPs, both in terms of transitions and double peaks, compared with the sequences EU014385, AY07272 and AY072724 used as references for Sub-assemblages AI, AII and AIII, respectively (see Table 2).

The ML phylogenetic tree based on the β-giardin gene confirmed the assignment of our isolates within the two clades representing the AII and AIII sub-assemblages (Figure 2). Twelve of our 16 isolates were identical to the reference sequence AY072723 for Sub-assemblage AII and to KM190678 and KY612248 sequences used for comparative purposes; they were reported, respectively, from humans in Italy, beavers in Canada and humans in Brazil. As for Sub-assemblage AIII, the reference isolates AY072724 (humans from Italy), FJ560590 (humans from France), FN386481 (waste water from Spain) and MT542767 (humans from Brazil) were identical to 4 of our 8 samples. In Table 3, the genotyping results linked to the sampling sites are reported.

Genetic variants among the sub-assemblages have been also identified, as highlighted by 6 polymorphic sites with 5 singleton variable sites and 1 parsimony informative site resulted from the polymorphic sites analysis. In addition, the haplotype analysis based on the current *bg* dataset revealed 7 different haplotypes, with *hp1* as the most common haplotype for AII sub-assemblage and *hp2* as the most detected for AIII sub-assemblage. The network spatial distribution showed a substantial sharing of the two most common *hp1* and *hp2* haplotypes among different sampling localities (Figure 3).

4 | DISCUSSION

In the present study, along with prevalence rates, the first data regarding sub-assemblages of *G. duodenalis* from colony stray cats in Italy are reported, with the aim to better understand the possible risks represented by these animals for human health in the study area. As for prevalence, our results reveal a high overall value (35.3%). Bouzid et al. (2015) in a meta-analysis conducted more than 68 epidemiological investigations worldwide and reported a 12% overall prevalence of *G. duodenalis* in cats, regardless of the involved cat category, although a very high heterogeneity between studies from different geographical localities was evidenced. In Europe, prevalence values ranging from 2.3% in owned and stray cats from Greece (Symeonidou et al., 2018) to 22.4% in shelter ones from Germany (Cirak & Bauer, 2004) are reported. Moreover, Epe et al. (2010) in a study on symptomatic cats involving seven countries of Western Europe showed a 20.3% overall prevalence, with highest values in Belgium and Germany (26.3% and 24.6%, respectively). In Italy, an analogous high rate was only recorded by Zanzani et al. (2014) from household cats in the north of the country (from 24.7% to 36.8%), whereas lower values were observed in central Italy by Paoletti et al. (2011), reporting a 6.1% prevalence from domestic and feral cats. Similarly, 7.5% and 8.5% positive animals were observed in owned cats by Mancianti et al. (2015) and by Tamponi et al. (2017), respectively. More recently, from regions partially overlapping those of the present study, Sauda et al. (2019) registered 10.6% prevalence among cats from public and private shelters. It is difficult to interpret the reasons for these heterogeneities, such as the high prevalence recorded in the present study. As stated by previous authors (Bouzid et al., 2015; Montoya et al., 2018), the detection method is probably the strongest driver influencing prevalence variations of *G. duodenalis* in cats. Because of its considerable effect on estimated prevalence, the immunofluorescence technique here adopted, a highly sensitive tool for the detection of *G. duodenalis* in stool samples, could likely explain our outcome.

Age and gender were not statistically associated with *G. duodenalis* infection, comparably with previous data (Epe et al., 2010; Montoya et al., 2018; Piekara-Stępińska et al., 2020; Tamponi et al., 2017); however, they are still present in different studies where younger cats (<6 months or <12 months of age) are considered at higher risk of positivity (Bouzid et al., 2015; Epe et al., 2010; Nagamori et al., 2020; Pallant et al., 2015; Sauda et al., 2019; Symeonidou et al., 2018; Tamponi et al., 2017; Zanzani et al., 2014). Regarding age, our findings could be affected by a bias due to the difficulty in recording accurate dates of birth in stray cats. However, at the colony level, it could be hypothesized that colonies with higher population size and younger cats could be more prone to be infected by *G. duodenalis*.

Also for genotyping, studies published so far reveal conflicting results, thus affecting a full agreeing of possible cats' role as a source of human *Giardia* infections. On the one hand, only Assemblage F in colony cats was found, thus supporting the opinion that this host likely represents little or no risk to humans (De Lucio et al., 2017; Fayer et al., 2006). On the contrary, Ramírez-Ocampo et al. (2017) in their meta-analysis reported that in more than 3,351 reviewed articles, one-third of the examined cats harbour the zoonotic Assemblage A. In the present study, the molecular analysis revealed,

TABLE 1 Descriptive analysis and association between direct immunofluorescence assay outcome for *Giardia duodenalis* and individual and colony variables in cats from central Italy

		Positive cats		Negative cats		
Individual variable		***N***	**%**	***N***	**%**	
Overall		**47**	**35.34 (47/133)**	**86**	**64.66 (86/133)**	***p*-value**
Age (years)	Mean	1.6		1.9		
	Median	2		2		
	SD	(±) 1.2		(±) 1.5		
	Min	0		0		
	Max	4		9		
Age (years)	≤1	22	16.54 (22/133)	37	27.82 (37/133)	.674
	>1	25	18.8 (25/133)	49	36.84 (49/133)	
Sex	Female	27	20.45 (27/132)	56	42.42 (56/132)	.337
	Male	20	15.15 (20/132)	29	21.97 (29/132)	
	Not reported			1		
BCS	Normal	43	33.59 (43/128)	80	62.5 (80/128)	.302
	Underweight	2	1.56 (2/128)	1	0.78 (1/128)	
	Overweight	0		2	1.56 (2/128)	
	Not reported	2		3		
		Positive colonies		**Negative colonies**		
Colony variables		***N***	**%**	***N***	**%**	
Overall		**16**	**64 (16/25)**	**9**	**36 (9/25)**	***p*-value**
Population size	Mean	13.4		12		
	Median	12.5		14		
	SD	(±) 6.2		(±) 6.2		
	Min	2		2		
	Max	30		20		
Cat population	≤13 cats	10	41.67 (10/24)	3	12.5 (3/24)	.390
	>13 cats	6	25 (6/24)	5	20.83 (5/24)	
	Not reported			1		
Age (years)	Mean	1.7		2.4		
	Median	1.9		2		
	SD	(±) 1.08		(±) 1.17		
	Min	0		1.1		
	Max	4		4.7		

Note: Positive colonies: Colonies with at least one positive cat.

Negative colonies: Colonies with no positive cat.

Abbreviation: *SD*, standard deviation.

p -value was calculated by using the chi-square test and for the variable cat population (≤ or >of 13 cats) by using Fisher's exact test.

except for the two mixed infection A + B, the exclusive presence of Assemblage A in all positive cats, as previously observed in Papini et al. (2007). Also, in Zanzani et al. (2014), a large percentage of the sampled cats harboured Assemblage A.

Giardia duodenalis genotyping at the sub-assemblage level allowed us to better identify zoonotic isolates and to define the potential risk of zoonotic transmission in the study area. Previous data showed that AII sub-Assemblage is predominantly found in humans, thus considered zoonotic (Ballweber et al., 2010; Cacciò et al., 2002; Skhal et al., 2017; Sprong et al., 2009), whereas while AIII has been detected in humans only occasionally, also in Italy (Cacciò et al., 2002), infecting preferentially wild hoofed animals (De Liberato et al., 2015; Rafiei et al., 2020; Sprong et al., 2009) and sometimes cats (Lebbad et al., 2010). In our data, a potential risk of zoonotic transmission could be deduced from the clusters obtained in the phylogenetic analysis, which grouped human and cat isolates

TABLE 2 Genetic heterogeneity within the isolates, with the presence of several SNPs compared with the reference sequences

Assemblage	Sub-assemblage	Reference sequence	Isolate Id.	Single-nucleotide polymorphisms	Double peaks	GenBank ID
A	AII	AY072723	C91129	A550**G**	-	MW969800
		AY072723	C98196	C521**T**	-	MW969801
		AY072723	C27889	C506**T**	-	MW969802
		AY072723	C54031	-	G445 (G/A), C506 (C/T), A592 (A/G), G647 (G/T), A688 (A/G)	MW969799
	AIII	AY072724	C27890	A593**G**	-	MW969805
		AY072724	C91131	G631**A**	-	MW969807
		AY072724	C47716	-	A446 (A/G), A593 (A/G)	MW969804
		AY072724	C23232	-	A497 (A/G), C521 (C/T), T524 (T/C)	MW969806
	AI/AIII	EU014385/AY072724	C52044	-	C443 (C/T), G684 (G/A)[a]	MW969808

[a]Diagnostic position.

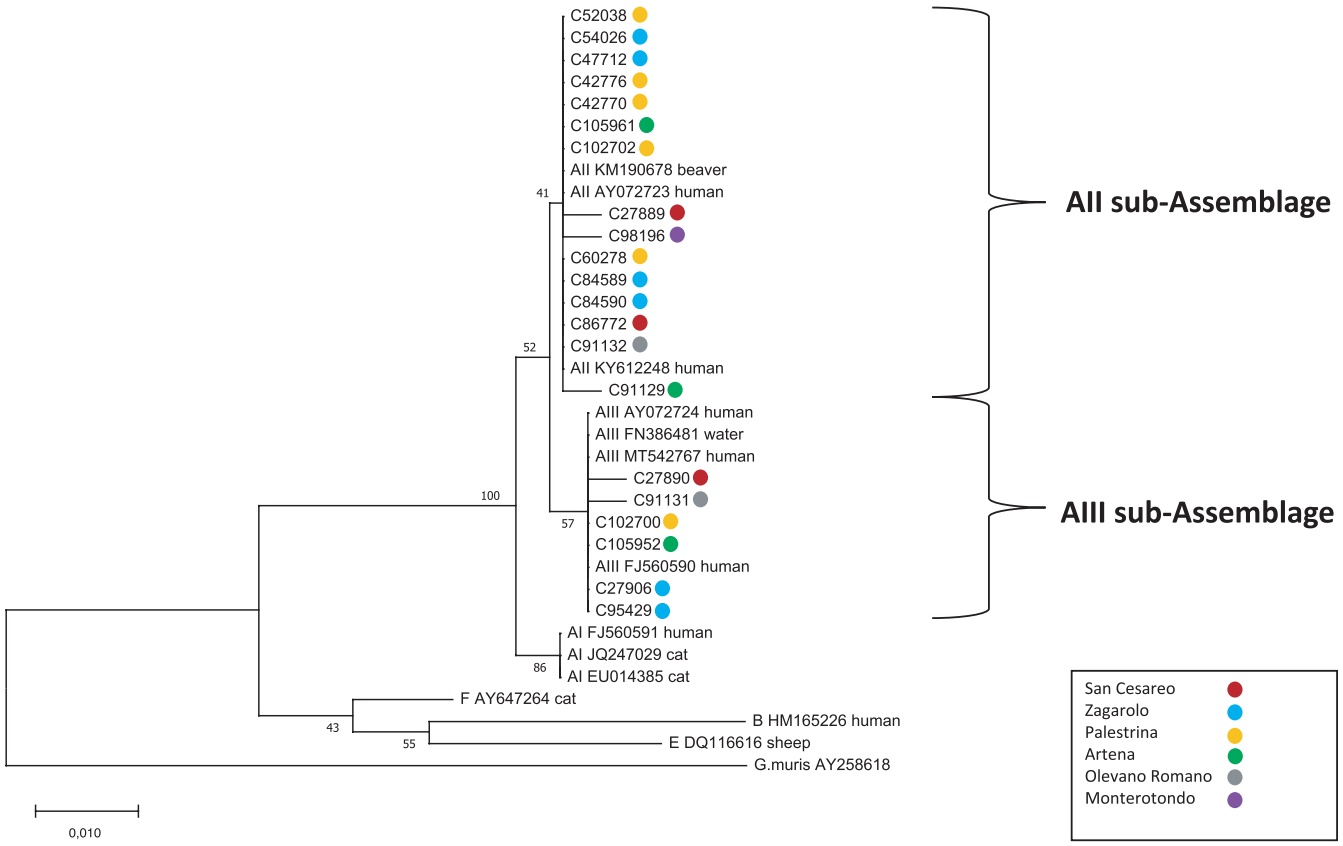

FIGURE 2 Ml phylogenetic tree based on β-*giardin Giardia duodenalis* sequences using the Tamura–Nei model. Numbers on the tree nodes indicate bootstrap values >50%. Accession numbers of sequences retrieved from GenBank are indicated. Colour dots represent sampling municipality as in Figure 1. Scale represents the distance in millions of years for the differentiation of each branch

belonging to the same sub-assemblage (both AII and AIII). Even if, from an epidemiological point of view, the reference sequences used are not linked to our isolates, these data could suggest that a possible zoonotic transmission of these *Giardia* isolates may occur.

The high level of polymorphism detected within AII and AIII sub-assemblages, as evidenced by the identification of multiple haplotypes with a heterogeneous pattern of SNPs, is not unexpected. It is known how isolates belonging to the same sub-assemblage are not identical and the sub-assemblages themselves could be considered as clusters of high-related isolates (Ryan & Cacciò, 2013). The various detected haplotypes shared among different cat colonies did not evidence any pattern of spatial

TABLE 3 Results of *Giardia duodenalis* genotyping at municipality and colony level

Colony municipality	*N*. positive colonies (*N* = 16)	*N*. microscopic positive cats (*N* = 47)	SSU-rDNA assemblage (*n*° isolates)	β-giardin sub—assemblage (*n*° isolates)
Artena	1	9	A (4)	AII (1); AII[a] (1); AIII (1); negative (1)
			Mixed A/B (1)	Negative (1)
			Not tested[b] (4)	*Not tested*[b] (4)
Cave	1	2	A (2)	Negative (2)
Gallicano nel Lazio	1	1	A (1)	Negative (1)
Monterotondo	1	2	A (2)	AII[a](1); negative (1)
Olevano Romano	1	2	A (2)	AII (1); AIII[a] (1)
Palestrina	3	13	A (12)	AI/AIII[a] (1); AII (4); AIII (2); negative (5)
			Negative (1)	AII (1)
San Cesareo	4	8	A (8)	AII (1); AII[a] (1); AIII[a] (2); negative (4)
Valmontone	1	2	*Not tested*[b] (2)	*Not tested*[b] (2)
Zagarolo	3	8	A (6)	AII (4); AII[a] (1); AIII (1)
			Mixed A/B (1)	Negative (1)
			Negative (1)	AIII[a] (1)

[a]Isolate presenting allelic pattern variability.

[b]Not enough material for DNA extraction.

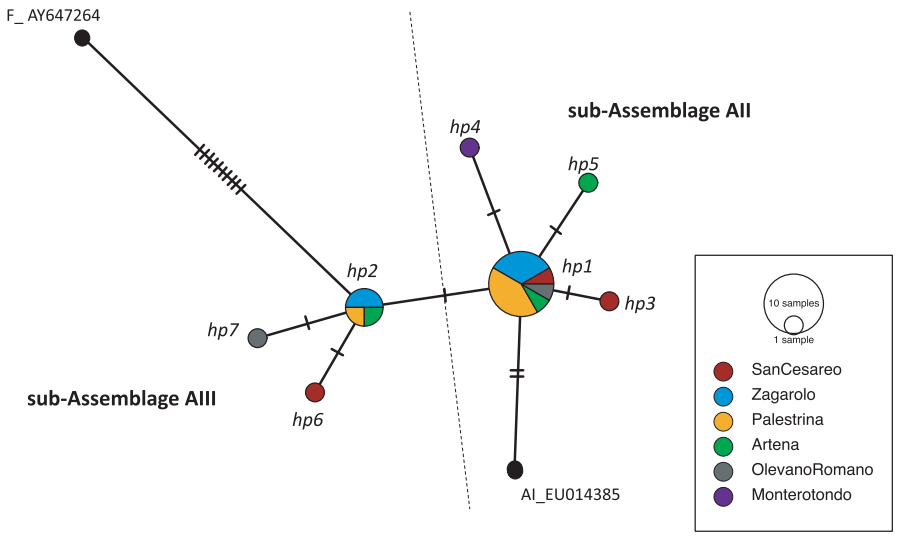

FIGURE 3 Median-joining network of β-*giardin* sequences dataset; AY647264 and EU14385, respectively, represent Sub-assemblage AI and Assemblage *F* (black nodes). Each circle indicates a unique haplotype (*hpN*), and its size is proportional to the number of isolates identified. Hatch marks represent mutations. Correspondence between haplotypes and our deposited sequences as follows: *hp1*→MW969798; *hp2*→MW969803; *hp3*→MW969801; *hp4*→MW969802; *hp5*→MW969800; *hp6*→MW969807; *hp7*→MW969805. Each sample area is indicated with a different colour

segregation, as indicated in the constructed haplotype network. This result allowed us to better understand how wide the circulation of *Giardia* isolates occurs within and between the different colonies, confirming the well-known nature of *G. duodenalis* as a ubiquitous protozoan (Garcia-R et al., 2017). The presence of ambiguous AI/AIII sub-assemblage in one isolate complies with previous studies (Cacciò et al., 2002; Lalle et al., 2005; Skhal et al., 2017), and it could be attributed to diverse sources of infection, fairly probable in stray cats, thus supporting the complex dispersal patterns of this organism.

In conclusion, the present study enables a step forward in terms of *G. duodenalis* prevalence data and molecular characterization in stray cat colonies from central Italy, allowing a reconsideration of cats, especially stray cats, as a possible source of human giardiasis and upgrading the possible sanitary relevance of cat colonies in well-defined geographical areas. These pieces of

evidence underline the importance of more systematic sampling of local fauna, along with high-resolution genotyping of *G. duodenalis* sequences, to provide insights into the zoonotic potential of different genotypes, transmission dynamics and host specificity, necessary to better identify the pathways of human giardiasis at different epidemiological scales.

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CONFLICT OF INTERESTS

The authors declare they have no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings are in possess of the Authors.

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