

Original article

Amblyomma sparsum Neumann 1899 on migratory birds from Africa: First records in Italy

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ARTICLE INFO

Keywords:

Amblyomma sparsum

Migratory birds

Italy

Ventotene

Mitochondrial molecular markers

ABSTRACT

Migratory birds play an important role in transporting ixodid ticks and tick-borne pathogens between continents. During the Boreal spring, migratory birds reach Europe, mainly from sub-Saharan Africa or from northern African countries but not much is known about the diversity and ecology of the ticks they spread. From 2017 to 2022, in the framework of two consecutive projects focused on sampling migratory birds from Africa to Europe, a total of 27 immature *Amblyomma* ticks were collected from migratory birds, belonging to 8 species, captured on the Island of Ventotene, an important stop-over site in the Mediterranean Sea. In the absence of adult specimens, morphological identification was limited to assigning these ticks to the *Amblyomma* genus. In this study, sequencing and comparative analysis of three mitochondrial molecular markers (12S rDNA, 16S rDNA, COI) were performed to achieve taxonomic identification. Sequences obtained from Ventotene specimens matched at 100% identity with *Amblyomma sparsum*. In conclusion, this study documented that immature stages of this species belonging to the *Amblyomma marmoreum* complex reached the Pontine Islands for six consecutive years. The entry of alien tick species and their potentially transmitted pathogens deserves further study, also in light of the globally ongoing climate change.

1. Introduction

Tick-bird association contributes to the dissemination of ticks and related pathogens over long distances, including urbanized areas to which large and medium-sized mammals have limited access (de la Fuente et al., 2015; Heylen et al., 2019). By traveling over long distances and across geographical barriers such as mountains, seas, and deserts, many avian species may be implicated in the spread of zoonoses, either as reservoirs, hosts, or carriers of infected ectoparasites, causing potential water-borne, tick-borne, and insect-borne diseases (Georgopoulou and Tsiouris, 2008). In Europe, similarly to other parts of the globe, migratory birds play an important role in the transportation of ixodid ticks (Acari: Ixodidae) and tick-borne pathogens (Hasle, 2013). During

the Boreal spring, migratory birds reach Europe, mainly from sub-Saharan Africa (trans-Saharan or long-distance migrants), or from northern African countries (intra-Palaearctic or short-distance migrants) where they spend the non-breeding season, but not much is known about the diversity and ecology of the ticks they spread (Laakkonen et al., 2009; Hornok et al., 2016). For this reason, studies on ticks hosted by migratory birds require investigations to gain an understanding of the potential dispersal of tick species from remote regions as well as the pathogens they can harbour. Between 2017 and 2022, more than 2500 ticks were collected as part of two consecutive projects focused on sampling migratory birds during their migration from Africa to Europe (Toma et al., 2021). The majority of these ticks were collected on the island of Ventotene (Lazio region, Italy) and identified at the species

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level. For twenty-seven immature specimens in the collection, morphological identification was limited to assigning these ticks to the genus *Amblyomma* and then molecularly identifying them as belonging to the *A. marmoreum* complex (Koch, 1844). In this study, we describe the molecular identification at the species level of these exotic hard ticks, widespread in Africa but not present in the European fauna, found on migratory birds captured on the island of Ventotene.

2. Material and methods

2.1. Tick collection

Fieldwork was carried out from 2017 to 2022, between March and May, during bird-ringing activities on Ventotene Island (Toma et al., 2021). This small Italian island is an important stop-over site in the Mediterranean for a huge number of migratory birds, mainly passerines, that reach the island after a non-stop flight from Africa during their spring migration (Spina et al., 1993). Birds were caught by experienced ringers and identified at the species level, sexed, aged, measured, marked with metal rings according to standard ringing procedures, and inspected for the presence of tick parasites (Mancuso et al., 2019). All the specimens were collected from each bird using a tick twister or tweezers and stored in Eppendorf tubes containing 70 % ethanol.

2.2. Morphological and molecular tick identification

The *Amblyomma* genus is not part of the European tick fauna, hence the specimens were identified based on literature that was specific to African tick fauna (Walker et al., 2003; Apanaskevich et al., 2008) and in consultation with experts from the University of Pretoria. Nevertheless, ticks collected from wild birds are generally immature and their morphological identification at the species level is challenging (Pascucci et al., 2019). The image acquisition of the immature specimens that were analysed (Fig. 1) was performed at the Science Department of Roma Tre University, using focus stacking light microscopy. Serial stacks of images were obtained with a Zeiss Axio Zoom V16 stereomicroscope equipped with a Plan-NEOFLUAR Z 1X objective lens and an axiocam 503 digital colour camera. Specimens were placed in a 5 mm petri dish and completely covered in 70 % ethanol. Images were later processed using Adobe Photoshop software.

After morphological identification, ticks were singularly homogenized using the TissueLyser LT (Qiagen, Germany), and nucleic acids were extracted with BioSprint 96 One-For-All Vet Kit (Qiagen), according to each manufacturer's protocol. The specimens were analyzed through amplification and sequencing of a fragment of the following molecular markers: 12S rDNA (Beati and Keirans, 2001), 16S rDNA (Black and Piesman, 1994), and mitochondrial cytochrome *c* oxidase subunit 1 (COI) (Lv et al., 2014). All PCR products were purified and directly sequenced at Eurofins Genomics (Ebersberg, Germany) using the same primers as those used for PCR in both forward and reverse directions. The sequences obtained were compiled using DS Gene v1.5 software (Accelrys Inc., San Diego, CA, USA) and analyzed using NCBI's Basic Local Alignment Search (BLAST) tool for the identification of tick species. Alignment of homologous sequences of *Amblyomma* specimens generated in this study and those available in GenBank was performed by DS Gene v1.5 software (Accelrys Inc., San Diego, CA, USA). Evolutionary analysis was conducted in MEGA11 by using the Maximum Likelihood method and Tamura 3-parameter model. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. The sequence of 16S from *Rhipicephalus sanguineus* (KC203362) was used as an outgroup (Fig. 2).

3. Results

In total, 27 immature specimens of *Amblyomma* sp. (20 nymphs and 7 larvae) were collected from 2017 to 2022 from migratory birds

belonging to 8 different passerine species caught for ringing activities on the island of Ventotene (Table 1). An accurate morphological characterization of the nymphs was carried out using a better-quality stereo microscope supported by efficient image editing software. Through the use of this tool, the eyes, previously barely visible, appeared flat and smooth set in the profile of the scutum, or "flush in the surface of the scutum", as reported by Theiler and Salisbury (1959) in the first description of *A. marmoreum*. However, in the absence of adult specimens, morphological identification was initially limited to assigning these ticks to the *Amblyomma* genus. To achieve identification at the species level, a molecular approach based on amplification and sequencing of three molecular targets was used. The length of the obtained sequences was 379 base pairs (bp) for 12S rRNA, 451 bp for 16S rRNA, and 825 bp for COI. By molecular analysis, all 27 ticks showed identical sequences to each other for each of the molecular targets analyzed (Table 1).

Comparative analysis using the Blast tool displayed significant matches between our sequences and the mitogenome sequence of *Amblyomma sparsum* (accession number OQ842962), only very recently submitted to the NCBI GenBank (Cotes-Perdomo et al., 2024). Indeed, except for very few differences in the primer regions, the analyzed sequences COI, 12S, and 16S showed 100 % identity with the corresponding fragments present in the mitogenome of *A. sparsum*. In addition, our sequences showed a high identity percentage with the 16S (100 % - 99.41 %, OL352904-OL352906), 12S (100 %, OL352895), and COI (100 %, OL339466) of specimens collected from migratory birds in Malta identified as a species of the *A. marmoreum* complex (Hornok et al., 2022). Conversely, the sequences obtained for ticks collected during this study displayed from about 94 % to 97.5 % identity with the respective ones of *Amblyomma nuttalli* from Zambia (OL741736) (Kelava et al., 2023) and from Kenya (SRR121168527 personal communication, Nardi et al., 2021). The identity percentage is instead further lower (< 96 %) when compared with the sequences of *A. marmoreum* sensu stricto (KY457515-KY457516). Furthermore, the sequence of the 12S target shared a genetic identity of 100 % and 99.7 % with sequences (KC817413 and KC817417, respectively) of two *Amblyomma* nymphs collected from migratory birds on Ventotene in 2014, which had not been identified at the species level at that time (Toma et al., 2014).

Finally, a common region (340 bp) of the 16S rRNA gene from Neotropical and Afrotropical *Amblyomma* species retrieved from the GenBank database was used to reconstruct a maximum-parsimony phylogenetic tree (Fig. 2). This cladistic analysis confirmed that *Amblyomma* specimens from Ventotene constituted a monophyletic clade with *A. sparsum* and ticks of the *A. marmoreum* complex from Malta.

4. Discussion

The *Amblyomma marmoreum* complex is a group of triphasic species that includes *A. marmoreum* sensu strictu, *Amblyomma sparsum*, *Amblyomma nuttalli*, *Amblyomma falsomarmoreum*, and probably other species according to Guglielmone et al. (2017). *Amblyomma marmoreum* s.s., the species that gives the name to the complex and known as the South African tortoise tick, is quite common and widely distributed across South Africa (Horak et al., 2006). This tick, like many *Amblyomma* species, is known to be primarily vector of *Ehrlichia ruminantium* (formerly *Cowdria ruminantium*), the causative agent of heartwater, a disease affecting domestic and wild ruminants in sub-Saharan Africa and some of the West Indian islands (Bezuidenhout, 1987; Oberem and Bezuidenhout, 1987). However, some species of the *A. marmoreum* complex are also involved in the infectious cycles of zoonotic pathogens, such as *Rickettsia* spp. belonging to the spotted fever and the typhus group (Cotes-Perdomo et al., 2024; Sánchez-Montes et al., 2019). Humans, like other large mammals, can be parasitized by this group of ticks as already reported in the literature (Tarragona et al., 2021; Horak et al., 2006).

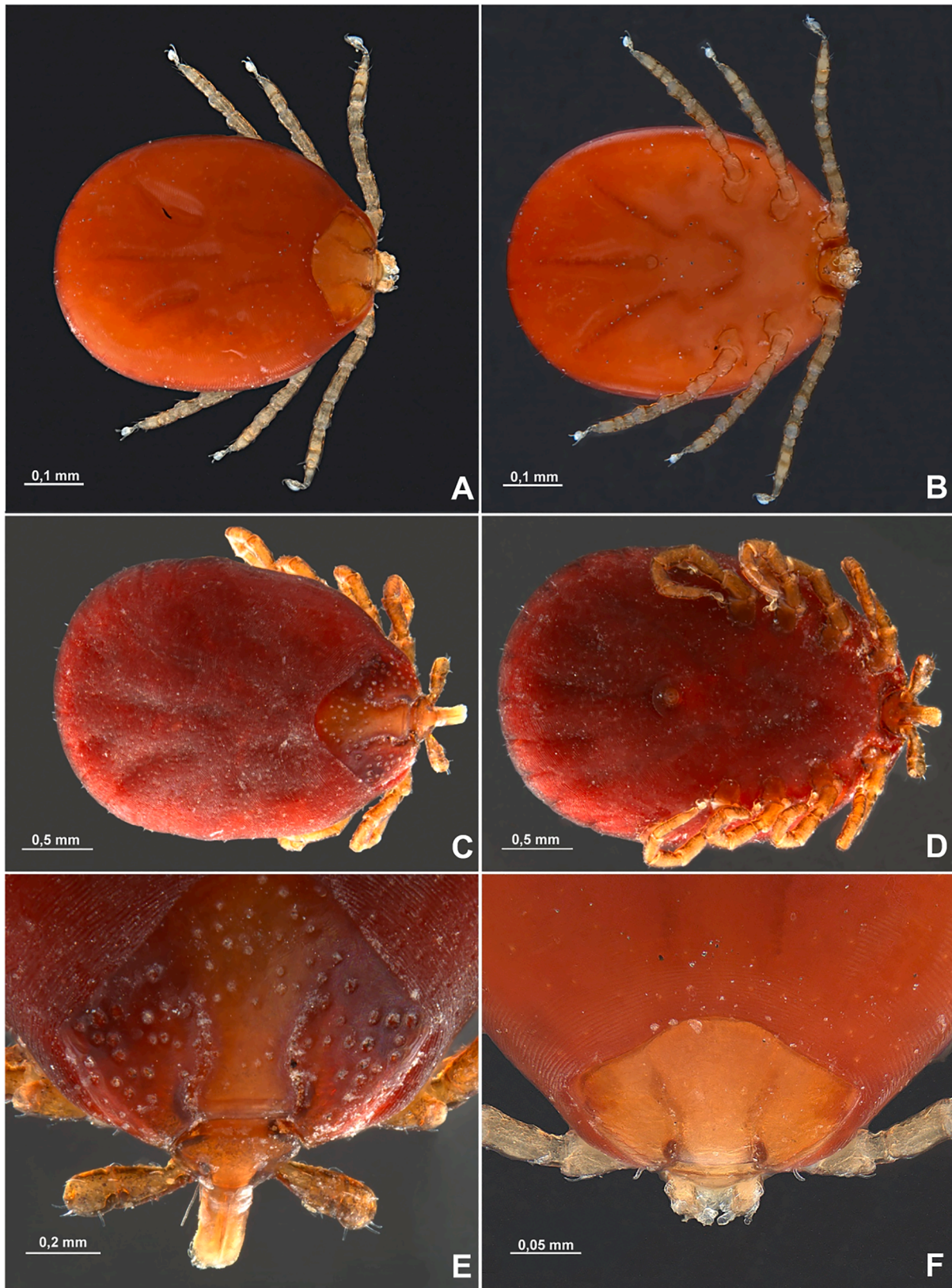


Fig. 1. Morphology of the immature specimens analysed in this study. A: larva ventral view; B: larva dorsal view; C: Nymph dorsal view; D: nymph ventral view; E: nymph scutum; F: larva scutum.

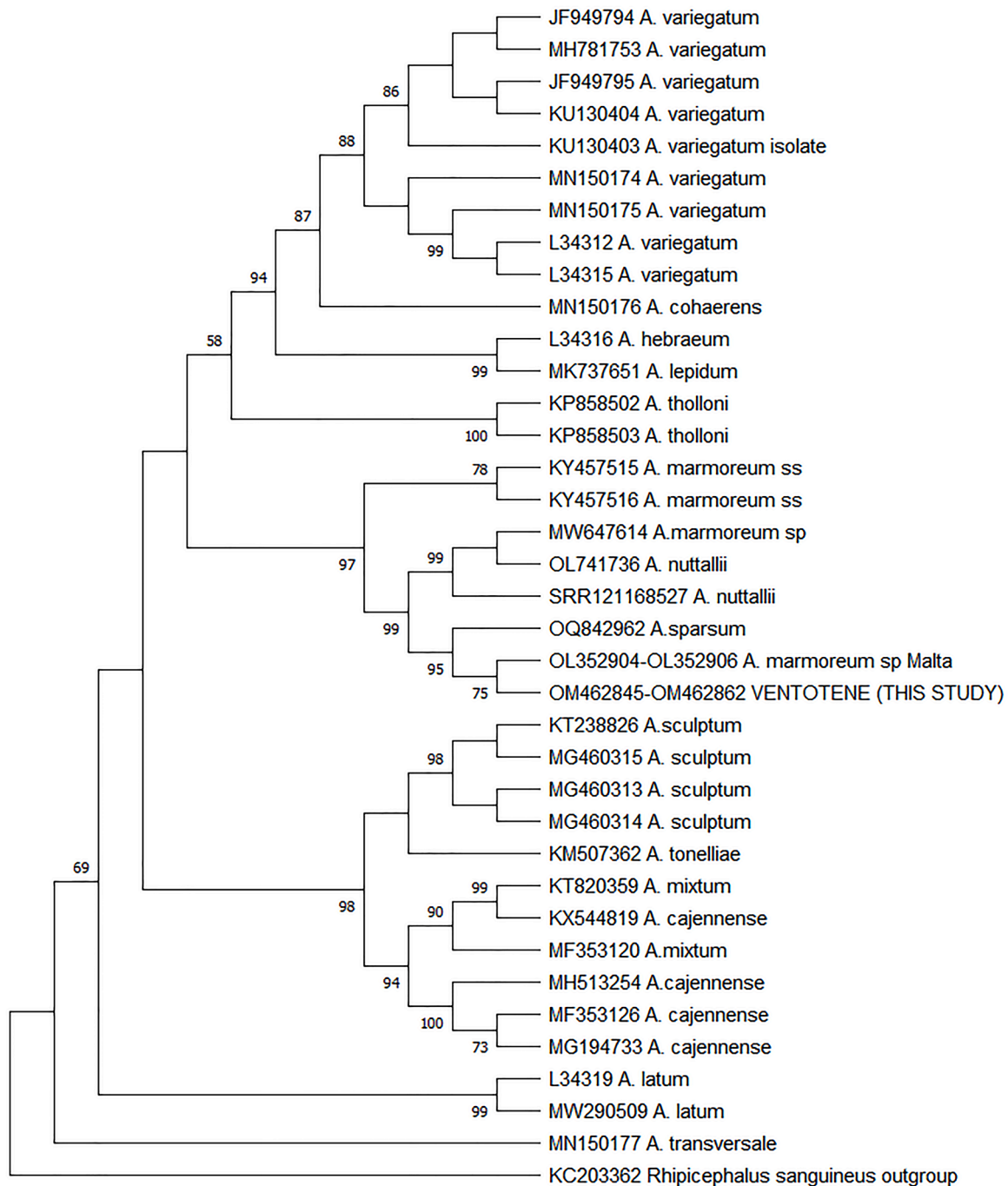


Fig. 2. Phylogenetic analysis based of 16S RNA sequences.

The evolutionary history was inferred by using the Maximum Likelihood method and Tamura 3-parameter model (Tamura, 1992). Branches corresponding to partitions reproduced in less than 50 % of bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test 1000 replicates are shown next to the branches (Felsenstein, 1985). Initial tree(s) for the heuristic search were obtained by applying the Neighbour-Joining method to a matrix of pairwise distances estimated using the Tamura 3 parameter model. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.9694)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 42.29 % sites). This analysis involved 53 nucleotide sequences. In total, there were 340 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (Tamura et al., 2021).

Amblyomma marmoreum was first comprehensively described by Theiler and Salisbury (1959) and then by Norval in 1975, who described its biology, ecology, and seasonality. Although the adults of these species feed mainly on reptiles, the immature stages show a very limited degree of host specificity. Nymphs and larvae feed on a wide range of vertebrate hosts: they have commonly been found parasitizing on both domestic (cattle, sheep, goats, dogs) and wild animals, such as reptiles,

ground-feeding birds, ungulates, canids, felids, and lagomorphs (Walker, 1991). Ticks of the *A. marmoreum* complex exhibit a preference for grasslands with scrub or tree cover and require moisture, but not in very arid habitats (Theiler 1962; Norval 1975). Such climatic and environmental features are not so different from those occurring in Mediterranean countries or the Southern United States where *A. marmoreum* has spread from southern Africa after being introduced to

Table 1

Specimen information and GenBank accession numbers for DNA sequences generated in this study. Note: sequences of specimens from 2022 collection were not submitted to GeneBank due to the 100 % identity with those from the previous years.

Collection Year	ID specimen (stage)	Host species	Molecular Markers		
			12S	16S	COI
2017	V17/Z329 (nymph)	Whinchat (<i>Saxicola rubetra</i>)	OM522963	OM462845	OM463605
2017	V17/Z536 (nymph)	Icterine warbler (<i>Hippolais icterina</i>)	OM522964	OM462846	OM463606
2017	V17/Z628 (nymph)	Willow warbler (<i>Phylloscopus trochilus</i>)	OM522965	OM462847	OM463607
2018	V18/Z239 (larva)	Tree pipit (<i>Anthus trivialis</i>)	OM522966	OM462848	OM463608
2018	V18/Z341 (larva)	Tree pipit (<i>Anthus trivialis</i>)	OM522967	OM462849	OM463609
2018	V18/Z342 (larva)	Tree pipit (<i>Anthus trivialis</i>)	OM522968	OM462850	OM463610
2018	V18/Z365 (larva)	Tree pipit (<i>Anthus trivialis</i>)	OM522969	OM462851	OM463611
2018	V18/Z366 (larva)	Tree pipit (<i>Anthus trivialis</i>)	OM522970	OM462852	OM463612
2018	V18/Z503 (larva)	Spotted flycatcher (<i>Muscicapa striata</i>)	OM522971	OM462853	OM463613
2018	V18/Z750 (nymph)	Spotted flycatcher (<i>Muscicapa striata</i>)	OM522972	OM462854	OM463614
2018	V18/Z878 (nymph)	Icterine warbler (<i>Hippolais icterina</i>)	OM522973	OM462855	OM463615
2019	V19/Z13 (nymph)	Willow warbler (<i>Phylloscopus trochilus</i>)	OM522974	OM462856	OM463616
2019	V19/Z482 (nymph)	Icterine warbler (<i>Hippolais icterina</i>)	OM522975	OM462857	OM463617
2020	V20/Z37 (nymph)	Icterine warbler (<i>Hippolais icterina</i>)	OM522977	OM462859	OM463619
2021	V21/Z103 (nymph)	Common nightingale (<i>Luscinia megarhynchos</i>)	OM522978	OM462860	OM463620
2021	V21/Z133 (nymph)	European pied flycatcher (<i>Ficedula hypoleuca</i>)	OM522979	OM462861	OM463621
2021	V21/Z408 (larva)	Spotted flycatcher (<i>Muscicapa striata</i>)	OM522980	OM462862	OM463622
2022	V22/013 (nymph)	Tree pipit (<i>Anthus trivialis</i>)	-	-	-
2022	V22/024 (nymph)	Tree pipit (<i>Anthus trivialis</i>)	-	-	-
2022	V22/044 (nymph)	Tree pipit (<i>Anthus trivialis</i>)	-	-	-
2022	V22/051 (nymph)	Whinchat (<i>Saxicola rubetra</i>)	-	-	-
2022	V22/060 (nymph)	Whinchat (<i>Saxicola rubetra</i>)	-	-	-
2022	V22/063 (nymph)	Whinchat (<i>Saxicola rubetra</i>)	-	-	-
2022	V22/119 (nymph)	Wood warbler (<i>Phylloscopus sibilatrix</i>)	-	-	-
2022	V22/124 (nymph)	Icterine warbler (<i>Hippolais icterina</i>)	-	-	-
2022	V22/265 (nymph)	Spotted flycatcher (<i>Muscicapa striata</i>)	-	-	-
2022	V22/270 (nymph)	Icterine warbler (<i>Hippolais icterina</i>)	-	-	-

Florida on imported reptiles (Allan et al., 1998; Burrige et al., 2000). Indeed, *Amblyomma* ticks were found in 28 of the 32 reptile breeding facilities investigated in 18 counties of the state, and specimens belonging to the *A. marmoreum* complex were found in 8 of the facilities, with *A. sparsum* found in one of these (Burrige et al., 2000). However, the characterization of species within this complex is still controversial due to the high morphological similarity, especially for immature species. In fact, according to Theiler and Salisbury (1959), there are disagreements between the text descriptions of the nymphs of three species from the *A. marmoreum* complex (*A. marmoreum*, *A. nuttalli* and *A. sparsum*) and the corresponding figures. Consequently, specimens of *A. marmoreum* can be easily confused with *A. sparsum*, *A. nuttalli*, or *A. falsomarmoreum*, and vice versa (Guglielmone et al., 2023).

The first record of this group of species in the Mediterranean occurred during one of the springs in the two-year period 2016–2017, when a specimen morphologically described as *A. marmoreum* was found in Italy on a bird captured on Ponza Island, close to Ventotene (Battisti et al., 2020). Subsequently, specimens described as *Amblyomma* sp. were collected on migratory birds in Ponza in 2019 (Rollins et al., 2021) and other specimens attributed to the *A. marmoreum* complex were found infesting birds captured in the Maltese Archipelago during the spring seasons of 2020 and 2021 (Hornok et al., 2022). To date, there is no evidence of local populations of *A. marmoreum* in Europe. It is likely that, given the distance they have to travel, ticks transported by migratory birds arrive in Europe in small numbers, do not find suitable hosts to complete their life cycle, or do not find suitable environmental conditions. Little is known about the environmental requirements of this species. We can only report that Horak et al. in 2006 described data on seasonality in South Africa, where the peak abundance of all stages on turtles occurs between January and May, with a peak of larvae and nymphs between March and April, the same period in which we found these early stages annually on migratory birds.

In the present study, 27 immature stages of *Amblyomma* sp. were found on seven species of trans-Saharan migratory birds caught in Ventotene from 2017 to 2022, as a part of two different projects (Toma et al., 2021; Mancuso et al., 2023). Until the end of 2020, the

mentioned specimens had only been identified at the genus level, due to their unclear morphology. Subsequently, a more accurate stereomicroscopic examination of surface morphology of the nymphs allowed the attribution of the samples to the *A. marmoreum* complex. Contextually, we faced numerous difficulties in molecularly identifying the collected ticks. Indeed, molecular-based identification has shown important limitations due to the degree of taxon coverage offered by public databases and the presence of problematic records, probably resulting from incorrect taxonomic assignment. This is mainly due to the many difficulties associated with the morphological identification at the species level in immature stages and fully engorged ticks, the similarity of morphology between different species, and the damage to the physical characteristics of specimens during collection.

Therefore, we have only very recently correctly identified at the species level specimens collected in Ventotene as *A. sparsum*, based on the phylogenetic relationship with the sequences obtained by Cotes-Perdomo et al. (2024).

The nucleotide sequences obtained from four out of the five nymphs collected in Ventotene and reported in the study by Toma et al. (2014) show 100 % nucleotide identity with those obtained in this study, while only one (the sequence KC817417) exhibits a single nucleotide difference. This observation moves back the first discovery of *A. sparsum* in our country by several years. In conclusion, this record confirmed that immature ticks of *A. sparsum* reach the Pontine Islands and probably other islands in the Mediterranean every spring, at least during the study period, but work is still ongoing to identify which other species of the *A. marmoreum* complex are involved. In addition to the identification of the exotic ticks that arrived in Italy, it is also worth mentioning the discovery of one of these specimens which was found to be infected with *Rickettsia tamurae* during screening for the pathogens (Mancuso et al., 2023). This *Rickettsia*, which is widespread in the Asian continent and often found in association with *Amblyomma testudinarium*, was described for the first time outside its original range. Although the pathogenicity of this bacterium in both animals and humans remains poorly understood, it belongs to the spotted fever group which is responsible for diseases that are increasingly recognized as public health threats worldwide

(Zhang et al., 2023).

To date, no data are available about the possibility that individuals of *A. sparsum* or other species belonging to the *Amblyomma marmoreum* group could have dropped off the host bird elsewhere in Italy during the yearly bird migratory wave from Africa. For this reason, the introduction of allochthonous tick species and their potentially transmitted pathogens deserves further study and attention, also in the light of the globally ongoing climate change.

Funding

This research was in the frame of the projects ‘Emerging and re-emerging zoonoses along the routes of migratory birds. An integrated approach to investigate the potential route of introduction and spread’ (IZS AM 04/19 RC) and ‘Risk of introduction and spread of vector-borne viruses in Italy’ (IZS AM 03/14/ RC) both funded by the Italian Ministry of Health. Partially funded by EU funding within the NextGeneration EU-MUR PNRR Extended Partnership initiative on Emerging Infectious Diseases (Project no. PE00000007, INF-ACT).

Author statement

I affirm that all the authors have read and accepted the manuscript. I also affirm that there is no conflict of interest and the content of this manuscript or a major portion thereof has not been published in a refereed journal, and it is not being submitted for publication elsewhere.

CRedit authorship contribution statement

Michela Menegon: Writing – original draft, Software, Methodology, Data curation, Conceptualization. **Elisa Mancuso:** Writing – original draft, Methodology, Investigation, Data curation, Conceptualization. **Marco Di Luca:** Writing – review & editing, Writing – original draft. **Francesca Casale:** Methodology, Investigation, Data curation. **Luis Neves:** Writing – review & editing, Writing – original draft. **Andeliza Smit:** Writing – review & editing, Writing – original draft. **Francesco Severini:** Writing – review & editing, Writing – original draft. **Michele Castelli:** Formal analysis. **Andrea Di Giulio:** Formal analysis. **Silvio G. d’Alessio:** Methodology. **Maria Goffredo:** Resources, Funding acquisition. **Federica Monaco:** Resources, Project administration, Funding acquisition. **Luciano Toma:** Writing – original draft, Supervision, Methodology, Investigation, Data curation, Conceptualization.

Data availability

Data will be made available on request.

Acknowledgments

We gratefully acknowledge the Riserva Naturale Statale “Isole di Ventotene e S. Stefano” for the logistic and financial support during the work on the island of Ventotene; to Dr. Sara Riello, Dr. Andrea Ferri, and all the collaborators and volunteers for their valuable contributions during sampling sessions; (Results from the Progetto Piccole Isole ISPRA). Moreover, a special thanks to Dr. Juan Esteban Uribe for kindly providing the *A. sparsum* sequence before being publicly released and to Dr. Massimo Scacchia for collaborative contacts with the University of Pretoria.

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