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On the way between Africa and Europe: Molecular taxonomy of ticks collected from birds in Malta

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ABSTRACT

The Maltese Archipelago is situated in the middle of the Mediterranean Basin, between Europe and Africa, therefore representing an important stopover site for migratory birds between these two continents. Despite this, up-to-date information is not available on tick species associated with birds in Malta. Therefore, in this study, birds mist-netted for ringing by BirdLife Malta were examined for the presence of ticks between September, 2019 and May, 2021. Ticks were identified morphologically and molecularly, using three genetic markers.

During the study period, 57 individuals of 22 bird species were found tick-infested, from which altogether 113 ixodid ticks were collected. The majority of developmental stages were nymphs, but 13 larvae and one female were also found. These ticks belonged to nine species: *Ixodes cumulatimpunctatus* (*n*=1), *Ixodes ricinus* (*n*=2), *Ixodes acuminatus* (*n*=2), *Ixodes frontalis* (*n*=5), *Ixodes festai* (*n*=1), one species of the *Amblyomma marmoreum* complex (*n*=8), *Hyalomma rufipes* (*n*=78), *Hyalomma marginatum* (*n*=7) and *Hyalomma lusitanicum* (*n*=1). Eight *Hyalomma* sp. ticks could only be identified on the genus level. Regarding seasonality, all Palearctic *Ixodes* species were carried by birds exclusively in the autumn (i.e., north to south), whereas *H. rufipes* (with predominantly Afrotropical distribution) was exclusively collected in the spring (i.e., carried south to north). Two tick species that occurred on birds in Malta, i.e., a species of the *A. marmoreum* complex and *I. cumulatimpunctatus* are only indigenous in the Afrotropical zoogeographic region. This is the first finding of the latter tick species in Europe, and four tick species were identified for the first time in Malta.

In conclusion, the diversity of tick species regularly arriving in Europe from Africa is most likely higher than reflected by data obtained in Mediterranean countries of mainland Europe. Most notably, ticks of the genus *Amblyomma* appear to be underrepresented in previous datasets. Ticks of the subgenus *Afrixodes* (represented by *I. cumulatimpunctatus*) might also be imported into Europe by migratory birds.

1. Introduction

In the era of climate change and globalization, the emergence and establishment of diseases in new geographic regions deserve increasing attention. In particular, blood-sucking arthropod species (so-called vectors) and infectious agents transmitted by them (i.e., vector-borne pathogens) are expected to undergo the most dramatic changes in terms of geographic ranges (Rocklöv and Dubrow, 2020). In the temperate zone of Europe, where new vectors and vector-borne pathogens will likely become established from the southern direction (from Africa), ticks are regarded as the most important disease transmitters (Rochlin and Toledo, 2020).

The Maltese Archipelago is situated in the middle of the Mediterranean Basin, between Europe and Africa, in the Central European route (or the Adriatic Flyway) of bird migration (Denac et al., 2010). Along this migratory route, the Maltese Islands represent the last stopover site for birds heading from Europe to their wintering grounds in Africa, and it is also the first land where birds arrive from Africa when entering

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Europe. Migratory birds are important long-distance carriers of ticks, and thus can play a significant role in intra- and intercontinental dispersal of tick-borne pathogens (Hornok et al., 2012). Nevertheless, apart from a brief report of *Ixodes ricinus* and *Ixodes frontalis* from two birds (Sultana and Gauci, 1977), more recent data on tick species associated with avian hosts are not available from Malta.

In order to compensate for this lack of data, in this study birds mist-netted for ringing by BirdLife Malta during an almost two-year-long period were examined for the presence of ticks. During the study nearly 20 000 birds were handled. Collected ticks were examined morphologically, and consequently analyzed with molecular methods based on three genetic markers.

2. Materials and methods

2.1. Sample collection

In this study, birds mist-netted for ringing by BirdLife Malta were examined for the presence of ticks between September 2019 and May 2021. During this period 19,637 birds were handled on four premises: Buskett, Simar, Comino, Ghadira (including habitats of woodland, wetland and garrigue). Ticks were removed from the skin with fine tweezers and stored in 96% ethanol. Palearctic *Ixodes* species were identified morphologically, *Hyalomma lusitanicum* molecularly, while other species by using both molecular and morphological characters based on the following sources: for the genus *Ixodes* Schulze (1943), Arthur (1963, 1965), Senevet and Rodhain (1968), Matthysse and Colbo (1987), Estrada-Peña et al. (2017, 2018); for the genus *Amblyomma* Theiler and Salisbury (1959), Mans et al. (2019); and for the genus *Hyalomma* Apanaskevich and Horak (2008), Apanaskevich et al. (2008), Sands et al. (2017). Pictures of ticks were made with a VHX-5000 digital microscope (Keyence Co., Osaka, Japan).

2.2. DNA extraction

Ticks were disinfected on their surface with sequential washing for 15 s in 10% NaClO, tap water and distilled water. DNA was extracted with the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instruction, including an overnight digestion in tissue lysis buffer and Proteinase K at 56°C. In case of 15 specimens selected for preliminary morphological identification, only two legs were used for DNA extraction (i.e., to preserve these as voucher specimens for later comparison). An extraction control (tissue lysis buffer) was also processed in each set of tick samples to monitor cross-contamination.

2.3. Molecular taxonomic analyses

The cytochrome *c* oxidase subunit I (*cox*1) gene was chosen as the first target for molecular analysis, in case of the 15 specimens selected for preliminary morphological identification. The PCR was modified from Folmer et al. (1994) and amplifies an approx. 710-bp-long fragment of the gene. The primers HCO2198 (5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3') and LCO1490 (5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3') were used in a reaction volume of 25 µl, containing 1 U (stock 5 U/µl) HotStarTaq Plus DNA Polymerase, 2.5 µl 10× CoralLoad Reaction buffer (including 15 mM $MgCl_2$), 0.5 µl PCR nucleotide Mix (stock 10 mM), 0.5 µl of each primer (stock 50 µM), 15.8 µl ddH2O and 5 µl template DNA. For amplification, an initial denaturation step at 95 °C for 5 min was followed by 40 cycles of denaturation at 94 °C for 40 s, annealing at 48 °C for 1 min and extension at 72 °C for 1 min. Final extension was performed at 72 °C for 10 min.

Another PCR was used to amplify an approx. 460-bp-fragment of the 16S rDNA gene of Ixodidae (Black and Piesman, 1994), with the primers 16S+1 (5'-CTG CTC AAT GAT TTT TTA AAT TGC TGT GG-3') and 16S-1 (5'-CCG GTC TGA ACT CAG ATC AAG T-3'). Other reaction components, as well as cycling conditions were the same as above, except for annealing at 51 °C. This method was used in case of the 15 specimens selected for preliminary morphological identification, as well as with those samples which yielded negative results in the 12S rRNA PCR.

In addition, a conventional PCR reaction was used with the primer pairs T1B (5′ - AAA CTA GGA TTA GAT ACC CT - 3′) and T2A (5′ - AAT GAG AGC GAC GGG CGA TGT - 3′) to amplify an approx. 360-bp-long fragment of the 12S rRNA gene from all DNA extracts (Beati and Keirans, 2001; Bitencourth et al., 2016). The PCR was modified with the following conditions: 5 µl of extracted DNA were added to 20 µl of reaction mixture containing 1 U HotStar Taq Plus DNA Polymerase (stock 5U/µl) (QIAGEN GmbH, Hilden, Germany), 0.5 µl dNTP Mix (stock 10 mM), 0.5 µl of each primer (stock 50 µM), 2.5 µl of 10x Coral Load PCR buffer (15 mM $MgCl_2$ included), 14.8 µl DW and 1.0 µl extra $MgCl_2$ (stock 25 mM). An initial denaturation step at 95 °C for 5 min was followed by 5 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s and extension at 72 °C for 30 s and 30 cycles of denaturation at 94 °C for 30 s, annealing at 53 °C for 30 s and extension at 72 °C for 30 s. Final extension was performed at 72 °C for 7 min (Burkman, 2009; Bitencourth et al., 2016).

2.4. Sequencing and phylogenetic analyses

In all PCRs non-template reaction mixture served as negative control. Extraction controls and negative controls remained PCR negative in all tests. Purification and sequencing of the PCR products were done by Biomi Ltd. (Gödöllő, Hungary) and at the Biological Research Center (Szeged, Hungary). Quality control and trimming of sequences were performed with the BioEdit program. Obtained sequences were individually compared to GenBank data by the nucleotide BLASTN program (https://blast.ncbi.nlm.nih.gov). New sequences were submitted to GenBank (*cox*1: OL339466-OL339477, 16S rRNA: OL352898-OL352924, 12S rRNA: OL352890-OL352897). Sequences from other studies (retrieved from GenBank) included in the phylogenetic analyses had nearly 100% coverage with sequences from this study. This dataset was resampled 1,000 times to generate bootstrap values. Phylogenetic analyses were conducted with the Neighbor-Joining method using p-distances and Maximum Likelihood method with Tamura-Nei model selected by the program MEGA version 7.0 (Kumar et al., 2016).

2.5. Ethical permission

All songbirds were handled and released by ringers of BirdLife Malta licensed under various permits issued by Maltese Government authorities, including the Environment and Resources Authority (ERA) and the Wild Bird Regulation Unit (WBRU). During handling and release of birds the regulations of Legal Notice 79/2006 (dealing with the Conservation of Wild Birds) were always maintained.

3. Results

During the study period 57 individuals of 22 bird species were observed as tick-infested, from which altogether 113 ixodid ticks were collected. The majority of developmental stages were nymphs (*n*=99), but 13 larvae and one female were also present. On most birds (*n*=40) only a single tick was found. The maximum number of ticks removed from the same bird was 11 *Hyalomma* specimens (seven larvae and four nymphs) from a greater whitethroat (*Sylvia communis*) caught in April. However, the mean intensity of tick infestation on this bird species was low (22 ticks from 11 birds, i.e., 2 ticks/bird), similarly to the cumulative intensity of 1.98% (113/57) taking into account all birds.

Based on morphological and molecular characteristics, as outlined below, the ticks belonged to three genera and nine species: *Ixodes cumulatimpunctatus* (*n*=1), *Ixodes ricinus* (*n*=2), *Ixodes acuminatus* (*n*=2), *Ixodes frontalis* (*n*=5), *Ixodes festai* (*n*=1), one species within the *Amblyomma marmoreum* complex (*n*=8), *Hyalomma rufipes* (*n*=78),

Hyalomma marginatum (*n*=7) and *H. lusitanicum* (*n*=1). Eight further *Hyalomma* sp. ticks could only be identified on the genus level (Table 1).

3.1. Morphological characteristics

Considering key morphological characters, the nymph identified as *I. cumulatimpunctatus* had broad, heart-shaped, almost smooth scutum (except for a fine reticulation); linear cervical grooves; trapezoid basis capituli, with prominent posterolateral corners; palpal segments II-III similar in length; small areae porosae (exceptionally present in the nymph stage); auriculae divided into an anterior and a broad, tooth-like backward directed posterior part; closed, oval anal groove; internal spur and prominent external spur on coxae I-III (Fig. 1). *Ixodes ricinus* nymphs had prominent internal spur on their first coxae. The *I. acuminatus* nymph had forward extended frontal area of basis (around the base of hypostome) and, similarly to the female, cornuae on the basis capituli and pointed internal spur on coxae II-III (Supplementary Fig. 1); *I. frontalis* larvae and nymphs had frontal "bumps" on the basis, nearly parallel sides of palpi, and nymphs had auriculae divided into anterior-posterior parts, as well as prominent external spurs on all coxae (Supplementary Fig. 2); the larva identified as *I. festai* had sharp internal spur on coxae I (Supplementary Fig. 3.a), the lateral teeth of its hypostome were posterolaterally directed (leaving a gap between individual teeth) and the dental formula was 3/3 only apically (Supplementary Fig. 3.b); nymphs of the *A. marmoreum* complex had numerous large, deep punctuations on the lateral fields of the scutum which had 1.27 breadth to length ratio (Supplementary Fig. 3.c); *H. rufipes* and *H. marginatum* nymphs had broadly rounded or slightly squared narrower posterior scutal margin, thus broader or narrower scutum (breadth to median length ratio: 1.4 or 1.35) and lemon-shaped or dorsally extended spiracular plates, respectively (Supplementary Fig. 4).

3.2. Molecular analysis of cox1 gene sequences

Concerning *Ixodes* species, there is no *cox*1 sequence of *I. cumulatimpunctatus* available in GenBank. The *cox*1 sequence (OL339467) of the nymph of this species (collected from a tree pipit, *Anthus trivialis*) in Malta, had the closest, but only 85.3% (550/645 bp) sequence identity to *I. frontalis*, reported from Hungary (KU170506). The *I. ricinus* nymph (OL339471) collected from a willow warbler (*Phylloscopus trochilus*) had 100% (641/641 bp) sequence identity with three specimens reported from Switzerland (AY945422, AY945432, AY945434). The *I. acuminatus* nymph (OL339473, from Eurasian skylark, *Alauda arvensis*) had the highest, 99.4% (632/636 bp) sequence identity with a specimen from Romania (JX394200). However, the *I. acuminatus* female (OL339474, from meadow pipit, *Anthus pratensis*) had a lower, 98.4% (626/636 bp) sequence identity with another specimen from Romania (JX394202). Three larvae of *I. frontalis* (OL339470) showed 100% (636/636 bp) sequence identity with haplotype-B of this species from Hungary (KU170501). The amplification of the *cox*1 gene fragment from *I. festai* was not successful.

Among metastriate ticks, three *Amblyomma* nymphs collected from tree pipits (*A. trivialis*) had 1 bp difference in the amplified part of their *cox*1 gene (OL339466, OL339476) which showed the highest but only 91.9% to 95.9% (592/644 to 610/636 bp) identity to sequences of *A. marmoreum* reported from South Africa (KY457515, KY457516, MW513957, MW513958). The *cox*1 sequence of *H. lusitanicum* (OL339475, from a Spanish sparrow, *Passer hispaniolensis*) was 99.8% (645/646 bp) identical to sequences (EU827719, EU827725) from Spain, and 98.8% (574/581 bp) identical to the sequence of *H. lusitanicum* from Portugal (KU130609). The five *H. rufipes* nymphs analyzed for this genetic marker belonged to three haplotypes: one (OL339472) from a greater whitethroat (*Sylvia communis*) had 100% (637/637 bp) sequence identity to a specimen from Kenya (MW243658), another (OL339468) from a sedge warbler (*Acrocephalus schoenobaenus*) had 99.8% (644/645 bp) sequence identity to a specimen from the Netherlands (MW495248), whereas three further ones (OL339477) from the same wood warbler (*P. trochilus*) had 100% (645/645 bp) sequence identity to a tick collected from Eurasian reed warbler (*Acrocephalus scirpaceus*) in the Netherlands (MT757612) and 100% (617/617 bp) sequence identity to a specimen from Israel (KY548845). The only *H. marginatum* (OL339469, from a common kingfisher, *Alcedo atthis*) analyzed in its *cox*1 gene showed 100% (645/645 bp) sequence identity to an adult tick collected in France (KX000650) and 100% (644/

Table 1
Tick species and developmental stages according to avian host species and month of collection.

Tick species or group	Number of avian hosts	Species of avian hosts (number of ticks*)**	Number of ticks	Month of collection**
Ixodes cumulatimpunctatus	1	ANT TRI (1)	1	April
Ixodes ricinus	2	ERI RUB (1)^{a} PHY TRO (1)^{b}	2	September^{b}, October^{a}
Ixodes acuminatus	2	ALA ARV (1)^{a} ANT PRA (1F)^{b}	2	October^{a}, November^{b}
Ixodes frontalis	3	ERI RUB (1+3L)^{a} PHO OCH (1)^{b}	5	October^{a}, November^{b}
Ixodes festai	1	ERI RUB (1L)	1	November
Amblyomma marmoreum complex	7	ANT TRI (2+1+1+1+1+1+1)	8	March, April, May
Hyalomma lusitanicum	1	PAS HIS (1)	1	September
Hyalomma rufipes	33	ACR ARU (5+9)^{a} ACR SCH (1+1+1+4+4+1)^{b} ACR SCI (3)^{c} CUR IBE (1)^{b} FIC ALB (1)^{a} FIC HYP (2L+4)^{b} HIR RUS (1)^{c} LAN SEN (1)^{a} MOT FLA (1)^{b} PHO PHO (2^{a}+3^{b}) PHY SIB (6^{b}+1^{a}+1^{a}+1^{a}) PHY TRO (1^{b}+3^{a}) SYL COM (1^{b}+2^{a}+1^{b}+1^{b}+1^{b}+1^{b}+7L^{b}+3^{b}+1^{b}+1^{b}) UPU EPO (1)^{c}	78	March^{c} April^{b} May^{a}
Hyalomma marginatum	5	ALC ATT (1)^{a} FAL TIN (1)^{b} SYL COM (1)^{b} PHY TRO (2)^{c} ACR SCH (2)^{b}	7	April^{b}, May^{c}, August^{a}
Hy. rufipes or *marginatum*	7	ACR SCH (1+1+2) PHY SIB (1+1) SYL COM (1+1)	8	April

*nymph, if otherwise not indicated (L = larva, F = female)
**within a row the same superscript letter indicates corresponding data of avian host species and month of collection
Abbreviations of bird species: ACR ARU = great reed warbler (*Acrocephalus arundinaceus*), ACR SCH = sedge warbler (*Acrocephalus schoenobaenus*), ACR SCI = Eurasian reed warbler (*Acrocephalus scirpaceus*), ALA ARV = Eurasian skylark (*Alauda arvensis*), ALC ATT = common kingfisher (*Alcedo atthis*) ANT PRA = meadow pipit (*Anthus pratensis*), ANT TRI = tree pipit (*Anthus trivialis*), CUR IBE = western subalpine warbler (*Curruca iberiae*), ERI RUB = European robin (*Erithacus rubecula*), FAL TIN = common kestrel (*Falco tinnunculus*), FIC ALB = collared flycatcher (*Ficedula albicollis*), FIC HYP = European pied flycatcher (*Ficedula hypoleuca*), HIR RUS = barn swallow (*Hirundo rustica*), LAN SEN = woodchat shrike (*Lanius senator*), MOT FLA = yellow wagtail (*Motacilla flava*), PAS HIS = Spanish sparrow (*Passer hispaniolensis*), PHO OCH = black redstart (*Phoenicurus ochruros*), PHO PHO = common redstart (*Phoenicurus phoenicurus*), PHY TRO = willow warbler (*Phylloscopus trochilus*), PHY SIB = wood warbler (*Phylloscopus sibilatrix*), SYL COM = common whitethroat (*Sylvia communis*), UPU EPO = Eurasian hoopoe (*Upupa epops*).

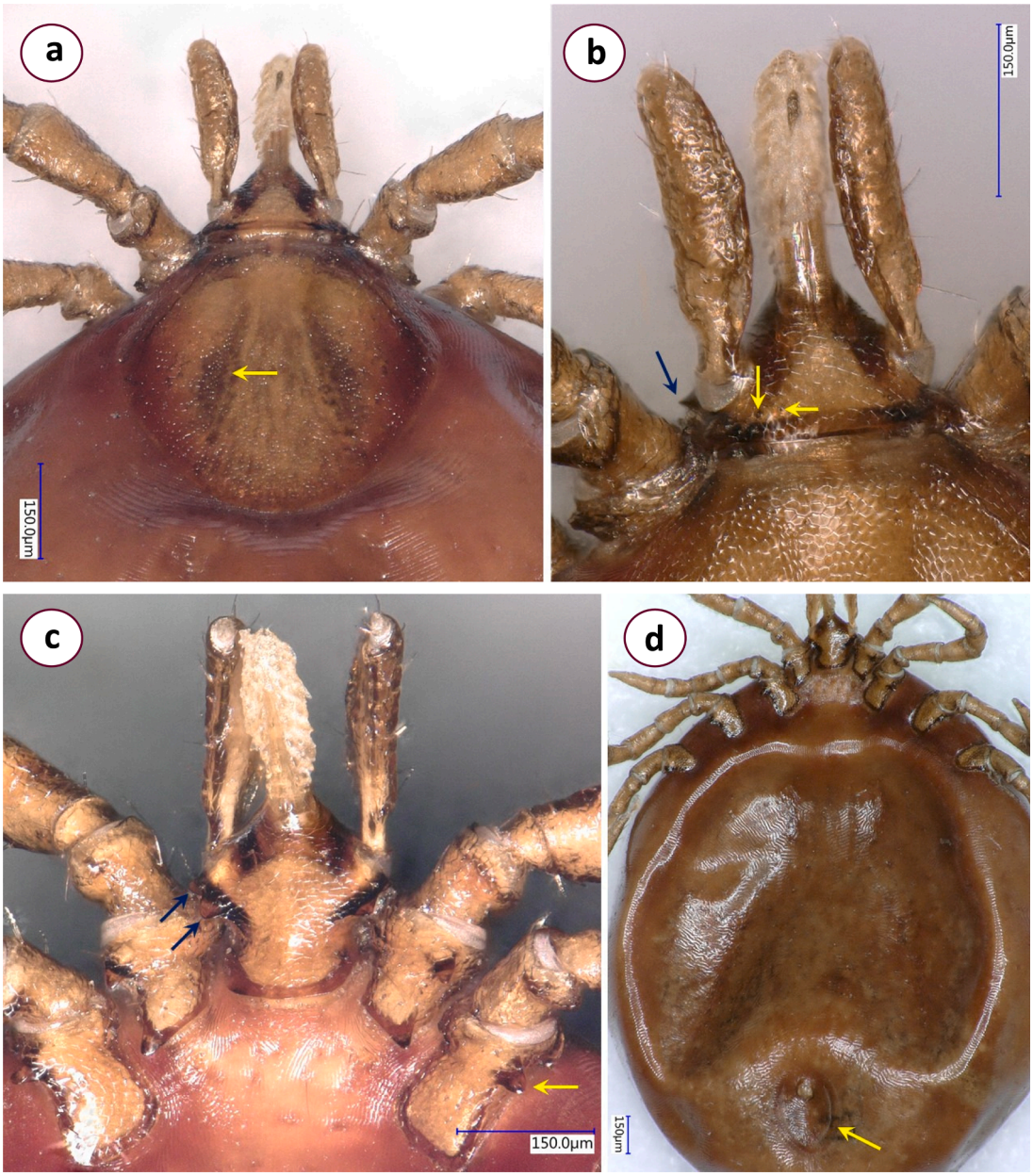

Fig. 1. Key morphologic characters of *Ixodes cumulatimpunctatus* nymph: (a) broad, heart-shaped scutum; linear cervical grooves (arrow); (b) trapezoidal basis capituli, with prominent posterolateral corners (blue arrow) and areae porosae (yellow arrows); (c) auriculae divided into anterior part and broad, caudally directed posterior part (blue arrows), with prominent external spurs on coxae I-III (yellow arrow); (d) closed, oval anal groove (arrow).

644 bp) to another from Turkey (MW366628).

The species identities and the above relationships were confirmed by the *cox*1 phylogenetic analysis (Fig. 2). Within Prostriata, subgenera *Afrixodes* and *Trichotoixodes* (including sequences of *I. cumulatimpunctatus* and *I. frontalis*, respectively) clustered as sister groups (Fig. 2).

3.3. Molecular and phylogenetic analyses of 16S rRNA gene sequences

Ixodes cumulatimpunctatus from Malta (OL352898) had 98.7% (370/375 bp) sequence identity to a conspecific tick collected from pig in the Democratic Republic of Congo (MN959774). Both *I. ricinus* nymphs collected from birds in Malta (OL352910) had 100% (390/390 bp) sequence identity with a specimen reported from Slovakia (GU074589). The *I. acuminatus* nymph (OL352911) had the highest, 99.2% (380/383 bp) sequence identity with a specimen from Spain (MH645515) and 99.7% (360/361 bp) with another from France (MH708166). The *I. acuminatus* female (OL352912) showed a higher, 99.7% (382/383 bp) and the same 99.7% (360/361 bp) sequence identities in these comparisons, respectively. Interestingly, three larvae and a nymph of *I. frontalis* had three different 16S rRNA haplotypes (OL352907-OL352909), with 99.8-100% (i.e., 401/401-402 bp) sequence identity to haplotype-B of this species from Hungary (KU170519). The only larva of *I. festai* (OL352913) had 97.9% (369/377 bp) sequence identity to *I. festai* reported from a dunnock (*Prunella modularis*) and a greenfinch (*Carduelis chloris*) in Hungary (KU170521, KU170522), while only 95.1% (329/346 bp) identity to *I. ventalloi* reported from Portugal (MG210719: questing male) and 96.1% (365/380 bp) identity to the neotype of *I. ventalloi* from Spain (KY231931).

The seven nymphs of the *A. marmoreum* complex analyzed in this context belonged to three 16S rRNA haplotypes (OL352904-OL352906) with only 2 bp difference from each other. These ticks showed up to

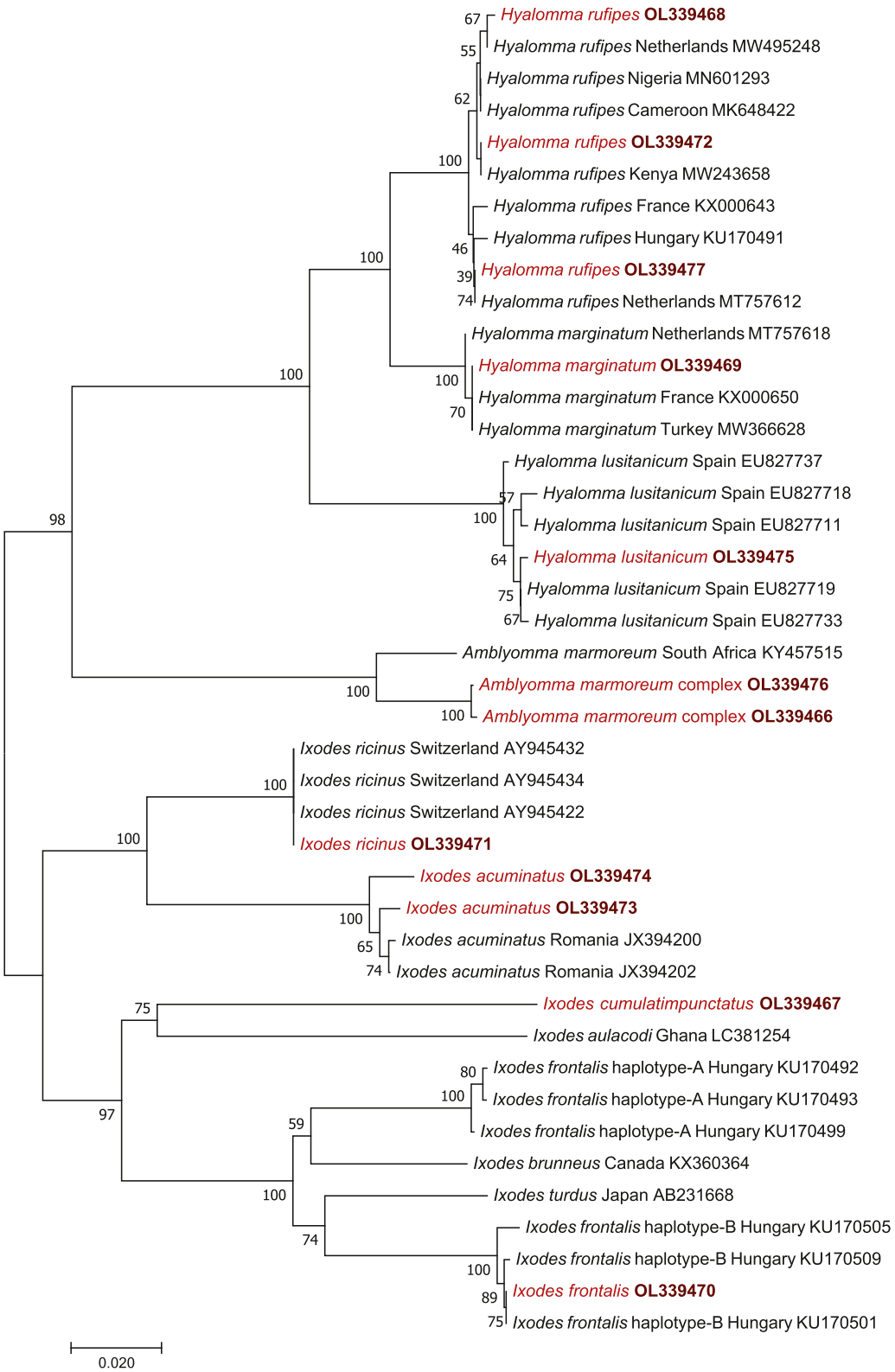

Fig. 2. Neighbor-Joining phylogenetic tree of ixodid ticks based on the *cox*1 gene sequences. In each row, after the species name, the country of origin and GenBank accession number are shown. Sequences from this study are indicated with red fonts and bold accession numbers. The scale-bar indicates the number of substitutions per site.

99.2% (395/398 bp) sequence identity to a tick reported under the name *A. marmoreum* from hinge-back tortoise (*Kinixys* sp.) (MW290507), and 93.8% to 95.2% (376/401 to 380/399 bp) identity to further sequences of *A. marmoreum* from South Africa (KY457515, KY457516). The 16S rRNA haplotype of *Hyalomma lusitanicum* (OL352914) collected in this study was 100% (403/403 bp) identical to a specimen of this species removed formerly from a rabbit in Malta (MG855659), and 99.5-99.7% (392/394 to 331/332 bp) identical to sequences of this species

(KU130444, KU130445) available from Portugal and Italy, respectively. Twenty-seven *H. rufipes* specimens had five 16S rRNA haplotypes (OL352899-OL352903), showing 99.7% (382/383 bp) to 100% (383/383 bp) sequence identities to *H. rufipes* collected from camels in Egypt (MK737649, MK737650). The *H. marginatum* nymph was 100% (384/384 bp) identical in its 16S rRNA sequence with a conspecific tick reported from Algeria (KP776645).

The phylogenetic tree of 16S rRNA sequences (Fig. 3) confirmed the above species identities. The clustering of the three *I. festai* sequences and that of *I. ventalloi* type specimen received high (100%) bootstrap support. Interpreting the phylogenetic tree in a geographical context, along the Adriatic Flyway, the results were consistent for two tick species collected from robins (*E. rubecula*) in Malta: sequences of both *I. frontalis* and *I. festai* from this study clustered with conspecific sequences reported from Hungary (Fig. 3). Haplotypes of *H. rufipes* and *H. marginatum* showed close phylogenetic relationship with conspecific haplotypes from north Africa (Egypt, Algeria), while the haplotype of *H. lusitanicum* with another from the Iberian Peninsula (Fig. 3).

3.4. Molecular analysis of 12S rRNA gene sequences

The only *Ixodes* species that yielded product in the 12S rRNA PCR was *I. frontalis*, with two haplotypes (OL352896, OL352897). These showed 99.7-100% (323/323 and 323/324 bp) sequence identity to questing nymphs of this species reported in GenBank from Portugal (MF621227, MF370635). Nymphs of the *A. marmoreum* complex (n=7 in this analysis) had identical 12S rRNA haplotype (OL352895), with 96.7% (328/339 bp) sequence identity to *A. marmoreum* reported from South Africa (KY457515). The great majority (n=48) of *H. rufipes* that were positive in the 12S rRNA PCR (OL352890) showed 100% (341/341 bp) sequence identity to a tick collected from an unreported avian host species in Italy (MW175439). In addition, there were four specimens with three divergent haplotypes (OL352891-OL352893) that had 99.7% (340/341 bp) sequence identity to the above specimen from Italy (MW175439). Finally, six *H. marginatum* nymphs yielded identical 12S rRNA sequences (OL352894), with 100% (335/335 bp) identity to *H. marginatum* reported in GenBank from Israel (KT391046).

3.5. Seasonality and host associations of ticks

All Palearctic *Ixodes* species were carried by birds in the autumn (in September, October and November), whereas *Amblyomma* and *Hyalomma* species (with the exception of a single *H. marginatum* collected in August and the only *H. lusitanicum* collected in September) were collected in the spring. Eight *Hyalomma* nymphs, which could not be unanimously assigned to *H. rufipes* or *H. marginatum*, were found in April (Table 1).

Host-associations are summarized in Table 1. All eight nymphs of the *A. marmoreum* complex were removed from tree pipits (*A. trivialis*), and the nymph of *I. cumulatimpunctatus* was also found on this bird species. Among *Hyalomma* species, *H. lusitanicum* was collected from a Spanish sparrow (*P. hispaniolensis*), whereas *H. rufipes* and *H. marginatum* from 14 and five bird species, respectively (Table 1).

Excluding those ticks which could only be identified to the genus level (as belonging to either *H. rufipes* or *H. marginatum*), co-infections of birds with two species were found in three cases. The nymph of *I. cumulatimpunctatus* and a nymph of the *A. marmoreum* complex occurred on the same tree pipit (*A. trivialis*) sampled in April. In addition, *H. rufipes* infested birds simultaneously with *H. marginatum* on two occasions in April (seven larvae and three nymphs of *H. rufipes* with a single nymph of *H. marginatum* on a common whitethroat, *S. communis*; or a single nymph of *H. rufipes* with two nymphs of *H. marginatum* on a sedge warbler, *A. schoenobaenus*).

4. Discussion

To the best of our knowledge, this is the first molecular analysis of ticks from birds in Malta. It is important to note in advance that in case of the Afrotropical tick species identified on the species or group level in this study, both (1) morphological and (2) molecular characters were used. In particular, the *I. cumulatimpunctatus* nymph was identified on the species level, because (1) its morphological characters were consistent with those in its original description (Schulze, 1943: except for the apparent lack of small internal spur on coxa IV), and (2) it had up to 98.7% 16S rRNA sequence identity to adults of this species (Ngoy et al., 2021). Nymphs of the *A. marmoreum* complex collected in this study were conspecific based on the minor difference between their *cox*1 and 16S rRNA gene sequences. However, their species could not be identified with certainty due to the following reasons: (1) the scutum had a punctuation pattern similar to *A. marmoreum, A. sparsum* and *A. nuttalli*, but its shape (breadth to length ratio: 1.27) was closest to that of the latter species (1.14, 1.22 and 1.29, respectively: Theiler and Salisbury, 1959). (2) Molecularly, nymphs of the *A. marmoreum* complex in this study had up to 99.2% 16S rRNA gene sequence identity to a tick (MW290507) reported under the name *A. marmoreum* by Mofokeng et al. (2021). It is noteworthy that in the latter study the pictured female tick shown as *A. marmoreum* resembles *A. nuttalli* based on its enamel pattern (Theiler and Salisbury, 1959; Horak et al., 2018). At the same time, the sequence identity of *Amblyomma* nymphs in this study was lower (91.9-95.9% in the *cox*1 and 93.8-95.2% in the 16S rRNA gene) in comparison with *A. marmoreum* (KY457515, KY457516) reported by Mans et al. (2019). Therefore, nymphs found on birds in Malta probably belong to *A. nuttalli*, but in the absence of available GenBank sequence of this species we cannot confirm it molecularly.

All three genetic markers analyzed in this study (the *cox*1, 16S and 12S rRNA genes) are important targets of PCRs used for barcoding and species identification of ixodid ticks (Lv et al., 2014). Concerning the results of molecular-phylogenetic analyses, sequence data of the 16S rRNA gene proved to be the most useful, in part because the *cox*1 PCR did not yield product with several specimens during a preliminary trial (e.g., for *I. festai*, consistently with Hornok et al., 2016). However, in order to identify *Hyalomma* specimens on the species level, the 12S rRNA test was chosen on account of its suitability to distinguish between *H. rufipes* and *H. marginatum* (Beati and Keirans, 2001; Roth et al., 2019). The ratio between these two species (i.e., higher proportion of *H. rufipes* than *H. marginatum*) was similar here (78 *H. rufipes* vs 7 *H. marginatum*) to what was reported from three Italian islands 700 km north of Malta (409 *H. rufipes* vs 93 *H. marginatum*: Toma et al., 2021).

Compared to bird tick studies reported from Central and Western Europe in general, the estimated frequency (prevalence: 19 637 birds examined, 57 found tick-infested) and the maximum level (intensity) of tick infestation was low among birds in Malta. In particular, during the study period, 113 hard ticks were collected. The maximum number of ticks removed from the same bird was 11, and the mean intensity of tick infestation was low (2 ticks per bird) in comparison with other studies (e.g., up to 20 ticks/bird in Hornok et al., 2016). The majority of life cycle stages were nymphs, but 13 larvae and one female were also found. The predominance of nymphs in the study material is in line with the results of a recent study from three Italian islands approx. 700 km north of Malta (Toma et al., 2021), but in contrast to the predominance of larvae more northward along the Central European (Adriatic) Flyway in Hungary (Hornok et al., 2016). On the other hand, ticks removed form birds in Malta represented nine species, thus the species diversity was higher than in the significantly larger material (n=3339 ticks, but only five species) reported in Central Europe, Hungary (Hornok et al., 2016). Considering tick species, this is the first finding of *I. cumulatimpunctatus* in Europe, and at the same time its northernmost record. Further four species (*I. cumulatimpunctatus, I. acuminatus*, one member of the *A. marmoreum* complex and *I. festai*) were found in this study for the first time in Malta (see Hornok et al., 2020).

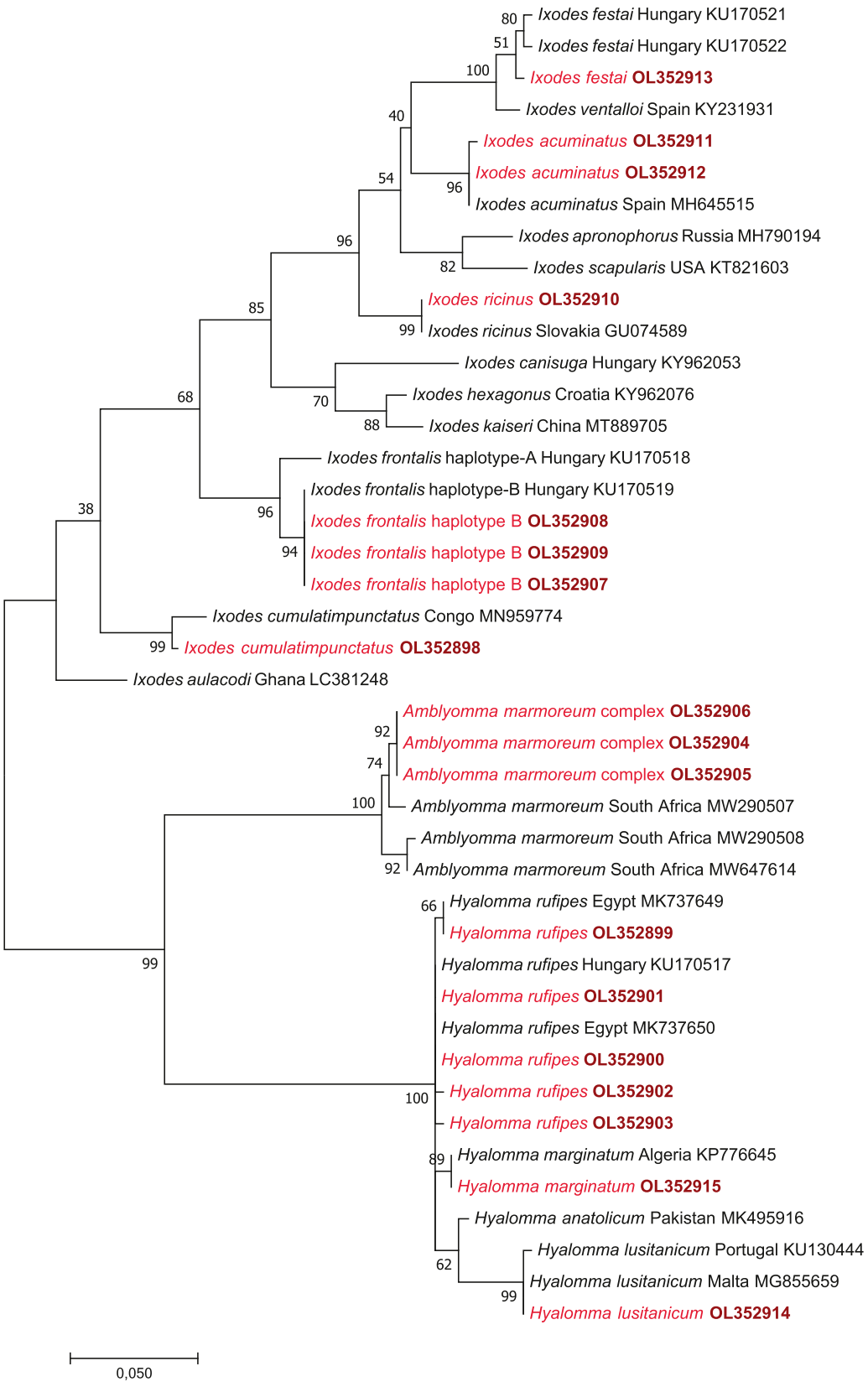

Fig. 3. Maximum Likelihood phylogenetic tree of ixodid ticks based on the 16S rRNA gene sequences. In each row, after the species name, the country of origin and GenBank accession number are shown. Sequences from this study are indicated with red fonts and bold accession numbers. The scale-bar indicates the number of substitutions per site.

Regarding those Palearctic genera of Ixodidae which were not collected in this study, it is not surprising that no *Dermacentor* specimens were found on birds in Malta, because (a) even north of the Mediterranean Basin these very seldom infest avian hosts (e.g., Hasle et al., 2009), and (b) south of Malta, in the Afrotropical geographic zone only two *Dermacentor* species occur and these typically infest large mammals (Keirans, 1993). While *Rhipicephalus sanguineus* sensu stricto is indigenous in Malta (Licari et al., 2017), the most likely reason for the absence of this species on birds of this study is that it very rarely associates with avian hosts (Szabó et al., 2012). On the contrary, *Haemaphysalis* species are common on birds along the Adriatic Flyway north of the Mediterranean Basin (e.g., Hornok et al., 2016), but they were not found among ticks collected during this study in Malta. This may indicate that the northward carriage of African *Haemaphysalis* species occurs less frequently in comparison with *Amblyomma* and *Hyalomma* species, most likely due to host preference either for mammals or for resident birds instead of migratory passerines (as exemplified by *H. leachi* and *H. hoodi*: Sylla et al., 2018).

In this study, 22 bird species were found tick-infested. Taking into account the routes of their migration, discounting six resident or short-distance migrant bird species, the majority of involved species (n=16) are long-distance migrants (Supplementary Text 1). Thus, it can be expected that all bird species of the latter category may bring immature ticks from Sub-Saharan Africa to Europe annually during spring migration.

Regarding seasonality, all Palearctic *Ixodes* species were carried by birds in Malta exclusively in the autumn (i.e., when migratory birds are heading southwards to their wintering grounds in Africa), whereas *H. rufipes* (with predominantly Afrotropical distribution) was exclusively collected in the spring (i.e., when migratory birds are heading northwards from their wintering grounds in Africa). During springtime two further tick species were identified on migratory birds here: a member of the *A. marmoreum* complex and *I. cumulatimpunctatus*. The latter species inhabits the Afrotropical zoogeographic region, including Western, Central, Eastern Africa (Ivory Coast, Congo, Rwanda, Uganda, Tanzania: Matthysse and Colbo, 1987; Guglielmone and Robbins, 2018; Ngoy et al., 2021). Species of the *A. marmoreum* group are also exclusively indigenous in the Afrotropical zoogeographic region (Guglielmone et al., 2014; Horak et al., 2018). For instance, *A. marmoreum sensu stricto* occurs only in regions of Southern Africa (Horak et al., 2006), but the geographical range of *A. nuttalli* extends towards the north and it shows a wider distribution including Western, Central and Eastern Africa (Theiler and Salisbury, 1959; Matthysse and Colbo, 1987). Interestingly, the occasional introduction of ticks from the *A. marmoreum* complex into the Palearctic region has long been known (Santos Dias, 1989), but these were not regarded as potentially invasive, capable of establishment in Europe (Guglielmone et al., 2014). Nevertheless, as shown here, ticks of the *Amblyomma marmoreum* group arrive in Europe more frequently in Malta than in Italy (Battisti et al., 2020).

Considering the results of morphological and molecular analyses, it is interesting to note that while *I. frontalis* and *I. cumulatimpunctatus* belong to different subgenera (*Trichotoixodes* and *Afrixodes*, respectively), they share important morphological features in the nymphal stage as observed here (divided auriculae, prominent external coxal spurs) and they showed close phylogenetic clustering in both *cox*1 and 16S rRNA phylogenetic analyses.

The larva identified here as *I. festai* was morphologically also similar to larvae of *I. ventalloi* described in other studies, based on the sharp internal spur on coxae I (*I. festai*: Arthur, 1963; Arthur, 1965; Senevet and Rodhain, 1968; *I. ventalloi*: Estrada-Peña et al., 2018). However, the teeth on the hypostome of the larva in this study were posterolaterally directed (leaving gap between the individual teeth) and the dental formula was 3/3 only apically (as in Arthur, 1963, 1965; Senevet and Rodhain, 1968), unlike in the case of *I. ventalloi* with 3/3 dental formula to mid-length and caudally directed teeth without gap between them (Estrada-Peña et al., 2018). Morphological differentiation was confirmed by sequence comparisons and phylogenetic analysis of the 16S rRNA gene, according to which the larva collected from a robin in Malta was much more closely related to *I. festai* females identified previously on birds in Hungary than to *I. ventalloi*. Females of these two species can be distinguished more easily based on the caudally directed auriculae and straight internal spurs on coxae I of *I. festai* (Contini et al., 2011; Hornok et al., 2016), unlike in *I. ventalloi* with medially curved auriculae and laterally curved internal spurs on coxae I (Latrofa et al., 2017; Estrada-Peña et al., 2018). Last but not least, the host preference of these two species appears to be quite different: *I. ventalloi* prefers lagomorphs, carnivores and rodents as hosts, and is occasionally reported from galliform birds (Estrada-Peña et al., 2018), whereas *I. festai* is a bird-associated tick species (Contini et al., 2011) frequently reported from passerines (Arthur, 1965; Papadopoulos et al., 2001; Hornok et al., 2016).

Thus, interspecific morphological similarities between *I. festai* and *I. ventalloi* and molecular differences within *I. ventalloi* collected from birds as reported (Toma et al., 2021) necessitate further studies to clarify the taxonomic status of these two species. Similarly, intraspecific sequence heterogeneity within the *A. marmoreum* complex and in the case of *I. cumulatimpunctatus*, as shown here between the specimen(s) from Malta and those already reported in Sub-Saharan Africa, justifies further morphological-molecular analyses to investigate the taxonomy of their groups.

Both *I. acuminatus* specimens of this study showed sequence divergence from ticks reported outside the Central European (Adriatic) Flyway (98.4–99.4% identity in the *cox*1 gene to conspecific ticks reported in Romania, and 99.2–99.7% identity in the 16S rRNA gene to specimens collected in Spain, France). Since *I. acuminatus* is a tick species almost exclusively associated with small to medium-sized mammals (Estrada-Peña et al., 2017), i.e., ground-bound hosts, this may indicate a certain degree of latitudinal genetic isolation of its populations across Europe, with only sporadic events of gene flow with avian hosts in a longitudinal direction.

On the contrary, sequence homogeneity of *H. rufipes* and *H. marginatum* within a broad geographical range (encompassing Western, Northern, Central and Southern Europe, as well as North Africa and the Middle East: Israel, Turkey) reflect that their immatures are frequently associated with birds, ensuring gene flow within and, via common wintering ground, across different flyways passing through the Mediterranean Sea.

New tick-avian host associations recognized in this study include the first finding of *I. cumulatimpunctatus* on tree pipit (*A. trivialis*), *I. acuminatus* on Eurasian skylark (*A. arvensis*) and meadow pipit (*A. pratensis*), *I. festai* on robin (*E. rubecula*), *H. lusitanicum* on Spanish sparrow (*P. hispaniolensis*) and *H. rufipes* on swallow (*H. rustica*).

Ticks with Afrotropical distribution, i.e., the nymph of *I. cumulatimpunctatus* and all nymphs of the *A. marmoreum* complex were collected from tree pipits (*A. trivialis*) in Malta. This bird species is known to breed in the whole Palearctic, while its wintering grounds are primarily located in the savanna-belt of Western, Central and Eastern Africa (O'Connor et al., 2018). They migrate on a broad front, thus tree pipits sampled in Malta along the Adriatic Flyway most likely departed from Western Africa, where both *I. cumulatimpunctatus* and *A. nuttalli* occur (see above). The single nymph of *I. cumulatimpunctatus* was shown here, to the best of our knowledge, for the first time to infest any songbird species in the order Passeriformes and in particular in the family Motacillidae (hitherto reported only from cuculiform and galliform birds: Guglielmone et al., 2014). The exact duration of feeding has not been reported for developmental stages of *I. cumulatimpunctatus*, but the tick in this study was collected in Malta early in April, and its host, the tree pipit (*A. trivialis*) is known to arrive from Africa in the east Mediterranean within half month (Zduniak and Yosef, 2011). This may be sufficient for the transportation of this tick species to the Mediterranean region (as shown here) on rare occasions even from Sub-Saharan Africa where it is indigenous (Arthur, 1965). Or, as an alternative

explanation, there may be not yet discovered populations of this tick species north of the Sahara. Recently, a single specimen of *A. marmoreum* has been reported from a migratory bird (the same species as in this study: tree pipit, *A. trivialis*) in Italy (Battisti et al., 2020). In this study, all nymphs of the *A. marmoreum* complex were removed from tree pipits (*Anthus trivialis*), meaning a strong association with this avian host species. Therefore, based on the present results, the tree pipit appears to be a relatively frequent importer of the relevant tick species from Africa into Europe in the mid-Mediterranean region, unlike in the east-Mediterranean region (where *Hyalomma* species are most frequently recorded from tree pipits: Hoogstraal et al., 1963). It is noteworthy that although *A. marmoreum* is a three-host tick species, its nymph stage feeds for around 47 days (Horak et al., 2018) and this may allow its long-distance transportation by avian hosts in Africa towards the north (as it was reported in Italy by Battisti et al., 2020). On the other hand, while *A. nuttalli* is also a three-host tick species, its nymphs only feed for 7−10 days (Horak et al., 2018). However, this may still suffice for its arrival into the Mediterranean region, since *A. nuttalli* was found on thrush nightingale (*Luscinia luscinia*) in Cyprus (Kaiser et al., 1974).

Interestingly, the willow warbler (*Phylloscopus trochilus*) was already reported as the (only) host of *I. ricinus* in Malta (Sultana and Gauci, 1977), in line with the present findings, but both bird species identified here as hosts of *I. frontalis* (the robin, *E. rubecula*, and the black redstart, *P. ochruros*) are new in the context of Malta (since a single specimen of this tick species was reported under the name *Ixodes pari* from an Eurasian blackcap, *Sylvia atricapilla*: Sultana and Gauci, 1977). The majority of (four out of five) *I. frontalis* was collected from robins in Malta, similarly to what was reported in Hungary (Hornok et al., 2016).

Importantly, the same haplotype of *H. lusitanicum* reported previously in Malta from a rabbit (Hornok et al., 2020) was identified here from a Spanish sparrow (*P. hispaniolensis*). This bird species has a distribution range including the Western Mediterranean (Eroukhmanoff et al., 2013) where this tick species is indigenous (Estrada-Peña et al., 2017). Taking into account that the bird-associated nymph collected in Malta was genetically closely related to the aboriginal population of this tick species in the Iberian Peninsula, and the Spanish sparrow (although formerly thought to be a resident bird species: Supplementary Text 1) was observed to migrate through Malta (Sultana, 2009), these data suggest that *H. lusitanicum* may have arrived in Malta from Southwestern Europe.

All ticks carried by birds in Malta, identified here on the species or group level, are known to infest humans, either rarely/sporadically (*I. acuminatus, I. cumulatimpunctatus, I. festai, I. frontalis*, species in the *A. marmoreum* complex including *A. marmoreum* and *A. nuttalli, H. lusitanicum, H. rufipes*) or often (*I. ricinus, H. marginatum*) (Guglielmone and Robbins, 2018).

5. Conclusions

According to results of the present study, collection of ticks from birds in Malta and their identification on morphological and molecular-phylogenetic bases may provide first-hand data on what tick species may arrive along the Central European (or Adriatic) Flyway from Africa into Europe. This highlights the importance of continuously monitoring this situation, especially in the era of global warming which also affects Europe and as a result the emergence of new tick species and tick-borne pathogens is expected in the near future.

CRediT authorship contribution statement

Sándor Hornok: Conceptualization, Supervision, Methodology, Writing – review & editing. **Bernard Cutajar:** Methodology, Data curation, Writing – review & editing. **Nóra Takács:** Methodology, Visualization, Investigation. **Nicholas Galea:** Data curation, Investigation. **David Attard:** Data curation, Investigation. **Charles Coleiro:** Data curation, Investigation. **Raymond Galea:** Data curation, Investigation. **Gergő Keve:** Methodology, Software, Conceptualization, Writing – review & editing. **Attila D. Sándor:** Methodology, Software, Conceptualization, Writing – review & editing. **Jenő Kontschán:** Methodology, Software, Conceptualization, Writing – review & editing.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ttbdis.2022.102001.

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