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DNA barcoding of tick species (Arachnida: Ixodida) to support species identification and discovery of cryptic genetic diversity with emphasis on the British fauna

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Abstract

Ticks are of medical and veterinary concern due to their role as vectors of a wide range of pathogens, including viruses, protozoa, and bacteria. Accurate species identification is essential during tick surveillance and disease control programmes, as certain pathogens are associated with particular tick species. In this study, genetic variation of a partial sequence of the cytochrome *c* oxidase I (*COI*) gene was used for molecular identification of tick species to corroborate morphological identification. This also enabled investigation of cryptic diversity within tick species. Thirty species belonging to the genera *Amblyomma*, *Argas*, *Carios*, *Dermacentor*, *Haemaphysalis*, *Hyalomma*, *Ixodes*, *Ornithodoros*, and *Rhipicephalu*s were assessed. Tree-topology analysis confirmed discrete clustering of specimens according to species for the majority of taxa. Intraspecific genetic divergence ranged from 0 to 6.08%. In taxa where species complexes are known, separation of discrete groups were found. Several species yielded >2% intraspecific genetic divergence when compared to other taxa, suggesting potential cryptic diversity. DNA barcoding was an effective approach for the morphological identification of UK and non-native ticks, and for the detection of cryptic diversity within species.

Keywords

COI DNA barcoding – ticks – tick surveillance – cryptic genetic diversity

1 Introduction

Ticks are ectoparasites of which there are 17 genera and approximately 896 species worldwide, distributed in the three families Argasidae (soft ticks), Ixodidae (hard ticks) and Nuttalliellidae (Guglielmone *et al.*, 2010; Horak *et al.*, 2002). They transmit a range of pathogens to vertebrate species including humans and domestic animals globally, therefore they are of medical and veterinary significance and can be a considerable economic burden to the livestock industry. In Europe, examples of pathogens transmitted by ticks include bacteria, such as *Borrelia burgdorferi* Burgdorfer *sensu lato* (*s.l.*), *Anaplasma phagocytophilum* Gordon, and *Rickettsia conorii* Brumpt, piroplasms such as *Babesia divergens* M'Fadyean & Stockman and *Theileria equi* Laveran, and viruses such as tick-borne encephalitis virus (TBEV) and Crimean-Congo hemorrhagic fever virus (CCHFV) (Moraga-Fernández *et al.*, 2023). Due to their role as vectors of numerous pathogens, ticks are one of the main target vector groups within surveillance and control programmes (Cull et al. 2020; Eisen and Paddock, 2021; Jameson and Medlock, 2011; Johnson *et al.,* 2022; Kooyman *et al.*, 2022). All rely on correct identification of the tick species (Mathison and Pritt, 2014). Within the UK, in terms of tick-borne pathogen circulation, louping ill virus (LIV), *B. burgdorferi s.l.* and *A. phagocytophilum*, along with species of piroplasms (e.g. *Babesia*, *Theileria*), can cause infections in livestock. More recently, TBEV has been reported in England from *Ixodes ricinus* Linnaeus (Holding *et al.*, 2019, 2020a,b).

Traditional approaches to tick species identification rely heavily upon morphology-based procedures, which typically require specialised training and may not always provide an accurate resolution of a specimen's identity. This is influenced by the homogeneity between life stages of different species, the preservation state of specimens collected, and the presence of species complexes (Cywinska *et al.*, 2006; Estrada-Peña *et al.*, 2017; Hernández-Triana *et al.*, 2012, 2014, 2015; Packer *et al.*, 2009). Therefore, in order to improve the accuracy of species identification and address this taxonomic impediment, Hebert *et al.* (2003a,b) proposed that a fragment of the mitochondrial cytochrome *c* oxidase unit I (*COI*) gene should be used as a standardised DNA marker in support of species identification.

In the United Kingdom (UK), there are 20 tick species which are considered indigenous (Johnson *et al.*, 2022; Medlock *et al.*, 2009). Some, such as *Ix. ricinus* feed on a range of hosts and vector many pathogens (Gray *et al.*, 2021), thus they are often the focus of surveillance. Others have intimate associations with certain wildlife species, are rarely encountered or are of less significance. Non-native species are occasionally detected in the UK, for example *Amblyomma clypeolatum* Neumann, *Hyalomma aegyptium* Linnaeus, *Hyalomma marginatum* Koch, and the species complex *Rhipicephalus sanguineus* (Hansford *et al.*, 2018; Jameson and Medlock, 2011; McGinley *et al.*, 2020), although there is no evidence that any have established breeding populations. An adult male of another non-native species, *Hyalomma rufipes* Koch, was detected on a horse that had never travelled outside the UK suggesting that an immature larva or nymph of this species may have successfully moulted (Hansford *et al.*, 2019). More recently, an adult *Amblyomma* cf. *marmoreum* Koch/ *Amblyomma* cf. *sparsum* Neumann was found on an imported leopard tortoise (*Stigmochelys pardalis* Bell) (Phipps *et al.*, 2021).

Genetic markers such as 16S RNA, 12S rRNA and 18S rRNA genes, and more recently the *COI* gene, have been used for the molecular characterisation of tick species worldwide (e.g. Beard *et al.*, 2021; Dantas-Torres *et al.*, 2024; Földvári *et al.*, 2022; Grech-Angelini *et al.*, 2016; Hornock *et al.*, 2015, 2017, 2024; Kelava *et al.*, 2021; Takano *et al.*, 2023). In the UK, Gillingham *et al.* (2020a) applied *COI* DNA barcoding methodology to characterise the tick fauna in the Channel Islands, while other authors have employed this methodology for the molecular identification of tick species such as *Carios vespertilionis* Latreille (Lv *et al.*, 2018), *Dermacentor reticulatus* Fabricius (de Marco *et al.*, 2017), *Hy. rufipes* (Hansford *et al.*, 2019) and *Hy. marginatum* (McGinley *et al.*, 2020). However, data on all British tick fauna is not complete.

In this study, we developed a molecular library based upon the combination of *COI* DNA barcoding methodology and morphological traits to support species identification of both native and non-native species detected in the UK. The dataset generated also supported further analysis of DNA barcode variability using tree-topology methodologies to visualise taxa groupings, which could potentially reveal cryptic diversity.

2 Materials and methods

Collection and identification of specimens

Ticks were collected from different locations in the UK using standard methods for ticks surveillance, including flagging, or manual removal from the vertebrate host (www.gov.uk/guidance/tick-surveillance-scheme). Full information for each specimen and location can be found in the publicly available dataset project (DS-TICKUK) in BOLD (Barcoding of Life database).

Specimens collected were stored in 96% ethanol and retained chilled until processing. Morphological identification of UK species was carried out by expert identification and using the identification keys of Hillyard (1996) and Estrada-Peña *et al.* (2017). For non-native ticks, expert identification and inter-examiner agreement was applied to these specimens.

DNA extraction methods

DNA was extracted from all UK tick specimens by performing a modified hotshot technique (Montero-Pau *et al.*, 2008). In brief, 1–2 tick legs were placed directly into 50 µl of alkaline lysis buffer (NaOH and EDTA) in a 96-well plate, which was then sonicated in a water bath for 10 min. The plate was subsequently incubated in a thermocycler for 30 min at 94 °C, cooled for 5 min at 4 °C, after which 50 µl of neutralising buffer (Tris-HCL) was added to each well; the plate was then stored at –80 °C until further analysis. Exotic ticks were processed by homogenising whole specimens and then DNA extracted using QIAgen DNeasy Blood and Tissue kits (QIAgen, Manchester, UK) following manufacturer recommendations. The DNA of sixty-nine specimens from Spain was extracted using the ammonium hydroxide (NH_4OH) method as described by Ammazzalorso *et al.* (2015).

COI DNA barcoding region amplification

For molecular species identification using the *COI* DNA barcoding region, the protocols of Hernández-Triana *et al.* (2012, 2014) and Hebert *et al.* (2003a,b) were followed. Briefly, previously published primers LCO (forward) and HCO (reverse) (Folmer *et al.*, 1994) were used to amplify a 658 base pairs (bp) target region of the *COI* gene. PCR amplicons were obtained using cycling parameters as previously described by Hernández-Triana *et al.* (2017). All PCR products were visualised using SYBR™ Safe on a 1.5% agarose gel, and samples showing bands of the correct size were sequenced in both directions using the ABI PRISM® BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). All sequences were assigned to a particular tick species when identity was ≥98% with sequences of named species in GenBank at the National Centre for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov). Samples giving negative PCR results and/or no sequence data were not investigated further and were excluded from analysis.

Data analysis

Paired bi-directional sequence traces were aligned using the Sequencer (v4.5; Genes Codes Corporation, Ann Harbour, MI), GenDoc (v2.6.02) and ClustalX sequence analysis programmes to produce a single 658 bp consensus barcode sequence. Full details for each tick specimen and associated sequence information including accession numbers can be found in the Barcoding of Life Database (BOLD, www.v4.boldsystems.org) within the 'Human Pathogens and Zoonoses Initiative', Working Group 1.4 (accessed at http://dx.doi.org/10.5883/DS-TICKUK). Accession numbers for all sequences were obtained from NCBI (accession numbers: PPO47756-PPO48583; MW288388-MW288389; OR1399899-OR139965). For certain species, *COI* DNA barcode sequences freely available at NCBI and/or BOLD were used due to limitations of our own sequences from UK tick populations (see Supplementary Table S1 for accession numbers). Potential misidentifications in databases were addressed by ensuring that specimens were identified by a taxonomic expert and cross-checking sequence similarity using BLAST application in the NCBI and sequence similarity. Full nucleotide sequences were initially analysed in MEGA v6 (Tamura, 2007).

Maximum likelihood (ML) and Bayesian maximum clade credibility (MCC) methods were employed for visualisation of the different groupings between specimens across the assessed taxa. The ML tree was reconstructed using IQ-TREE v2.0.7 (Minh *et al.,* 2020) with 10,000 bootstrap approximations. The UFBoot2 programme was implemented within IQ-TREE v2.0.7 to evaluate branch support (Hoang *et al.*, 2018), while the ModelFinder option was included in the script to select the best fitting nucleotide substitution model (Kalyaanamoorthy *et al.*, 2017). The standard parameters in BEAST algorithm v1.7.5 (https://beast.community/) were used to construct the MCC tree from previously aligned sequences, following selection of HKY substitution and strict clock model and Yule process for speciation (Tree prior) to generate BEAST analysis file (.xml) in BEAUti (v1.7.5). Bayesian analysis was constructed using the .xml file generated, while the Beast log file was imported in Tracer 9 (v1.8) to confirm effective sample size (ESS) values (≥ 200) and convergence to the stationary distribution (presence of a horizontal plot). A final MCC tree was then generated in TreeAnnotator v1.7.5 (https://beast.community/). In both programmes, nucleotide sequence of the red mite (*Dermanyssus gallinae* De Geer) was used as an outgroup. All trees constructed were visualised and annotated in FigTree v1.4.4 (https://github.com/rambaut/figtree/). In addition, for DNA barcode sequences greater than 400 bp, a Barcode Index Number (BIN) was assigned, and each BIN was mapped according to specimens per species. The taxonomic discordance in the dataset was analysed using BOLD functionalities (Hernández-Triana *et al.*, 2017).

In order to determine whether divergence levels above 2% constitute one or more species, species delimitation calculations were performed. The inter-group (between groups) and intra-group (within groups) diversity was calculated using two methods, uncorrected *p*-distance (proportion of difference) and maximum composite likelihood model using MEGA11 (Tamura *et al.*, 2021). Both models accounted for both transitions and transversions. The K/θ ratio method was used to delimitate species within the groupings (Birky, 2013).

3 Results

Genetic diversity

In total, we analysed in MEGA 999 *COI* DNA barcode sequences for 30 species belonging to the genera *Argas, Carios*, *Amblyomma, Dermacentor, Haemaphysalis, Hyalomma, Ixodes*, and *Rhipicephalus* (Table 1). We were not able to analyse sequences of *Ixodes caledonicus* Nuttal, *Ixodes unicavatus* Neumann, and *Ixodes rothschildi* Nuttall and Warburton. Within these species, we also analysed selected non-native taxa that are currently considered of veterinary importance because they are expanding in distribution range worldwide or have been submitted by the Tick Surveillance Scheme carried out by the United Kingdom Health Security Agency (UKHSA) (Table 1).

The differentiation of tick species for all specimens showed a similar configuration in both ML and MCC trees, therefore only the collapsed MCC tree is shown (see Figure 1 collapsed, Supplementary Figure S1 uncollapsed ML tree, Supplementary Figure S2 uncollapsed MCC tree). For most species, individuals of the same species clustered together, although this was not the case for all taxa. Within the genus *Argas*, the first incongruence was found in *Argas reflexus* Fabricius where one specimen from Spain (voucher number/sample id B01-G779B) did not group with the other three specimens (voucher number/sample id B45-B5-1, A01-G779A, B44-B4-1) (Supplementary Figures S2 and S3). The same was observed for the species complex *Rh. sanguineus*, where *COI* sequences separated geographically in three distinct groups: Antigua and Barbuda, Grenada and Mexico, Greece and Spain. Similarly, sequences from *Ixodes vespertilionis* Koch differentiated geographically into three discrete groups from Spain, Slovenia, Montenegro and Serbia. Two species were grouped in the same clade

TABLE 1 List of tick species (in alphabetical order), average genetic diversity, country of collection, number of specimens with DNA barcodes, and BOLD BIN number.[1]

Species	Average intraspecific genetic diversity (%)	Country	*N*	BOLD BIN
Amblyomma americanum	0.64	Mexico, Pánama	4	BOLD:AAU2925
Argas persicus	0	Mexico	11	BOLD:ACG2419
Argas reflexus	0.20	Spain	5	n/a
Carios vespertilionis	0.03	United Kingdom, Spain	10	BOLD:ADG8369
Dermacentor marginatus	0.35	Spain	13	BOLD:AAL1447
Dermacentor reticulatus	0.05	Germany, Czechia, United Kingdom	95	BOLD:AAL1453
Haemaphysalis inermis	0.55	Croatia, Spain	5	BOLD: ACH7916
Haemaphysalis longicornis	1.31	Korea	11	n/a
Haemaphysalis punctata	0.14	Spain, United Kingdom	208	BOLD:ABX1743
Hyalomma aegyptium	2.04	Israel, Romania	9	BOLD:AAX2350
Hyalomma lusitanicum	0.43	Israel, Romania	5	BOLD:ACH4580
Hyalomma marginatum	0.24	Spain	15	n/a
Hyalomma rufipes	1.37	France, Cameroon, Malta, Iraq, Kenya, the Netherlands, South Africa, United Kingdom	12	BOLD: AAB4005
Ixodes acuminatus	0.66	Malta, Spain	12	BOLD:ACI1693

TABLE 1 List of tick species (in alphabetical order) (*cont.*)

Species	Average intraspecific genetic diversity (%)	Country	*N*	BOLD BIN
Ixodes* cf. *affinis***	5.28	Panamá	3	BOLD:ABY3619 BOLD:ADB0645
Ixodes apronophorus	0.76	Russia	14	BOLD:AEB6832
Ixodes arboricola	0.20	Spain, United Kingdom	9	BOLD:ADK8663
Ixodes canisuga	0.93	Spain, United Kingdom	42	BOLD:ABX1801
*Ixodes frontalis****	2.90	United Kingdom	49	BOLD:ADJ8108
Ixodes hexagonus	0.67	United Kingdom	68	n/a
Ixodes lividus	0	United Kingdom	2	n/a
Ixodes ricinus	0.11	United Kingdom	213	BOLD:AAZ2224
Ixodes trianguliceps	0.06	United Kingdom	5	BOLD:ADK9162
Ixodes uriae	0.71	Norway	6	BOLD:AAI7939
*Ixodes ventalloi****	2.75	Italy, United Kingdom	26	BOLD:ADX9940
Ixodes vespertilionis,**,****	6.08	France, Spain, Slovenia, Montenegro, Serbia	41	BOLD:ACV7519, BOLD:AEV4890, BOLD:ADK7202, BOLD:ACH9349
Ornithodoros maritimus	0.25	France	2	n/a
Rhipicephalus microplus	0.08	Mexico	8	BOLD:AAV0824
Rhipicephalus turanicus	0.67	China, Iran, Israel	12	BOLD:ACI089
Rhipicephalus sanguineus* species complex (as *Rh. linnaei* and *Rh. sanguineus senso stricto*), **, ***	5.63	Antigua and Barbuda, Grenada, Greece, Mexico, Spain	84	BOLD:AAI0467 [*Rh. linnaei* – Antigua and Barbuda, Grenada, Mexico]; BOLD:ACI0511 [*Rh. sanguineus senso stricto* Greece]; BOLD:AAU2924 [*Rh. sanguineus senso stricto* Spain]

[1] Mean intraspecific values of sequence divergence (Kimura2-Parameter distance) are shown in percentage (%). Species complexes (*), taxa with deep divergence (**) in the Bayesian maximum clade credibility and Maximum Likelihood trees, and taxa with well above 2% genetic divergence (***) are marked with asterisks; non-native species to the UK are highlighted in bold; (n/a) entry without assigned BIN number in BOLD.

within the collapsed MCC tree, *Ixodes apronophorus* Schulze and *Ixodes acuminatus* Neumann (Figure 1); nonetheless, specimens of these species showed a more discrete separation in the non-collapsed ML and MCC tree (Supplementary Figures S1 and S2).

The levels of sequence divergence were variable across the taxa, with conspecific individuals collected from a single site often exhibiting 0 to 0.10% divergence, while other specimens showed higher levels of divergence (Table 1). The average intraspecific genetic divergence measured 1.96% ranging from 0 to 6.08% (Table 1), while the interspecific divergence ranged between 4.59 to 35.55% (Supplementary Table S2). Interspecific genetic divergence values were higher between species

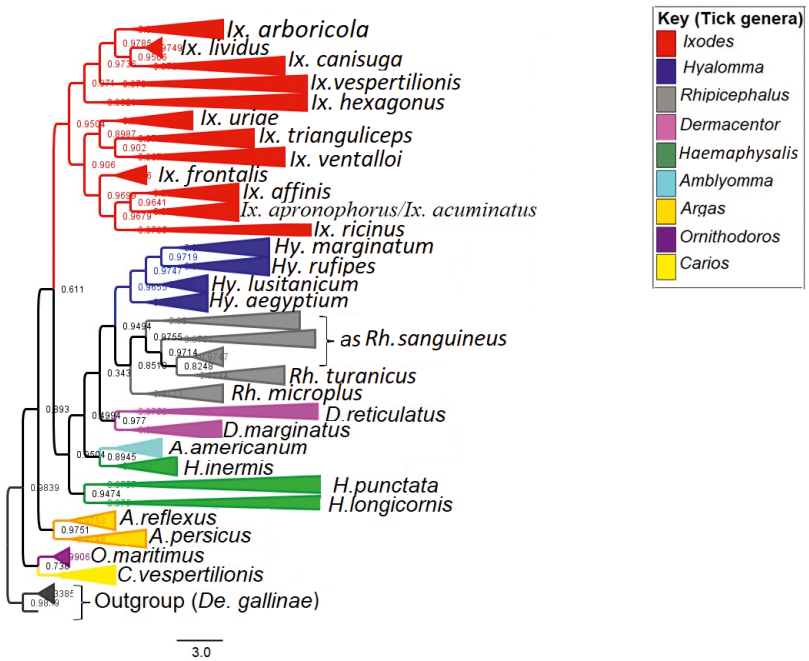

FIGURE 1 Bayesian maximum clade credibility (MCC) tree constructed (collapsed) using *COI* sequences (658 bp) from nine tick genera, rooted to red mite (*Dermanyssus gallinae*) (outgroup). Groups of various species of tick genera have been collapsed (triangular shapes) for simple presentation and clarity. The tree is drawn to display time scale and posterior probabilities on each node. Red: *Ixodes*; Blue: *Hyalomma*; Grey: *Rhipicephalus*; Cyan: *Dermacentor*; Green: *Haemaphysalis*; Sky blue: *Amblyomma*; Dark yellow: *Argas*; Purple: *Ornithodoros*; Yellow: *Carios*.

from different genera (Supplementary Table S2). The lowest level of genetic divergence was observed between the species *Hy. rufipes/Hy. marginatum* (4.59%) and *Ixodes lividus/Ixodes canisuga* (7.50%). The greatest level of divergence was observed between the species *Hy. aegyptium/A. reflexus* (35.55%), *Hy. rufipes/A. reflexus* (35.00%), followed by *Ornithodorus maritimus/Rhipicephalus microplus* (34.34%).

We detected the following species with well above 2% intraspecific genetic diversity, *Ixodes affinis* Neumann, *Ixodes frontalis* Panzer, *Ixodes ventalloi* Gil Collado, *Ix. vespertilionis* and the *Rh. sanguineus* species complex (Table 1). The species complex *Rh. sanguineus* showed a deep divergence in the MCC tree (Figure 1), as well as *Ix. vespertilionis*, which also differentiated into different discrete groups (Supplementary Figure S1 and Figure S2).

BIN discordance

The BIN count analysis using BOLD capabilities was only based on a subset of the total *COI* barcodes we analysed in MEGA. The dataset only included 828 barcode sequences this analysis [the remainder 171 sequences were not included in this analysis because they did not have a BIN assigned; this is because they originated from public sequences in the NCBI database, or made available for this study but they were not included in the BOLD dataset].

BOLD capabilities assigned a BIN to each of 828 *COI* barcodes we analysed (see Table 1 for BINs). The BIN count identified 20 BINs out of 19 morphological species only available for this analysis. Most species consisted of single BINs (Table 1). Species with two BINs are *Ix. acuminatus* (BOLD:ACI1693, BOLD:ABY3619), *Ix. vespertilionis* (BOLD:ADK7202, BOLD:ACH9349). In addition, specimens identified as *Rh. sanguineus* species complex consisted of three BINs (BOLD:AAI0467–Antigua and Barbuda, Grenada and Mexico; BOLD:ACI0511–Greece; BOLD:AAU2994–Spain), while *Ix. vespertilionis* have four BINs (BOLD:ACV7519, BOLD:AEV4890, BOLD:ADK7202, BOLD:ACH9349) (Table 1). Discordant BINs occurred at the species level, mainly because of the discrepancy in taxonomic information assigned to certain specimens within NCBI or BOLD databases, for example *Argas vespertilionis* vs *C. vespestilionis*.

Species delimitation analysis

When specimens belonging to the *Rh. sanguineus* species complex were assessed as a whole (*Rh. sanguineus* from Europe and the western hemisphere and *Rhipicephalus turanicus*), the divergence was 6.00% (uncorrected *p*-distance 0.06). When each group within these species was assessed individually, the intra group diversity reduced to 0.01 (<1%) or below (Supplementary Table S3a), whilst the inter-group diversity was more than 10-fold higher (Supplementary Table S3b).

For *Ix. frontalis*, *Ix. ventalloi* and *Ix. vespertilionis*, the overall *p*-distance of these species was 0.028, 0.027 and 0.055 (2.80%, 2.70% and 5.50%), respectively. The exception to this was *Ix. vespertilionis* which clustered into two clear groups, but there were also two samples (KR902756 and LC036330) which were divergent from the rest of the *Ix. vespertilionis* sequences. Therefore, for *Ix. vespertilionis* the analysis was performed twice by dividing the species into two groups with 'group one' containing the two divergent sequences and then by splitting the species into four groups, and keeping these stand-alone sequences separate. When treating *Ix. vespertilionis* as two groups the intra group diversity of 'group two' fell below 1%, but 'group one' remained high (8.10%). When these standalone sequences were separated from 'group one', the divergence fell to below 1% (Supplementary Table S3a). The inter-group diversity for *Ix. frontalis* and *Ix. vespertilionis*, when divided into four groups, was at least 10-fold higher than intra-group diversity. The inter-group diversity was higher for *Ix. ventalloi*, but it did not quite reach the 10-fold increase (Supplementary Table S3b). Similar results are observed when using uncorrected p-distance measures and a maximum composite likelihood model. For the *Ixodes* species, only *Ix. frontalis* passed the threshold to be considered separate species. This method performs a comparison of mean difference both within and between groups, with values greater that four indicating that two groups represent different species. As this method results in two values for θ, it is recommended that the larger value is used to determine the final K/θ ratio, which results in a more conservative ratio less likely to delineate species incorrectly.

4 Discussion

In this study, most morphologically identified species formed defined groups (Figure 1; Supplementary Figure S1 and Figure S2) based on *COI* DNA barcode sequences, supporting the use of DNA barcoding in combination with morphological traits as an effective approach for the identification of tick species, either for those considered native to the UK or species that have been introduced (Gillingham *et al.*, 2020b).

Interspecific genetic divergence between morphospecies ranged between 4.59 and 35.55%, whereas intraspecific genetic divergence within distinct species ranged from 0 to 6.08%. Most of the specimens within a known morphospecies were resolved in the MCC tree (Figure 1). However, some individuals in certain taxa such as *Rh. sanguineus* species complex (Figure 1) grouped in multiple branches. *Rhipicephalus sanguineus* species complex is recognised as a species complex composed of up to 12 valid species mainly distributed in the Afrotropical region with some species expanding to other zoogeographical regions (Bakkes *et al.*, 2021; Sánchez *et al.*, 2018). However, the fact that specimens did not cluster within discrete groups suggests a limitation of the *COI* gene as a genetic marker to differentiate between different populations within the *Rh. sanguineus* species complex. This finding is not surprising as several species within such species complexes are related (Bakkes *et al.*, 2021; Lv *et al.*, 2014; Nava *et al.*, 2015; Sánchez *et al.*, 2018; Sánchez-Montes *et al.,* 2021). Several revisions attempting to delineate *Rh. sanguineus* species complex combining morphological traits and molecular markers have been published (Nava *et al.*, 2015), including the most recent reviews from by Dantas-Torres *et al.* (2024) and Kelava *et al.* (2025) in which the complex was thoroughly reviewed and delineated. Certain lineages within the complex are referred to as the 'temperate lineage' and 'tropical lineage'; however, Šlapeta *et al.* (2022) re-described the adult stages of *Rhipicephalus linnaei* (Audouin) and characterised this species using molecular analysis. A male specimen of *Rh. linnaei* collected in Esna City, Egypt was selected as the neotype, and the authors proposed that this name should be applied to populations from the species complex *Rh. sanguineus* identified from tropical areas, which have been currently supported by Dantes-Torres *et al.* (2024). Applying this rationale and taking into consideration the geographic distribution of the specimens here identified as *Rh. sanguineus* species complex from Antigua and Barbuda, Grenada, and Mexico (BIN BOLD: AAU2924), the name *Rh. linnaei* must be applied to these *COI* sequences; furthermore, the sequences originating from Greece (BIN BOLD: ACI0511) and Spain (BIN BOLD:AAI0467) should be considered as *Rh. sanguineus sensu stricto*. However, we

believe that further studies still need to be conducted using larger molecular datasets and other molecular markers linked to morphological traits in this species complex, and that *COI* sequences freely available within the NCBI and/or BOLD databases need to be assigned to the correct species name (within the *Rh. sanguineus* species complex). The application of the K/θ ratio analysis to the *Rhipicephalus* sequences provides further support to the hypothesis that the specimens previously recorded as *Rh. sanguineus* are in fact separate species. The high level of divergence seen within the group as a whole indicates that *Rh. sanguineus* species complex contains multiple cryptic species. This is further supported by the finding that when each group is separated, the intra-group diversity drops to below 1% for all groups, and the inter-group diversity is more than 10-fold higher.

Another closely related species within the *Rh. sanguineus* species complex is *Rh. turanicus* Pomerantsev. The *COI* DNA barcoding sequences analysed in this study originate from China, Greece and Israel (Palearctic Region), and they are all closely grouped with the *Rh. sanguineus* species complex. Bakkes *et al.* (2020) reviewed *Rh. turanicus* using an integrative approach by applying morphological traits and molecular data (12S and 16S rDNA). The authors stated that *Rh. turanicus* was formed of two lineages, one Palearctic lineage and one Afrotropical lineage, and that the taxonomy was difficult because specimens from Cyprus and Zambia interbreed and can produce fertile hybrids. Nonetheless, the author proposed a new species name for the South African populations, and considered specimens from Southern Europe, the Middle East and Asia as *Rh. turanicus sensu stricto*, a rationale which has been adopted in this study. Similar trends have been observed in other vector groups such as mosquitoes and blackflies (Danabalan *et al.*, 2012; Hernández-Triana *et al.*, 2012, 2014, 2015, 2017).

We follow the classification of Mans *et al.* (2021) in recognising *Carios* as a valid genus within Argasidae (see Figure 1). In the UK, the genus *Ixodes* shows the greatest diversity and includes some of the most important tick vector species such as *Ix. ricinus* (Jameson and Medlock, 2011; Holding *et al.*, 2019). Although *Ix. ricinus* is widespread in the UK (Gandy *et al.*, 2023) and it can be collected in a range of different habitats, no cryptic genetic diversity was observed in this study. Specimens from other species within the *Ixodes* genus all clustered within the same group, except for *Ix. vespertilionis* (Supplementary Figures S1 and S2), where different clusters were observed supporting the hypothesis that it might be a species complex; another split was seen in some sequences from specimens identified as *Ix. frontalis* (Hornok *et al.*, 2015; 2024; Takano *et al.*, 2023). Both *Ix. acuminatus* and *Ix. apronophorus* are displayed as a single species clustered in the MCC tree (Supplementary Figure S1), although specimens are well-differentiated in the un-collapsed MCC tree (Supplementary Figure S1 and Figure S2). Our results are in concordance with previous studies, demonstrating that *Ix. apronophorus* is closely related to other species within the genus *Ixodes* (Rar *et al.*, 2020). The separation of *Ix.* cf. *affinis* in two groups based on only three *COI* sequences with a genetic diversity of 5.28% was surprising. There are limited numbers of available *COI* sequences for this species across Central America, and the two sequences downloaded from NCBI (accession number KF200161, KF200140) diverged from our own sequence (BOLD ID TKMEX012016-H11). However, since we do not have access to the vouchers from where these sequences originated, we are unable to comment further on their taxonomic status. An additional means of species delimitation in the genus *Ixodes* was employed by utilising the 4X ratio method (Birky, 2013). This method only showed that *Ix. frontalis* groups surpassed the ratio threshold of 4 to be considered as separate species. It is interesting to note that even though *Ix. vespertilionis* had the highest level of divergence, it was not distinguished as a separate species using the 4X ratio method.

Misidentifications were also found within immature stages of *Ix. ricinus* that matched with 100% identity to *Haemaphysalis punctata*. This species is a known vector species of piroplasms and possibly *Borrelia* bacteria (Phipps *et al.*, 2022) and it is found in southern England and Wales, as well as in Central Europe, Sweden, and Asia. However, in past decades its population has increased in Northern Europe (Phipps *et al.*, 2022), and a rise in records of *H. punctata* biting humans, companion animals and heavy infestations on sheep have also been recorded in the UK, especially in South Downs National Park (Medlock *et al.*, 2018b). Within our dataset, the use of *COI* DNA barcoding highlighted those specimens identified as *Ixodes* sp. (TKPH040-20, TKPH041-20), which grouped together with *Ix. hexagonus*. This was also confirmed by morphological examination. The latter supports the utility of DNA barcoding methodology in highlighting taxonomic problems within large *COI* DNA barcoding datasets (Hebert *et al.*, 2003a,b; Packer *et al.*, 2009).

An outbreak of *Babesia canis* (Piana and Galli-Valerio) (De Marco *et al.*, 2017) facilitated the genetic study of different populations of *D. reticulatus* in the UK, where specimens of this species were found in new areas along the coast. However, all specimens grouped together in

our analysis, and no cryptic diversity was found between the different populations we assessed.

In general, we detected species with well above 2% intraspecific genetic diversity such as *Ix. affinis*, *Ix. frontalis*, *Ix. ventalloi*, *Ix. vespertilionis*¸ and the species complex *Rh. sanguineus* (Table 1). The *Rh. sanguineus* showed a deep divergence in the MCC tree, which supports the current knowledge that it is species complex (Figure 1). Furthermore, it has been argued that high genetic diversity within *Ix. vespertilionis* might indicate that it is a species complex (Hornok *et al.*, 2015); this is also supported by our data since all *COI* DNA barcode sequences analysed differentiated into discrete groups (Supplementary Figure S1 and Figure S2). In addition, the only three available sequences from *Ix. affinis* differentiated into two different groups (Supplementary Figure S1 and Figure S2). No other species revealed such extensive divergence within the tree, including taxa for which expansion of their distribution is documented such as *D. reticulatus* or *H. punctata*, or species such as *Ix. ricinus* which is well distributed in the UK.

Regarding non-native tick species, although the adults of certain species are readily identifiable using morphological keys, the development of a molecular library for rapid species identification is useful when specimens are in a poor state of preservation or only immature forms are collected. This is an essential step for the rapid establishment of vector control measures (Versteirt *et al.*, 2015), particularly in the event of a non-native introduction of ticks in the UK via migratory birds or travellers returning to the UK, as in the case of *Hyalomma* species (Medlock *et al.*, 2018b; Hansford *et al.*, 2018).

The utility and limitations of *COI* in species identification have been reviewed in Hernández-Triana *et al.* (2012). Although it was not the objective of this paper to draw conclusions into the relationships of British ticks by analysing *COI* DNA barcoding sequences alone, we could comment on the clusters based on the tree topology obtained from MMC analysis (Figure 1) [also see Supplementary Figure S1 and Figure S2 for further view of tree topology]. All species we analysed were grouped in discrete clusters within the genus they are assigned to, with the exception of *Haemaphysalis inermis*, which was placed together with *A. americanum* in a group comprising *Hyalomma*, *Rhipicephalus*, and *Dermacentor* with high bootstrap values (Figure 1). We only have three sequences available for analysis for *H. inermis*, but our results agree with the hypothesis of Burger *et al.* (2013). In their study, the authors proposed a discrete structure in the relationship within the genus *Haemaphysalis* where two species, *Haemaphysalis parva* Neumann and *H. inermis*, were highly divergent from the remaining taxa. In their analysis, the authors provided evidence for the division of the Metastriata into two clades, *Amblyomma senso stricto* and the *Rhipicephalinae* (e.g. *Rhipicephalus*, *Hyalomma*, *Dermacentor*) including *Haemaphysalis* (plus *Bothriocroton* and *Amblyomma sphenodonti* Dumbleton).

In summary, our dataset demonstrated that five species showed at least above 2% intraspecific genetic divergence (Table 1) when compared to other taxa, and this might be indicative of cryptic diversity. For all other species, the variation in intra- and interspecific genetic values obtained in this study falls within ranges previously reported for *COI* DNA barcoding studies in ticks (Ondrejicka *et al.*, 2017; Krčmar *et al.*, 2022; Zhang and Zhang, 2014).

5 Conclusions

This study provides *COI* DNA barcoding data to support the molecular identification of native tick species in the UK, as well as several non-native species. We augment the barcoding data for UK tick species present in the UK such as *C. vespertilionis*, *Ix. acuminatus, Ix. arboricola*, *Ix. canisuga*, *Ix. hexagonus*, as well as other species of veterinary and public health importance such as *Rh. microplus* and the species complex *Rh. sanguineus*. Even though most specimens were differentiated by *COI* gene analysis, certain taxa could not be distinguished using this genetic marker within the genus *Rhipicephalus* (*Rh. sanguineus* species complex) and *Ixodes* (*Ix. vespertilionis*) supporting the hypothesis that they are a species complex. The latter supports the need for continuing research using alternative molecular markers in combination with morphological traits for accurate species delineation in ticks.

Supplementary material

Supplementary material can be found online at https://doi.org/10.52004/2054930X-20251022 under Supplementary Materials.

Figure S1. Maximum likelihood phylogenetic tree (non-collapsed) constructed using *COI* barcode sequences (658 bp) from nine tick genera, with 10,000 bootstrap approximations rooted to the red mite (*Dermanyssus gallinae*) (outgroup). Groups of various species of tick genera were not collapsed for clarity. Bootstrap values ≥ 70 are represented by a diamond node shape. Red: *Ixodes*; Blue: *Hyalomma*; Grey: *Rhipicephalus*;

Cyan: *Dermacentor*; Green: *Haemaphysalis*; Sky blue: *Amblyomma*; Dark yellow: *Argas*; purple: *Ornithodoros*; Yellow: *Carios* species. Sequences are named with NCBI/BOLD accession numbers, source and country of origin where available (e.g. GB: Great Britain; ES-Spain).

Figure S2. Bayesian maximum clade credibility (MCC) tree (non-collapsed) constructed using *COI* barcode sequences (658 bp) from nine tick genera, rooted to the red mite (*Dermanyssus gallinae*) (outgroup). Groups of various species of tick genera were not collapsed for reader confirmation. The tree is drawn to display time scale and posterior probabilities on each node. Red: *Ixodes*; Blue: *Hyalomma*; Gray: *Rhipicephalus*; Cyan: *Dermacentor*; Green: *Haemaphysalis*; Sky blue: *Amblyomma*; Dark yellow: *Argas*; purple: *Ornithodoros*; Yellow: *Carios* species. Sequences are named with NCBI/BOLD accession numbers, source and country of origin where available (e.g. GB: Great Britain; ES-Spain).

Table S1. Species name and accession number of COI barcodes sequences used in our dataset, which are publicly available in the NCBI or BOLD databases.

Table S2. Percentage of interspecific (between groups) pairwise K2P genetic divergence of unique tick COI DNA barcodes (658 bp).

Table S3. Genetic divergence calculations. (A) Intra-group divergence (d) Each of the groups identified within the *Rhipicephalus sanguineus* species group, *Ixodes frontalis*, *Ixodes ventalloi* and *Ixodes* as determined by both uncorrected *p*-distance and Maximum Composite Likelihood model. (B) Inter-group divergence within each of the identified groups as determined by both uncorrected *p*-distance and Maximum composite likelihood model. (C) shows the K/θ ratio used for species delimitation as set out by Birky (2013).

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Author contributions

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Conflict of interest

The authors declare no competing interests.

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Data availability

All data generated or analysed during this study are included in this published article [and its supplementary information files]. The datasets generated during and/or analysed during the current study are available in the https://portal.boldsystems.org/recordset/DS-TICKUK [also using the link https://doi.org/10.5883/DS-TICKUK].

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