

Leishmania spp. in Squamata reptiles from the Mediterranean basin

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Abstract

Leishmania tarentolae is a nonpathogenic trypanosomatid species isolated from geckoes in the Mediterranean basin. In Italy, *L. tarentolae* and *Leishmania infantum* occur in sympatry in areas where canine leishmaniosis (CanL) is endemic. Here, we investigated the prevalence and distribution of *L. tarentolae* in reptilian hosts and the presence of *L. infantum* in the same animals in southern Italy. From April 2020 to April 2021, lizards and snakes were captured in urban, peri-urban sites, natural parks, and dog shelters. Blood was individually sampled and assessed for the presence of *Leishmania* spp. by cytology and/or molecular biology. Captured lizards were euthanized and their tissues/organs were tested by duplex real-time-PCR for the detection of *L. infantum* and *L. tarentolae* and the positive ones were amplified by conventional PCR to confirm the species identification by sequencing. Samples were also screened by real-time PCR for *L. infantum* kDNA minicircle. Of the 259 collected Squamata reptiles, 34 (13.1%) specimens scored positive for *Leishmania* spp., with a prevalence of 10% for *L. tarentolae* ($n = 26$) and of 3.1% for *L. infantum* ($n = 8$). Positive lizards were mostly *Podarcis siculus* ($n = 26$) from dog shelters, with six specimens positive for *L. infantum*. In addition, *L. tarentolae* was detected in one *Podarcis filfolensis* lizard, and in five *T. mauritanica* geckoes (with one gecko and *P. filfolensis* lizard showing amastigote-like forms in thrombocytes), whereas *L. infantum* was detected in one *T. mauritanica*, and one *Hemidactylus turcicus*. Data herein reported demonstrate that saurian reptiles living in CanL endemic areas of the Mediterranean basin may be exposed to both *L. tarentolae* and *L. infantum* indicating a sympatric circulation of these species in different vertebrate hosts. Moreover, this study represents the first molecular evidence of *L. infantum* infection in European reptiles.

KEYWORDS

Leishmania infantum, *Leishmania tarentolae*, *Podarcis*, Squamata reptiles, *Tarentola*, zoonosis

1 | INTRODUCTION

Leishmania (Kinetoplastida, Trypanosomatidae) is a genus of protozoan parasites that infect mostly mammals, except for the subgenus *Sauroleishmania*, which includes parasites of lizards (Klatt et al., 2019). *Sauroleishmania* represents a sister-clade of the subgenus *Leishmania*, a group that englobes most medically and veterinary important species (Noyes et al., 1998). Indeed, leishmaniasis represents an important yet neglected disease in tropical, subtropical, and temperate regions of the world (Otranto & Dantas-Torres, 2013). The distribution of *Leishmania* is strictly related to their vectors, represented by different species of sand flies (Akhoundi et al., 2016; Alemayehu & Alemayehu, 2017). *Leishmania* spp. in the Neotropical regions are vectored by species of *Lutzomyia* and those in Palearctic and Afrotropical regions by *Phlebotomus* sand flies (Daoudi et al., 2020; Mann et al., 2021). Conversely, *Sauroleishmania* is vectored by *Sergentomyia*, a genus of sand fly for a long time considered solely herpetophilic, thus feeding mainly on cold-blooded animals (Mendoza-Roldan et al., 2020a; 2021a).

In Europe, zoonotic visceral leishmaniasis is caused by *Leishmania infantum*, dogs being its primary reservoirs, with a wide distribution throughout the Mediterranean basin (Otranto & Dantas-Torres, 2013; Martín-Sánchez et al., 2020). This species has also been detected in different species of vertebrates, such as lagomorphs (i.e., rabbits and hares) that play an important role as sylvatic reservoirs in endemic areas of Europe (Molina et al., 2012; Tsokana et al., 2016; Martín-Sánchez et al., 2021). Apart from *L. infantum*, no other pathogenic species of *Leishmania* is present in western and central Europe (Akhoundi et al., 2016). However, *Leishmania tarentolae*, a species of the subgenus *Sauroleishmania*, has a sympatric occurrence with *L. infantum* (Akhoundi et al., 2016; Klatt et al., 2019). In particular, *L. tarentolae* was isolated from the blood of *Tarentola mauritanica* geckoes collected near the city of Monopoli, Apulia (Pozio et al., 1986), a highly endemic region for canine leishmaniosis (CanL) in Italy (Mendoza-Roldan et al., 2020b). This species was also isolated from *Mediodactylus kotschy* (Pozio et al., 1983) in Italy and *Tarentola annularis* in Sudan (Elwasila, 1988). Moreover, recent studies have shown the interaction of these two *Leishmania* species in the before mentioned epidemiological context, where *Phlebotomus perfiliewi* and *Phlebotomus perniciosus* have been detected molecularly positive for *L. tarentolae* (Pombi et al., 2020; Mendoza-Roldan et al., 2021b) and *Sergentomyia minuta* for *L. infantum* in Italy (Tarallo et al., 2010; Latrofa et al., 2018a; Iatta et al., 2020; Pombi et al., 2020) as well as in other European endemic areas of CanL (Campino et al., 2013; Bravo-Barriga et al., 2016; Maia et al., 2015; González et al., 2020). Importantly, *S. minuta* displays a primarily anthrophilic feeding behavior, rather than just herpetophilic (Maia et al., 2015; Pombi et al., 2020; Abbate et al., 2020; Mendoza-Roldan et al., 2021b), giving the possibility of *L. tarentolae* to infect also mammals. Indeed, *L. tarentolae* was first molecularly identified in mummies from Brazil (Novo et al., 2015), and then in human blood donors from central Italy (Pombi et al., 2020) and inhabitants of the islands of the Pelagie archipelago (Iatta et al., 2021). In addition, *L.*

tarentolae was also detected in blood from other mammals such as dogs, suggesting its sympatric occurrence with *L. infantum* in the same kind of host (Mendoza-Roldan et al., 2021b). Thus, in areas where both species of *Leishmania* and their vectors circulate, non-permissive hosts (i.e., mammals for *L. tarentolae* and reptiles for *L. infantum*) may be infected or even co-infected. This event is reinforced by studies in China, where reptiles (i.e., lizards and snakes) scored positive to pathogenic species of *Leishmania* (i.e., *Leishmania donovani*, *Leishmania tropica*, *Leishmania turanica*) (Chen et al., 2019; Zhang et al., 2019). However, although *L. tarentolae* is widely used in biotechnology (i.e., recombinant protein production, vaccine candidate, RNA editing), due to its genetic similarities with *L. infantum*, combined with its non-pathogenic nature (Klatt et al., 2019), major knowledge gaps regarding the life cycle, transmission, seasonality, and hosts of this species still exist. Therefore, the aim of this study was to assess the prevalence and distribution of *L. tarentolae* in reptilian hosts from CanL endemic areas, as well as the presence of zoonotic *L. infantum* in reptiles.

2 | MATERIALS AND METHODS

2.1 | Study areas and sample collection

Squamata reptiles (i.e., lizards and snakes) were collected during a year (April 2020 to April 2021) from various locations of four southern regions of Italy (i.e., Apulia, Basilicata, Calabria, Sicily), endemic for CanL. Specifically, the study areas were as follows for each region: Apulia (site 1: Municipality of Bari: 41°06'24.9"N 16°52'27.2"E; site 2: Dog shelter, Brindisi municipality: 40°36'30.3"N 17° 59'40.0"E; site 3: Dog shelter, Lecce municipality: 40°25'09.6"N 18°09'56.1"E; site 4: Noicattaro municipality: 41°04'48.2"N 16°59'29.1"E; site 5: Torre a Mare, Bari municipality: 41°05'31.5"N 16°59'24.2"E; site 6: Campus of the Department of Veterinary Medicine, University of Bari "Aldo Moro": 41°1'31.584"N, 16°54'3.6288"E); Basilicata (site 7: Parco Regionale di Gallipoli Cognato, Piccole Dolomiti Lucane: 40°32'17"N, 16°07'20.17"E); Calabria (site 8: Dog shelter A.M.I.C.I., Municipality of Cassano allo Jonio: 39°43'50.7"N 16°17'56.9"E); and Sicily (site 9: Aeolian islands - Filicudi, Lipari, Salina, Vulcano: 38°29'16.1"N 14°56'07.8"E; site 10: Pelagie islands, Linosa: 35°52'15.8"N 12°52'19.1"E; Figure 1). Study sites were chosen based on previous reports of CanL (Otranto et al., 2017) having similar characteristics (i.e., dog shelters, peri-urban areas or natural parks) where different species of reptiles live in sympatry with the canine and human population. Most of these sites had a typical Mediterranean environment characterized by olive or orange trees, the presence of the typical "muretti a secco" (stone walls) where reptiles (i.e., *Podarcis* lizards, *Hierophis* snakes, and *Tarentola* and *Hemidactylus* geckoes, among others) and sand flies thrive (Figure 1). Study sites and distribution of *Leishmania* in reptiles were illustrated in maps generated using QGIS version 3.4.4-Madeira, to show the origin of the detected pathogens (Qgis Development Team, 2015).

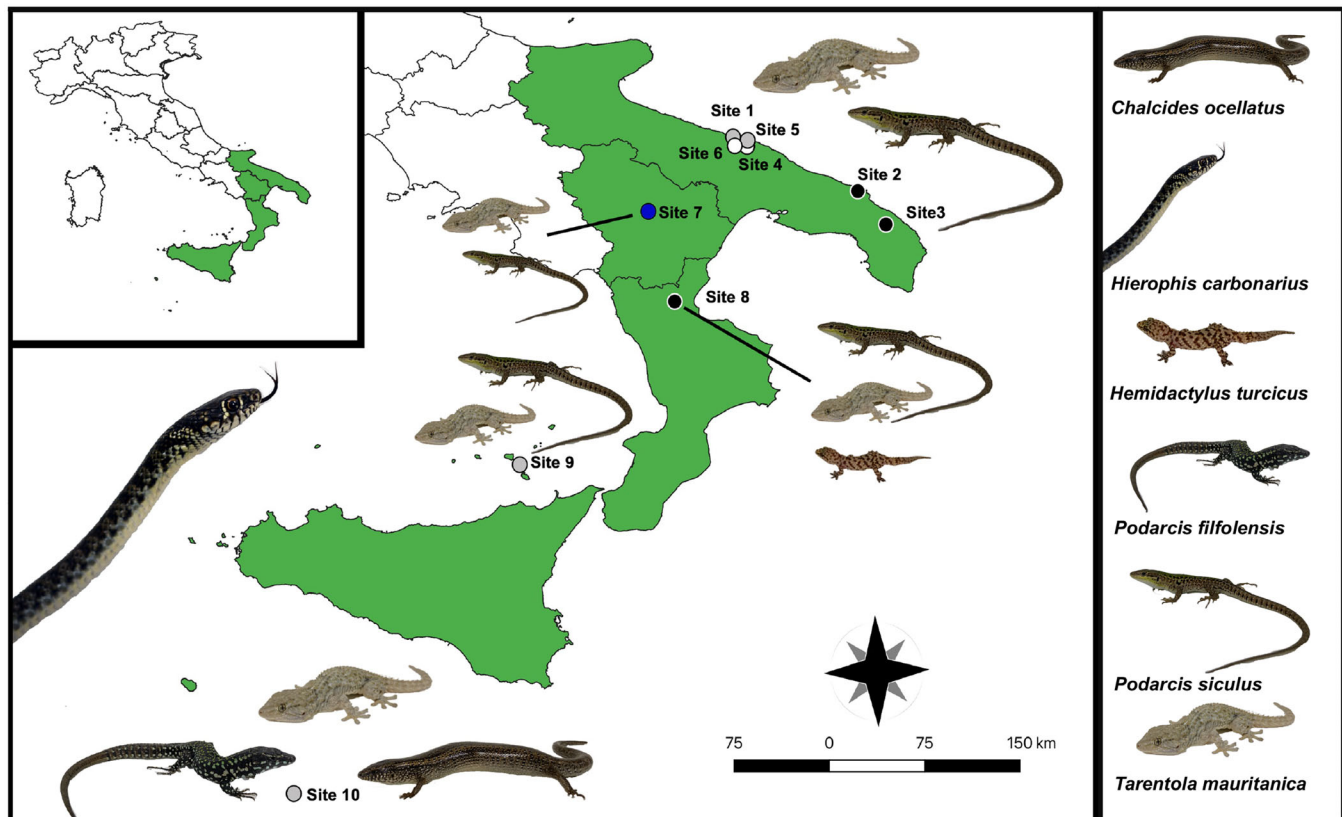


FIGURE 1 Regions and study sites where reptiles were collected in southern Italy. White circles represent peri-urban areas, gray circles urban areas, black circles dog shelters, blue circle natural park. *Hierophis carbonarius* were captured in all regions. Saurian species collected are shown for each region

2.2 | Reptile capturing and processing

Reptiles were captured by lassoing, by hand or in the case of snakes using herpetological hooks. Species of reptiles were identified using reference keys (Arnold, 2002) and physically examined to assess their biological and health status. All data were recorded in each animal's file. A small amount of blood (~100 μ l) was obtained via lizard tail fracture, or by cardiocentesis, when animals were adults and non-gravid females. In snakes, blood samples were collected from the ventral coccygeal vein and stored in EDTA tubes, later they were transferred to 1.5 ml tubes and stored at -20°C . For animals collected alive, blood smears were performed and then assessed for the presence of hemoparasites (Telford, 2009), using Diff-Quik stain (Skipper & De Stephano, 1989). Smears were rinsed in tap water to remove excess stain and later evaluated using an optical microscope (LEICA DM LB2, Germany). Captured lizards were humanely euthanized according to protocols (Warren, 2014) and dissected. Intestine, heart, kidneys, liver, lungs, spleen, and skeletal muscle were individually collected from each reptile and frozen at -20°C . Snakes and lizards that were collected already dead (i.e., by predation or roadkill), were also dissected and tissues were taken when possible.

2.3 | Molecular biology

Genomic DNA (gDNA) was extracted from lizard tissues (i.e., intestine, heart, kidneys, liver, lungs, spleen, skeletal muscle), as well as from reptile blood samples, using two commercial kits, GenUPgDNA and GenUPBlood DNA Kits (Biotechrabbit GmbH, Hennigsdorf, Germany), respectively, according to the manufacturer's instructions. All samples were tested by duplex real-time PCR (dqPCR) for the detection of *L. infantum* and *L. tarentolae* and considered positive with Ct values up to 38.0 and 38.6, respectively, as previously described (Latrofa et al., 2021), and of *L. infantum* kDNA minicircle (120 bp) by real time-PCR (qPCR), using a protocol described elsewhere (Francino et al., 2006). gDNA from an *L. infantum* isolate cultured in Tobie-Evans medium from an infected dog living in Italy (zymodeme MON-1) and *L. tarentolae* (strain RTAR/IT/81/ISS21-G.6c/LEM124) promastigotes were used as controls. gDNA extracted from the blood sample of lizard and dog negative for *Leishmania* spp. were used as negative control. For sequences analyses, *Leishmania* dqPCR positive samples were amplified by conventional PCR (cPCR) using primers L5.8S/LITSR targeting a partial region of the internal transcribed spacer 1 (ITS1, ~300 bp) (El Tai et al., 2001). Amplified products were examined on 2% agarose

gels stained with GelRed (VWR International PBI, Milan, Italy) and visualized on a Gel Logic 100 gel documentation system (Kodak, NY, USA). Amplicons were purified and sequenced in both directions using the same primers as for PCR, employing the Big Dye Terminator v.3.1 chemistry in an automated sequencer (3130 Genetic Analyzer, Applied Biosystems, Foster City, CA, USA). All sequences were aligned using the ClustalW program (Larkin et al., 2007) and compared with those available in GenBank using the BLASTn search tool (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). To determine the genetic relationship of *Leishmania* sequence, the representative ITS1 sequences obtained from reptiles, those previously obtained (Mendoza-Roldan et al., 2021b), and those of the reference strains of *L. tarentolae* and *L. infantum* were phylogenetically assessed along with those of other *Leishmania* spp. available in the GenBank database. Phylogenetic tree was inferred using the Maximum Likelihood (ML) method based on the Kimura 2-parameter model (Kimura, 1980), selected by best-fit model analysis and based on the lowest score obtained by Bayesian Information Criterion (BCI), using MEGA6 software (Kimura, 1980). Evolutionary analyses were conducted with 5000 bootstrap replications using MEGA6 software (Tamura et al., 2013). The corresponding ITS1 sequence of *Trypanosoma brucei* (Accession number: KU552356.1) was used as an outgroup.

3 | RESULTS

The Squamata reptiles sampled ($n = 259$), belonged to two suborders (i.e., $n = 252$ Sauria, $n = 7$ Serpentes), four families (i.e., $n = 7$ Colubridae, $n = 65$ Gekkonidae, $n = 163$ Lacertidae, $n = 24$ Scincidae), five genera, and six species (Table 1). Sixteen of these animals were collected dead (i.e., $n = 1$ *Hierophis carbonarius*, $n = 14$ *Podarcis siculus*, $n = 1$ *T. mauritanica*), most of them predated by dogs or cats. The number of reptiles captured in different regions (i.e., 120 Apulia, 11 Basilicata, 26 Calabria, 102 Sicily), along with their positivity to *Leishmania* spp. is reported in Table 1. In particular, 34 (13.1%) reptiles scored positive for *Leishmania* spp., specifically for *L. tarentolae* ($n = 26$; 10%) and for *L. infantum* ($n = 8$; 3.1%) in at least one tissue. Out of 2030 (i.e., $n = 243$ blood; $n = 1787$ tissue) reptile tissues examined by dqPCR and qPCR, 51 samples (i.e., $n = 7$ blood, $n = 1$ intestine, $n = 7$ heart, $n = 6$ kidneys, $n = 11$ liver, $n = 10$ lungs, $n = 8$ spleen, and $n = 1$ skeletal muscle) scored positive for *Leishmania* spp., corresponding to 34 reptiles. The liver had the highest number of positive samples, whereas the higher amount of *Leishmania* DNA was recorded from blood with a Ct value of 20.4 (Table 2). On the other hand, of the seven blood samples, five were positive for *L. tarentolae* (detected by dqPCR) and two for *L. infantum* in *P. siculus* and *T. mauritanica* (detected by qPCR). Most of the positive animals were captured from the Apulia region (21/34, 61.7%; Figure 2), and positive specimens were recorded in seven out of 10 study sites (i.e., 2, 3, 4, 6, 8, 9, 10), corresponding to three regions (Apulia, Calabria, Sicily; Table 1). Positive lizards were mostly *P. siculus* ($n = 26/34$; 76%) from dog shelters (site 2, 3, 8) or islands (site 9), with six individuals positive for *L. infantum*. In addition, *L. tarentolae* DNA was detected in one *P. filfolensis* lizard from Linosa island, with *Leishmania* spp. amastigote-like forms inside thrombocytes observed in the blood smear (Figure 3A).

Furthermore, five *T. mauritanica* geckos were positive to *L. tarentolae* DNA, with one gecko being positive in blood (site 8) showing also *Leishmania* spp. amastigote-like forms inside thrombocytes (Figure 3B-D). Conversely, *L. infantum* was molecularly detected in one *T. mauritanica* (site 4), and in one *Hemidactylus turcicus* (site 8) (Table 2). Overall, out of 243 blood smears, two presented amastigote-like forms. All samples from seven *H. carbonarius* snakes scored negative.

Leishmania tarentolae and/or *L. infantum* were detected from April to October 2020 (late spring–early fall), except for July for *L. tarentolae* and April and August for *L. infantum* (Figure 4). No animals were collected from November 2020 to January 2021 and they scored positive only in April 2021. The percentage of positivity for *L. infantum* was lower compared to that of *L. tarentolae* throughout the study period.

BLASTn analysis of ITS1 sequences confirmed the *L. tarentolae* species identification showing a nucleotide identity of 99.0% with the reference sequence (KU680858) available in the GenBank database and with *L. tarentolae* sequences obtained in previous molecular studies in Italy (Pombi et al., 2020; Mendoza-Roldan et al., 2021b). The phylogenetic analysis of ITS1 clustered all *L. tarentolae* representative sequences herein obtained in a species-specific clade (*Sauroleishmania*) and as a monophyletic clade with that of *Leishmania adleri*, excluding the other *Leishmania* species (bootstrap value of 82%; Figure 5). Consensus sequence obtained for *L. tarentolae* was deposited in GenBank database (Accession number: OL449848).

4 | DISCUSSION

This study indicates that saurian reptiles living in CanL endemic areas of southern Italy are exposed to both *L. tarentolae* and *L. infantum*. Moreover, the distribution of *L. tarentolae*, herein assessed for the first time, is widespread in peri-urban areas and dog shelters, infecting different types of lacertid lizards and geckoes. Chiefly, this study represents the first molecular evidence of *L. infantum* infection in European reptiles.

The six species of reptiles collected are considered synanthropic, as they are able to live in urbanized areas, interacting with human and domestic animal populations (Rugiero, 2004; Simbula et al., 2019). Particularly, *P. siculus* and *T. mauritanica*, the most captured individuals, are widespread throughout the Mediterranean basin being invasive in other countries and regions (Baldo et al., 2008; Medina et al., 2019; Tuniyev et al., 2020). Indeed, the dead reptiles examined were mostly *P. siculus* predated by dogs or cats in peri-urban areas of Apulia and Island of Sicily (i.e., the regions where 85.7% of reptiles were collected). The study sites within these two regions have in common a high density of reptiles, dogs, and cats, in confined areas such as shelters and islands, thus characterized by a high predatory pressure (Mori et al., 2019). This sympatric occurrence of reptiles, domestic animals, and humans may facilitate the transmission of parasites and pathogens among them by predation, direct contact, or by vectors, such as in the case of *Leishmania* spp. (Mendoza-Roldan et al., 2020b).

The molecular prevalence of *Leishmania* spp. in reptiles has been studied in other geographical areas (i.e., Northwestern China), with a

TABLE 1 Family and species of collected reptiles, indicating positivity for *Leishmania infantum* or *Leishmania tarentolae* in sites from regions of southern Italy along with the number of positive tissues per reptile

Family	Species (n)	Region	Site	Positive to <i>Leishmania</i> spp. /total (%)	Tissue (n = 2030)
Colubridae	<i>Hierophis carbonarius</i> (7)	Apulia	5	0/2	
		Basilicata	7	0/1	
		Calabria	8	0/1	
		Sicily	9	0/3	
Gekkonidae	<i>Hemidactylus turcicus</i> (6)	Calabria	8	1 <i>L. infantum</i> /6 (16.6)	1H
	<i>Tarentola mauritanica</i> (59)	Apulia	1	0/6	1B
			2	0/1	1LI
			3	0/5	
			4	1 <i>L. infantum</i> /12 (8.3)	
			5	0/1	
			6	1 <i>L. tarentolae</i> / 4 (25)	
		Basilicata	7	0/3	
		Calabria	8	1 <i>L. tarentolae</i> / 8 (12.5)	1B
		Sicily	9	2 <i>L. tarentolae</i> /14 (14.3)	2B
			10	1 <i>L. tarentolae</i> / 5 (20)	1LU
Lacertidae	<i>Podarcis filfolensis</i> (24)	Sicily	10	1 <i>L. tarentolae</i> / 24 (4.1)	1LI
	<i>Podarcis siculus</i> (139)	Apulia	2	1 <i>L. infantum</i> / 18 (5.5)	1LI
			3	10 <i>L. tarentolae</i> / 30 (33.3)	4H; 1I; 3K; 1Mu;
			4		4LI; 6LU; 4S
			5	1 <i>L. infantum</i> ;	1H; 1LU; 3K; 1S
			6	4 <i>L. tarentolae</i> / 18 (27.7)	1LI; 1LU; 1S
				0/9	
				1 <i>L. infantum</i> ; 2 <i>L. tarentolae</i> / 14 (21.4)	
		Basilicata	7	0/7	
		Calabria	8	1 <i>L. infantum</i> ; 2 <i>L. tarentolae</i> /11 (27.2)	2B; 2LI; 1LU; 1S
		Sicily	9	2 <i>L. infantum</i> ; 2 <i>L. tarentolae</i> / 32(12.5)	1B; 1H; 1LI; 1S
Scincidae	<i>Chalcides ocellatus</i> (24)	Sicily	10	0/24	
Total				8 <i>L. infantum</i> ; 26 <i>L. tarentolae</i> / 259 (13.1)	51 (7B; 7H; 1I; 6K; 1Mu; 11LI; 10LU; 8S)

Abbreviations: B, Blood; H, Heart; I, Intestines; K, Kidney; Mu, Muscle; LI, Liver; LU, Lung; S, Spleen.

prevalence up to 57.9% (183/316) in lizards (Zhang et al, 2019) and 46.6% (7/15) in snakes (Chen et al., 2019). The negative results on seven *H. carbonarius* snakes could be due to the low number of animals screened. In Italy, previous molecular data showed a prevalence of 35.7% for *L. tarentolae* in *P. siculus* in the Apulia region (Mendoza-Roldan et al., 2021b). The molecular diagnosis of *Leishmania* spp. was herein validated for the first time in Europe considering that in all previous studies only culture and cytological examination of tissues of reptiles were performed (Pozio et al., 1986). Few comprehensive epidemiological studies have been carried out on *Sauroleishmania*, though those run in the Palearctic region showed up to 30% of

presence of promastigotes in the culture of *Tenuidactylus caspius* (formerly *Gymnodactylus caspius*) (Belova, 1971). In Italy, *L. tarentolae* promastigotes were observed in 37% of examined *T. mauritanica* by blood culture or blood smear examination (Pozio et al., 1986). Conversely, in the present study, blood of *T. mauritanica* gecko with amastigote-like forms in thrombocytes, was molecularly positive (i.e., Ct 20.4), suggesting a high parasitic load. *Leishmania tarentolae* amastigote-like forms have also been observed in thrombocytes from *T. mauritanica* (Pozio et al., 1986), in leucocytes of *Cyrtodactylus kotschy* and *T. annularis* (Pozio et al., 1983; Elwasila, 1988), and in erythrocytes from *P. siculus* (Mendoza-Roldan et al., 2021b). However, cytological

TABLE 2 Number and percentage of reptiles and their tissues positive to *Leishmania infantum* and/or *Leishmania tarentolae* by dqPCR and qPCR. The mean (M), minimum (Min), maximum (Max) and standard deviation (Sd) values of the cycle of quantification (Ct) are reported

Reptiles (n = 259)	<i>Leishmania tarentolae</i>					<i>Leishmania infantum</i>				
	P (%)	Ct			P (%)	Ct			P/T (%)	
		Mean	Min-max	SD		Mean	Min-max	SD		
<i>C. ocellatus</i> (24)	0	–	–	–	0	–	–	–	0	
<i>H. carbonarius</i> (7)	0	–	–	–	0	–	–	–	0	
<i>H. turcicus</i> (6)	0	–	–	–	1 (16.6)	32 (LI) qPCR	–	–	1 (16.6)	
<i>P. filfolensis</i> (24)	1 (4.1)	37.6 (LI)	–	–	0	–	–	–	1 (4.1)	
<i>P. siculus</i> (139)	20 (14.4)	34.1 (B, H, I, K, Mu, LI, LU, S)	24.7 (LI)–38.4 (H)	3.0	4 dqPCR; 2 qPCR (4.3)	35.6 (B, LI, LU)	34.3 (LI)–36.3 (B qPCR)	0.8	26 (18.7)	
<i>T. mauritanica</i> (59)	5 (8.47)	32.1 (B, LI, LU)	20.4 (B) – 38.6 (LU)	6.3	1 (1.7)	35.2 (B) qPCR	–	–	6 (10.16)	
P (%)	26 (10)				4 dqPCR; 4 qPCR (3.1)				34 (13.1)	

Abbreviations: P, positive reptiles; P/T, Positive/Total; B, Blood; H, Heart; I, Intestines; K, Kidney; Mu, Muscle; LI, Liver; LU, Lung; S, Spleen.

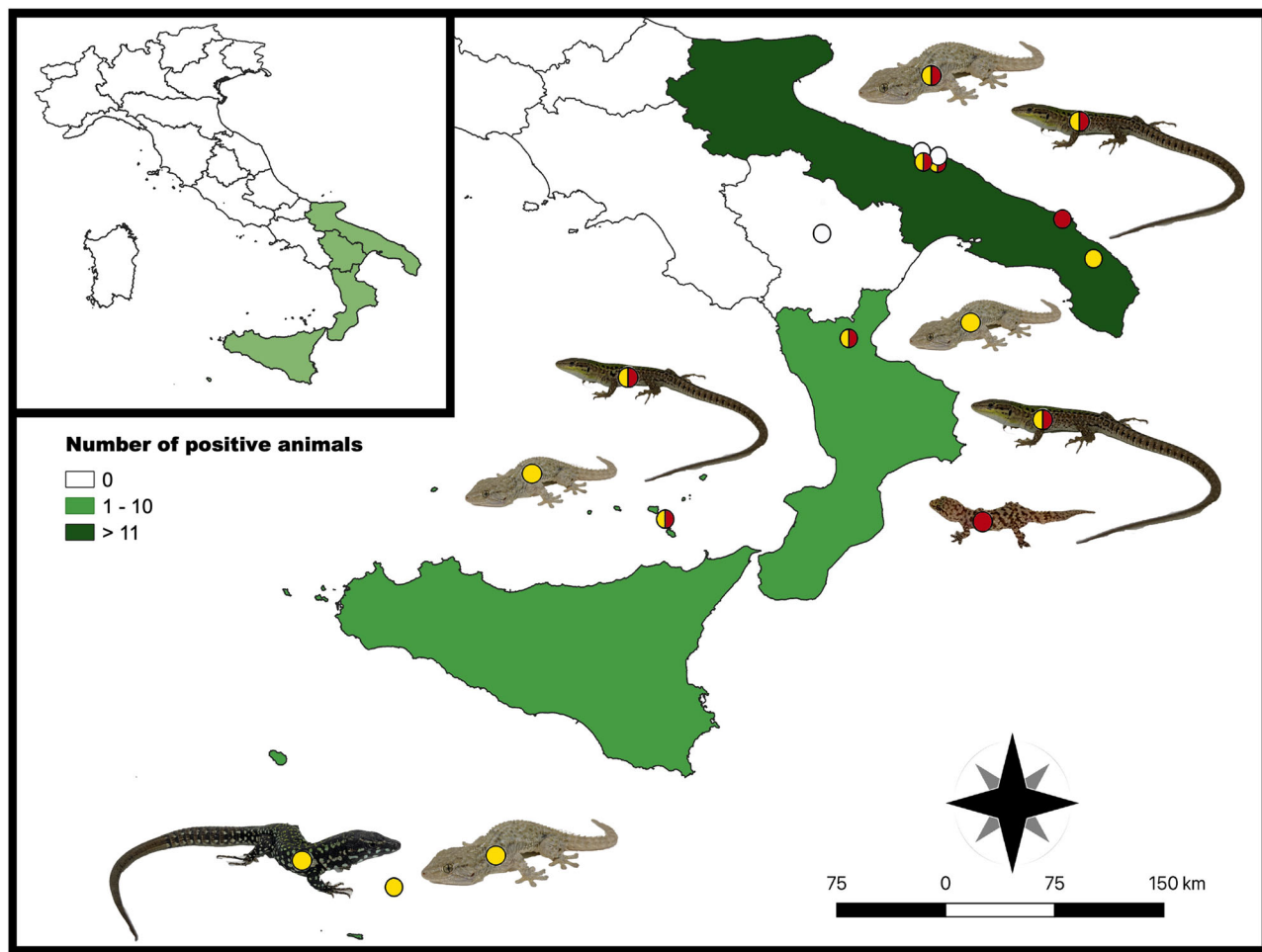


FIGURE 2 Distribution map of *Leishmania tarentolae* and *Leishmania infantum* molecularly detected from reptiles collected in four regions of southern Italy. White circles indicate negative sites, yellow circles *L. tarentolae* positive sites and reptiles, red circles *L. infantum* positive sites and reptiles, half yellow and half red circles *L. tarentolae* and *L. infantum* positive sites and reptiles

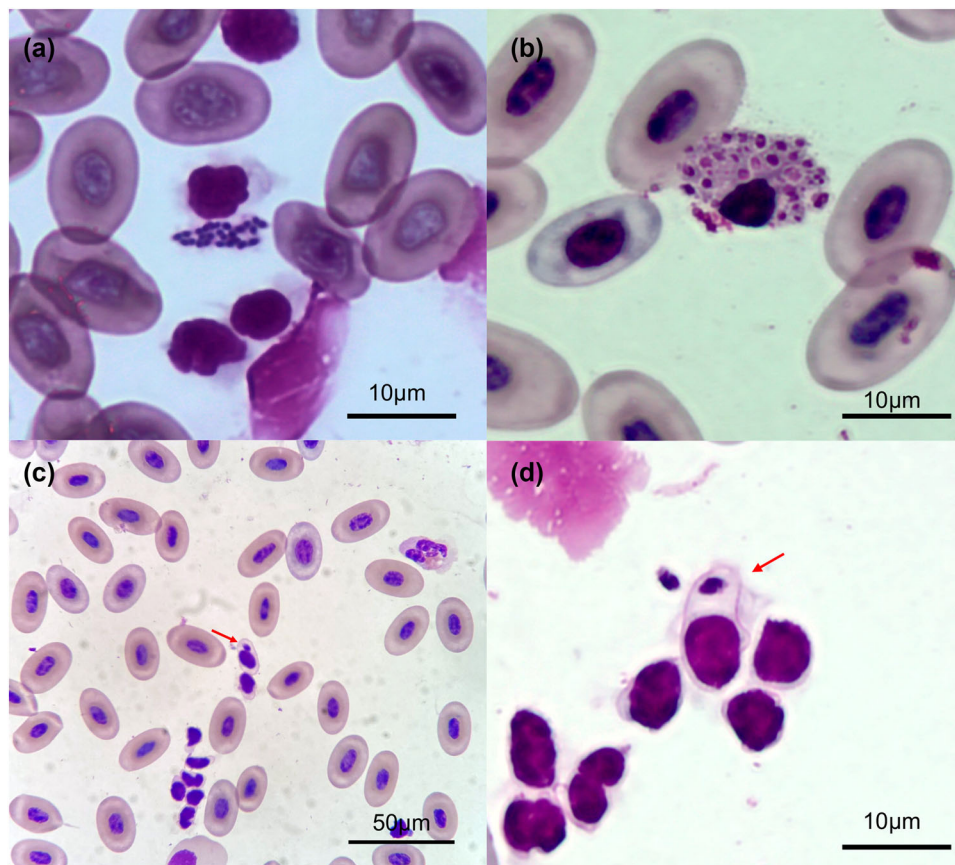


FIGURE 3 *Leishmania* spp. Amastigote-like forms in thrombocytes of a *Podarcis filfolensis* and *Tarentola mauritanica*. (A) Amastigote-like forms in thrombocyte of a *P. filfolensis*; (B–D) Amastigote-like forms in thrombocytes of a *T. mauritanica*. Red arrows indicate amastigote-like forms. Scale bars (A, B, D) 10 µm; C 50 µm

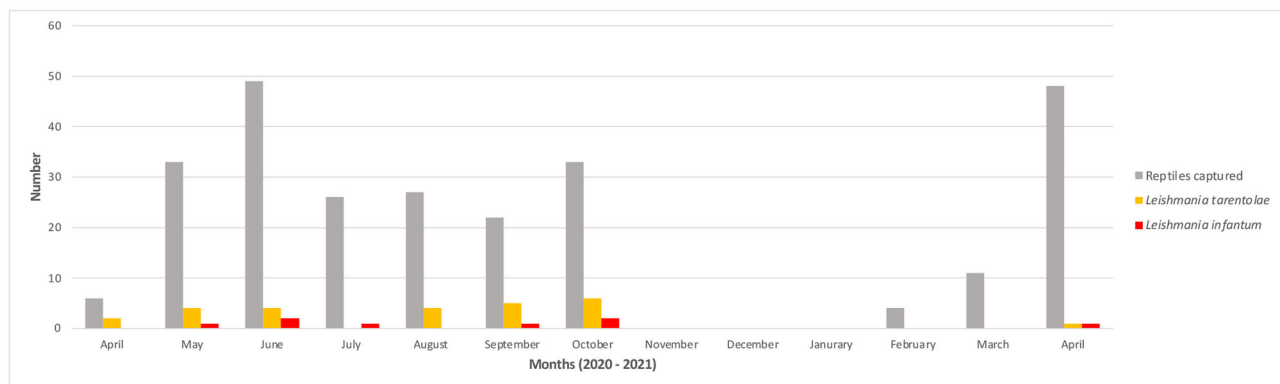


FIGURE 4 Seasonal variation represented by number of captured reptiles and number of positive animals to *Leishmania tarentolae* and *Leishmania infantum* per month, from April 2020 to April 2021

examination remains the less sensitive technique. The above has been demonstrated in former studies, where out of 3827 reptiles examined, only four blood smears revealed promastigotes or amastigote-like forms (Belova, 1971). Differently, both molecular techniques herein used are highly sensitive and specific, as suggested by the prevalence rate of positive blood (3%) and liver samples (4.2%), as well as the

results of previous studies carried out on different host tissues (Latrofa et al., 2018; Mendoza-Roldan et al., 2021b; Iatta et al., 2021).

Furthermore, the distribution and hosts-association of *L. tarentolae* herein assessed, suggests that this species is distributed throughout southern Italy, infecting more than just the main host *T. mauritanica*. Indeed, *P. filfolensis* is here described as a new lacertid host for this

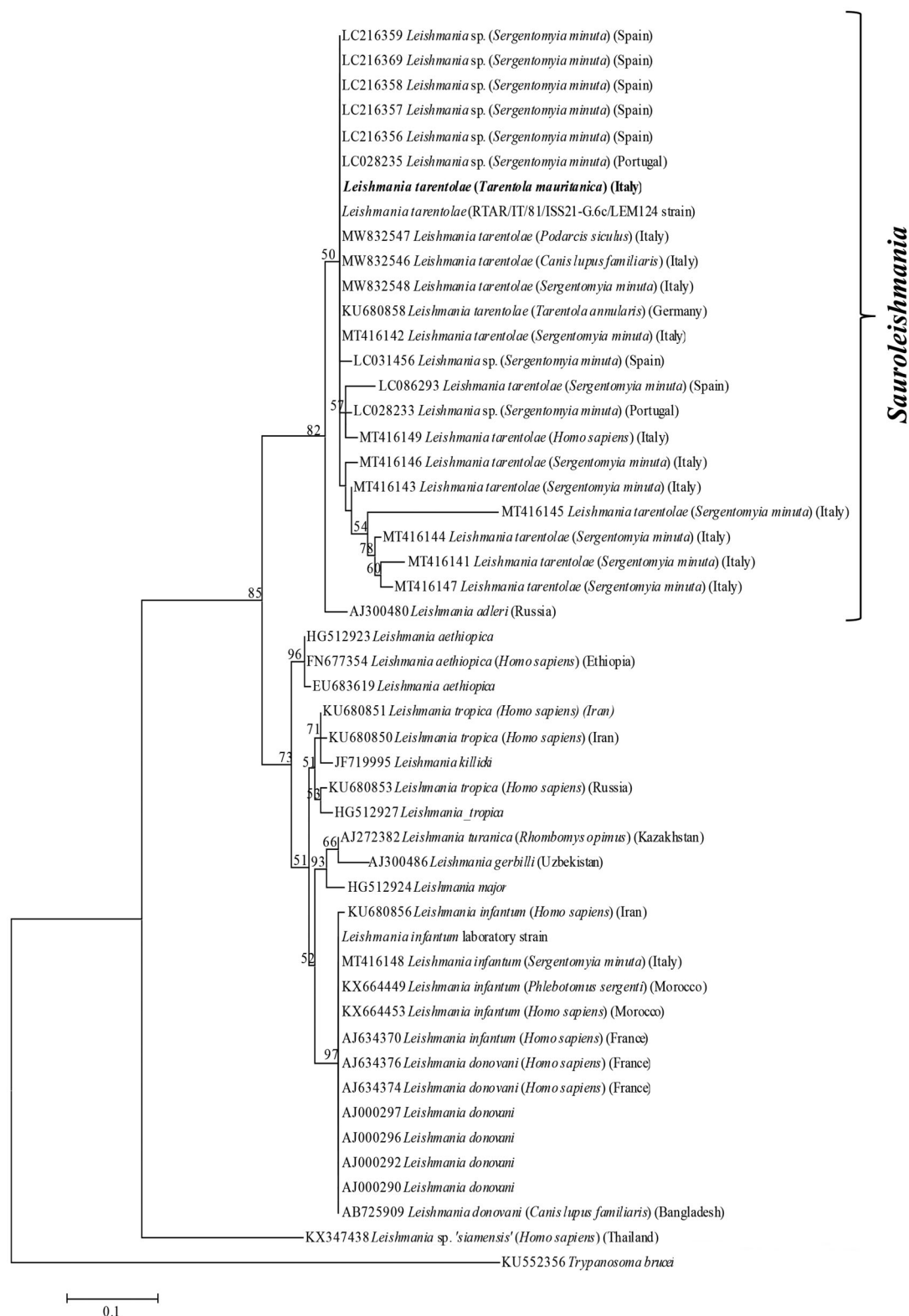


FIGURE 5 Phylogenetic tree based on *Leishmania* ITS1 sequences inferred using the maximum likelihood method based on the Kimura 2-parameter model. *Trypanosoma brucei* is used as outgroup. Scale bar indicates nucleotide substitution per site. *Leishmania* spp. sequenced in this study is in bold

species of *Leishmania* in the island of Linosa. In addition, phylogenetic analyses showed a clustering of *L. tarentolae* from *Tarentola* species and other hosts (i.e., lizards, dogs, sand flies, humans), which in addition formed an intraspecific clade of *L. tarentolae* clearly distinct from another *Sauroleishmania* species (i.e., *L. adleri*) as well as the remaining *Leishmania* spp. Finally, the phylogenetic results are in accordance with current classification of both species of *Leishmania* present in Europe (Akhoundi et al., 2016).

Moreover, the lower molecular prevalence of *L. tarentolae* compared to that previously reported in the same hosts (Mendoza-Roldan et al., 2021b), could be accounted by the season in which the parasite may cause an active infection. The prevalence of *L. tarentolae* infection in *P. siculus* collected in April 2020 (2/6; 33.3%) was similar to that observed in September and October representing the end of sand fly season, suggesting that animals may remain infected throughout the year. Data are in accordance with previous studies in which promastigotes were isolated throughout the year (Belova, 1971), therefore indicating that these hosts are infected for their lifespan. Moreover, negative results during the winter months (January and February 2021) could be due to a low number of individuals collected in this period, given the fact that reptiles from Europe go through hibernation/estivation or lethargy periods during the late autumn and winter months (November to March; Storey, 2006).

The molecular positivity to *L. infantum* in different kinds of tissues of three European reptiles is an unprecedented result. In Europe, many mammalian species (i.e., carnivores, cattle, bats, rodents, and lagomorphs) and even birds have been found molecularly or serologically positive to *L. infantum* (Cardoso et al., 2021), whereas this is the first survey carried out in Mediterranean reptiles. Indeed, the detection of samples (i.e., blood, heart, liver) positive to *L. infantum* using kDNA specific qPCR (Francino et al., 2006), which is routinely used in endemic areas of CanL, suggests recent infections with a low parasitic load. In addition, positive samples to this protozoan species were detected in reptiles from dog shelters and island environments, which are known to be endemic for CanL (Otranto et al., 2017; Mendoza-Roldan et al., 2020b). These areas share similar characteristics such as concentrated populations of dogs and cats, high abundance of sand flies and reptiles. Thus, the likelihood of infected sand flies feeding on reptiles is presumable. Indeed, a recent study demonstrated that *Phlebotomus* species, experimentally infected, may act as alternative vectors of *L. tarentolae* (Ticha et al., 2021). Also, *Phlebotomus* species have been found molecularly positive for *L. tarentolae* (Pombi et al., 2020; Mendoza-Roldan et al., 2021b), and *S. minuta* for *L. infantum* (Campino et al., 2013; Maia et al., 2015; Bravo-Barriga et al., 2016; Latrofa et al., 2018; Iatta et al., 2020; Pombi et al., 2020; González et al., 2020) and mammal blood (Pombi et al., 2020; González et al., 2020; Mendoza-Roldan et al., 2021b). Finally, reptiles have already been detected molecularly positive for mammalian pathogenic *Leishmania* spp. (Chen et al., 2019; Zhang et al., 2019), which could suggest the possibility of transmission to these non-permissive hosts. Conversely, the role of reptiles as reservoirs of *L. infantum* is still negligible and needs further studies.

5 | CONCLUSIONS

Saurian reptiles (i.e., lizards and geckoes) living in CanL endemic areas of the Mediterranean basin may be infected by *L. tarentolae* and *L. infantum*. Results of this study indicate that *L. tarentolae* is widely distributed in peri-urban areas and dog shelters in the southern regions of Italy, being able to infect its typical hosts (i.e., geckoes) and even lacertid lizards. This saurian-associated *Leishmania* circulates throughout the year, with active infections on reptiles during late spring and early fall. Overall, there are still many knowledge gaps in the life cycle and seasonal variation of all *Sauroleishmania* species. This study represents the first molecular evidence of *L. infantum* infection in three species of European reptiles, thus indicating sympatric circulation of these species with a potential overlap in vertebrate hosts in CanL endemic areas. The role of reptiles as reservoirs of *L. infantum* should be further investigated to understand the possible implications for the prevention and control of CanL in endemic areas.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Jairo Alfonso Mendoza-Roldan was associated with conceptualization, investigation, formal analysis, and wrote the original draft. **Maria Stefania Latrofa** was associated with investigation, methodology, and reviewed and edited the final manuscript. **Viviana Domenica Tarallo** was associated with investigation and methodology; **Ranju RS Manoj** was associated with methodology and reviewed and edited the final manuscript; **Marcos Antonio Bezerra-Santos** reviewed and edited the final manuscript and performed formal analysis; **Giada Annoscia** was associated with investigation and methodology; **Roberta Iatta** reviewed and edited the final manuscript and performed formal analysis; **Domenico Otranto** was associated with validation, supervision, and reviewed and edited the final manuscript.

ETHICS STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page have been adhered and all applicable international, national, and/or institutional guidelines for the care and use of animals were followed. Protocols for the collection of reptiles were approved by the ethical committee of the Department of Veterinary Medicine of the University of Bari, Italy (Prot. Uniba 12/20), as well as, authorized by the Ministry for Environment, Land and Sea Protection of Italy (Approval Number 0073267/2019), the *Societas*

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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