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Original article

Description of *Rhipicephalus hibericus* sp. nov. (Ixodoidea: Ixodidae), a species of the *Rhipicephalus sanguineus* group in southwestern Europe

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ABSTRACT

We describe all the life stages of *Rhipicephalus hibericus* n. sp., provide the types, and present molecular support for a new species of the *Rhipicephalus sanguineus* sensu lato group, present in southwestern Europe, that has been historically confused with *Rhipicephalus turanicus* Pomerantzev, 1940. A new name is proposed for this taxon because it was impossible to ascribe to types of already described species in the group, deposited for more than 100 years in natural history institutions. The males have a dorsum showing deep and coarse punctations (absent in *Rhipicephalus sanguineus* sensu stricto) and adanal plates with large punctations (absent in *R. sanguineus* s.s.); the tail of the spiracular plate is as wide as the closest festoon (half the width in *R. sanguineus* s.s.). Females have large punctations in dorsal fields, a wide spiracular plate, and a "V" shaped genital opening; such a combination of characters cannot be found in other species of the group. Immatures are described from specimens collected on hosts (Rodentia and Eulipotyphla). Both larvae and nymphs are markedly smaller than *R. sanguineus* s.s. Nymphs display long, backward-projected auriculae; larvae are almost half the size of *R. sanguineus* s.s. The new species can hybridize with *R. sanguineus* s.s. in laboratory colonies producing an unfertile F2, laying brown and dry eggs that did not hatch. Phylogenetic analysis of partial *coxI* gene sequences placed *R. hibericus* in a well-supported clade with other sequences of *R. sanguineus* s.l. from Portugal, as a sister clade of *R. sanguineus* s.s. The new species does not belong to the *R. turanicus* group of species. Both *12S* and *16S* partial gene sequences were not as precise in the correct phylogenetic placement of *R. hibericus*, in part probably due to the existence of erroneously identified sequences in GenBank©. This description, together with the previous reinstatement of *Rhipicephalus secundus* and *Rhipicephalus rutilus*, and the description of the neotypes of *R. sanguineus* s.s. should help researchers to adequately identify their collections. Our findings demonstrate that *R. turanicus* is absent in southwestern Europe. Old collections should be re-examined to provide the actual range of the new species.

1. Introduction

The tick *Rhipicephalus sanguineus* sensu stricto (Latreille, 1806) is, from a public health and economic perspective, one of the most important species of the *Rhipicephalus sanguineus* group. This group also includes *Rhipicephalus sulcatus* Neumann 1908, *Rhipicephalus rossicus* Yakimov and Kol-Yakimova 1911, *Rhipicephalus schulzei* Olenev, 1929, *Rhipicephalus pumilio* Schulze 1935, *Rhipicephalus pusillus* Gil Collado 1936, *Rhipicephalus secundus* Feldman-Muhsam 1952, *Rhipicephalus turanicus* Pomerantzev, 1940, *Rhipicephalus leporis* Pomerantzev, 1946, *Rhipicephalus guilhoni* Morel and Vassiliades 1963, *Rhipicephalus moucheti* Morel 1965, and *Rhipicephalus camicasi* Morel, Mouchet and Rodhain 1976 (Bakkes et al., 2020; Nava et al., 2018; Pegram et al., 1987a, 1987b; Walker et al., 2000). The name *R. sanguineus* s.s. has often been used to designate any population of ticks of the *R. sanguineus* group associated with dogs worldwide, without following any strict, formal, biological, morphological, or molecular criteria (Dantas-Torres et al., 2013; Nava et al., 2015).

The morphological descriptions of ticks denominated as *R. sanguineus* s.s. are based on specimens originating from different sites (Nava et al., 2018), resulting in some cases in a lack of agreement of the basic characters necessary for the recognition of the species (Burlini

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et al., 2010; Chitimia-Dobler et al., 2017; Coimbra-Dores et al., 2018; Dantas-Torres et al., 2013, 2017; de Oliveira et al., 2005; Labruna et al., 2017; Levin et al., 2012; Liu et al., 2013; Moraes-Filho et al., 2011; Nava et al., 2015, 2012; Sanches et al., 2016; Szabó et al., 2005; Zemtsova et al., 2016). For example, ticks from parts of southwestern Europe (Spain, Portugal, France) and southern Switzerland, identified morphologically as *R. sanguineus* s.s. and *R. turanicus*, respectively, were shown to be almost identical in terms of genomic sequencing to what has been named *Rhipicephalus sanguineus* sensu lato (Almeida et al., 2017; Beati and Keirans, 2001; Bernasconi et al., 2002; Dantas-Torres et al., 2017; Mangold et al., 1998; Moraes-Filho et al., 2011; Nava et al., 2018).

On the other hand, the taxon *R. turanicus* s.l. has been widely used to name a tick, morphologically different from *R. sanguineus* s.s., that is a common parasite of wild and domestic ungulates and carnivores in south-western Europe (Gray et al., 2013). *Rhipicephalus turanicus* Pomerantzev, 1940, was redescribed in detail, including measurements of all life stages, by Filippova (1997). The species is now known to exist in many parts of Asia and has been recently separated from another similar entity, existing in the Tropical region, namely *Rhipicephalus afranicus* (Bakkes et al., 2020). The situation is very different in the western Palearctic, where different lineages of *R. sanguineus* s.l. that have been historically called *R. turanicus* s.l. coexist and are difficult to differentiate due to a lack of adequate morphological or molecular criteria.

Specimens alleged to be *R. turanicus* were re-described by Manilla (1998) including explicit reference to a wide tail of the spiracular plate in males, and the "V" shaped genital opening in the females, with illustrations redrawn from Morel and Vassiliades (1962). However, the illustrations of the adanal plates of the male are inconsistent with the material drawn by Filippova (1997) and pictured by Dantas-Torres et al. (2017). Such a trait is considered an important feature for species discrimination. The criteria based on the shape and size of the female's genital opening were popularized by Morel and Vassiliades (1962) while studying specimens probably belonging to different species of the group from different sites in the Mediterranean region and sub-Saharan Africa, thus resulting in a wide variety of morphological variation. Nowadays, these criteria are rarely used for morphological separation of species, since they involve the dissection and mounting of the cuticle, and have been replaced by criteria based on molecular biology; however, molecular techniques still lack a background to separate the hypothetical "*R. turanicus*" of southwestern Europe with species of the *R. sanguineus* group.

Besides, *R. secundus* Feldman-Muhsam was synonymized with *R. turanicus* based on the morphology of the genital opening of females and a few other details (Morel and Vassiliades, 1962). The species was reinstated in 2022 (Mumcuoglu et al., 2022) after examination of part of the type material and other specimens identified as *R. secundus* by Feldman-Muhsam. Both morphological and molecular features supported the clear separation of both taxa. However, *R. secundus* is morphologically and molecularly far from the specimens collected in southwestern Europe (Mumcuoglu et al., 2022). Further studies by Gilot and co-workers published in the late XXth Century enhanced the view that *R. turanicus* exists in Western Europe and several studies about its ecology in France have been produced (Gilot et al., 1977, 1990; Gilot and Pautou, 1981). In recent years, other studies (Almeida et al., 2017; Coimbra-Dores et al., 2018; Silva, 2017) reinforced the notion that *R. turanicus* is absent in western Europe but did not provide a formal description of the existing taxon, sometimes collected with *R. sanguineus* s.s. One of the main features discriminating the male of *R. sanguineus* s.l. (or "*R. turanicus*" in southwestern Europe) is the width of the tail of the spiracular plate, which is as broad or slightly broader than the closest festoon in *R. turanicus* (while in *R. sanguineus* s.s. is always half as broad as the festoon). The widely distributed *R. sanguineus* s.l. in western Europe has this feature; in consequence, the name *R. turanicus* prevailed to name specimens of the genus presenting deep punctuations in the dorsum, wide tail of the male's spiracular plates, and variable shapes of adanal plates, even if they molecularly clustered near *R. sanguineus* s.s. Interestingly, the morphology of immatures was never considered as an additional clue for the definition of the species.

Efforts to compare the specimens of *Rhipicephalus* in western Europe with already named and further synonymized species kept in museums or academic institutions have been unsuccessful in missions carried out in the last 10 years, mainly due to the wide morphological variability of the species and the poor conservation state of the types of other species, as listed by Nava et al. (2018). This prevented the comparison of available material from southwestern Europe with species described in the late XIXth Century. Also, it was impossible to obtain DNA from stored specimens in museums, because the specimens are the types of a species and therefore must be preserved, or they are dry or in very old alcohol (not curated), rendering DNA amplification unsuitable.

This study describes all the life stages, provides the types, and presents molecular support for a new species of the *R. sanguineus* group present in southwestern Europe. This species is named *Rhipicephalus hibericus* n. sp. to disentangle all the ambiguous denominations of *R. sanguineus* s.l. in the target territory, and the impossibility of assigning it to an already described species. We also provide data regarding the hybridization in the laboratory between *R. sanguineus* s.s. and *R. hibericus* n. sp. Basic details of the known hosts, tick's seasonal activity, and the known distribution of the species in southwestern Europe are also presented.

2. Materials and methods

2.1. Collection of ticks

The specimens devoted to this study were collected by flagging (adults) or on hosts (immatures) around an area of about 10 km radius in La Cartuja Baja (41°36′16″N 0°49′21″W), Zaragoza province, Spain. Specimens were examined under a Nikon stereomicroscope.

2.2. Molecular characterization

Eleven adult ticks (6 males and 5 females) were prepared following Halos et al. (2004) and modified as follows. Briefly, half tick was placed in a 2-ml sterile microtube containing ten 0.1 mm sterile glass microbeads, and one 3.2 mm sterile stainless steel microbead (BioSpec). The tubes were cooled in liquid nitrogen for 1 min and immediately after crushed by shaking in a Mini Bead Beater-16 (Model 607-EUR, BioSpec) for 2 cycles of 1 min 30 s at 3450 rpm. The tubes were then briefly centrifuged at a maximum speed (10,000 x g) and the pellets were suspended in 180 μL of lysis buffer (ATL buffer, Qiagen). DNA was then extracted using Qiagen DNeasy Blood & Tissue kit (Qiagen, Valencia, CA, USA) following manufacturer instructions. The final elution volume was 100 μL. Three genes were targeted for sequencing: one protein-encoding mitochondrial gene (cytochrome oxidase I, *coxI*) and two mitochondrial ribosomal genes (*12S* and *16S* rDNA). Partial PCR amplifications of the *coxI* gene (approximately 680 bp) were performed using previously described primers LCO1490 (5′-GGT CAA ATC ATA AAG ATA TTG G-3′) and HCO2198 (5′-TAA ACT TCA GGG TGA CCA AAA AAT CA-3′) (Folmer et al., 1994). The primer forward (5′-AAA CTA GGA TTA GAT ACC CTA TTA TTT TAG-3′) and reverse (5′-CTA TGT AAC GAC TTA TCT TAA AGA GTG-3′) were subsequently used to amplify approximately 400 bp of *12S* gene (Szabó et al., 2005). Fragments of approximately 460 bp of the *16S* gene were: 16S+1 (5′-CCG GTC TGA ACT CAG ATC AAG T-3′) and 16S-1 (5′-GCT CAA TGA TTT AAA TTG CTG T-3′) as described in Mangold et al. (1998). PCR reaction conditions were carried out in a final volume of 25 μL containing 0.25 μL Taq-polymerase (1 U/μL), 1 μL Mg2+, 2.5 μL KCl 10x, 2.5 μL dNTP's, 2.5 μL of each primer [2 μM] and 5 μL of the extracted DNA. Cycling parameters for each gene were those defined in Szabó et al. (2005), Mangold et al. (1998) and Hebert et al. (2003), respectively. Successfully amplified DNA products were purified and sequenced by Macrogen (Madrid, Spain) following the Sanger method. Sequences were aligned

and edited using BioEdit Sequence Alignment Editor 7.2.5 (Hall, 1999), and consensus sequences were then scanned against the GenBank© database using BLAST. Unique *coxI, 12S* and *16S rRNA* sequences identified in our study were deposited in GenBank© under accession numbers OR965519, OR965523, PP234669–70, PP236415–16, PP261623 for *coxI*, OR946402-OR946409 for *12S rRNA*, and OR946396-OR946401 for *16S rRNA*, respectively.

Phylogenetic trees were inferred by using both the Maximum Likelihood method (ML) and a Bayesian approach. For the ML method, the best fitting evolutionary model was conducted with JModeltest2 (Darriba et al., 2012) and the best model selection was based on the Akaike Information Criterion (AIC). Support for the topologies was tested by bootstrapping over 1000 replicates with gaps excluded in the pairwise comparison. After the best model selection, phylogenetic trees were visualized in MEGA-X (Kumar et al., 2018) and edited using FigTree 1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/). Phylogenetic reconstruction was also carried out following a Bayesian approach, using Geneious (available at https://www.geneious.com). These analyses consisted of 100,000 generations starting from a random tree and four Markov chains with default heating values, sampled every 5,000th generation. Two separate runs were conducted for each analysis, and the first 10 % of sampled trees were discarded as burn-in, before obtaining a consensus tree. Sequences of *Rhipicephalus annulatus* and *R. australis* were used as outgroups in the tree using the *coxI* gene. *Rhipicephalus decoloratus, R. australis, R. microplus,* and *R. annulatus* were included as outgroups in the trees for *12S* and *16S*.

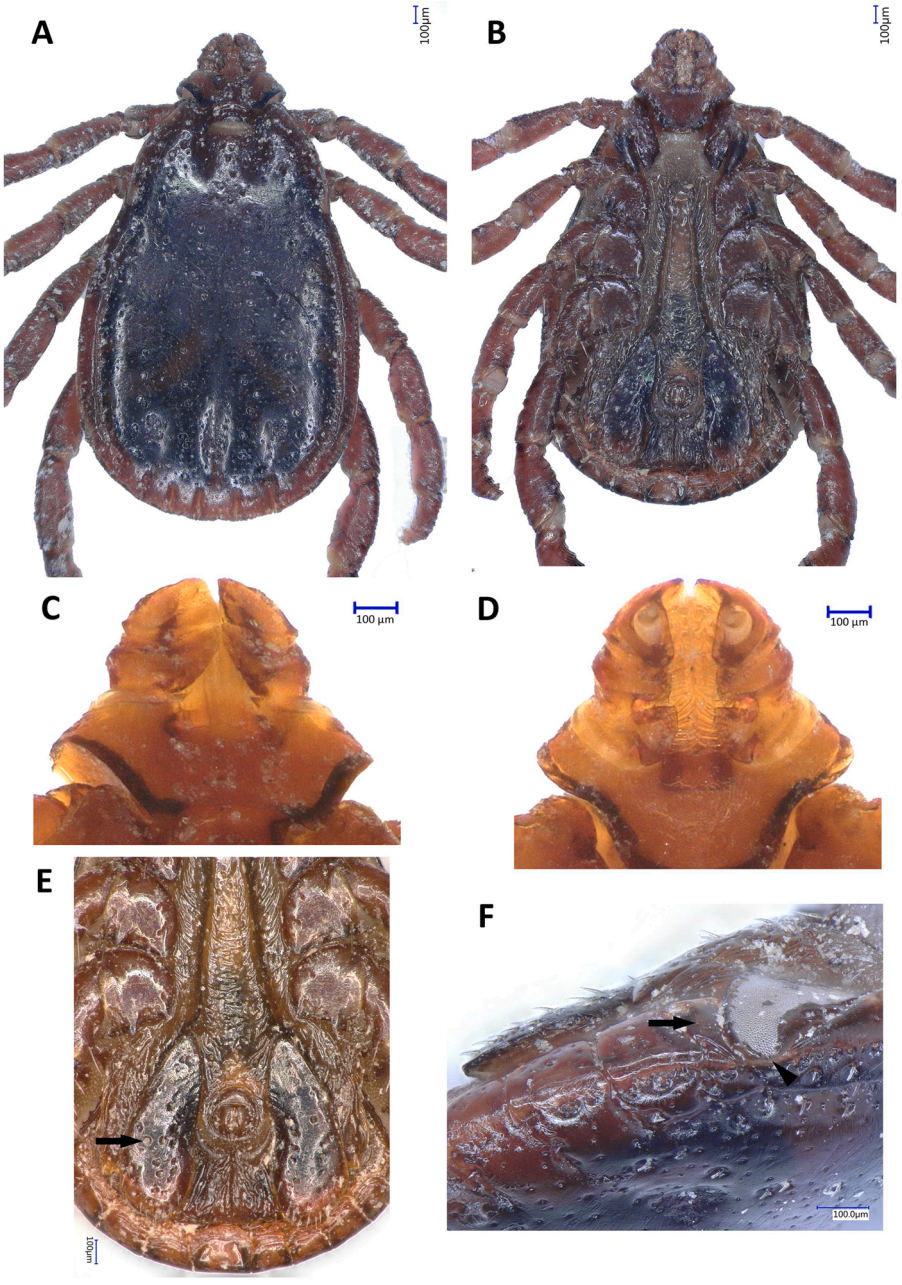

Fig. 1. Male type of *Rhipicephalus hibericus* n. sp. A: dorsal aspect. B: ventral aspect. C: capitulum, dorsal view. D: capitulum, ventral view. E: anus, anal plates, adanal plates and coxae III-IV; the image is intended to illustrate the shape of the adanal plates and the deep punctations (arrow). F: lateral view of the posterior part of the body showing the spiracular plate and the festoons (ventral part of the body is upwards) to note the relative width of the tail of the plate with the adjacent festoon (arrows).

2.3. Experimental hybridization with Rhipicephalus sanguineus s.s.

Adult ticks obtained from the colony that provided the neotype specimens of *R. sanguineus* s.s. (Nava et al., 2018) were allowed to feed together with adults of the alleged new species, and the results of the fertility of the resulting specimens were analyzed. Males and females of *R. hibericus* n. sp. were collected while questing in the type locality.

Ticks were fed on laboratory rabbits by using feeding chambers attached to the dorsum of the hosts. Alternate crosses of male x female of each species were done, always consisting of ten males or females of the two species feeding inside the same capsule. The offspring (F1) resulting from these crosses were fed again on rabbits to generate the F2. The hypothesis was that if F2 failed to emerge then we could suspect the existence of an infertile hybrid progeny. The ticks were kept at 25 °C and 83–86% relative humidity, with a daily photoperiod of 12 h light-12 h dark. The colonies were maintained with the agreement of the Commission for Animal Ethics of the Faculty of Veterinary Medicine, Zaragoza (2015–06656A), adhering to the European protocols for animal welfare.

3. Results

3.1. Description

***Rhipicephalus hibericus* n. sp.** (Figs. 1-4).

Material examined.

Ten males, ten females, 19 nymphs, 24 larvae.

Type specimen: holotype, male, collected by flagging on May 2022, in the mid-Ebro Valley (La Cartuja Baja, Zaragoza province, Spain; 41°36′16″N 0°49′21″W); paratype, female, collected with the male type (same site, same date). Paratypic series: 10 larvae and 10 nymphs collected feeding but slightly engorged on greater white-toothed shrew (*Crocidura russula*) from the same location as adults. All the material is deposited in the collection of the Veterinary Faculty at Zaragoza (Spain).

Description.

Male (Voucher specimen number: #Rh1; Fig. 1).

Body: outline broadly oval, broadest at the level of legs IV, slightly narrower anteriorly, with a slightly concave margin at the level of the eyes; total length 4.24 ± 0.41 mm, length from apices of scapulae to posterior body margin 3.75 ± 0.39 mm, maximum width 1.70 ± 0.08 mm (Fig. 1A). *Scutum*: inornate, yellowish- or reddish-brown coloration; punctations moderate in number, unequal in size, larger and more densely distributed in the anterior field, where they are deeper and larger; marginal grooves deep and with several punctations. The marginal grooves slightly reach the first festoon, but deep punctations behind the end of the marginal grooves may delimitate the first two festoons; the marginal grooves do not reach the eyes (Fig. 1A). Cervical grooves short, deep, and comma-shaped; posteromedian groove distinct, elongated, and V shaped, with deep punctations in the lateral parts of the groove; posterolateral grooves sub-circular, shorter than posteromedian groove, commonly of different size and aspect in most specimens; eyes flat, scapulae rounded (Fig. 1A). *Ventrally* with adanal plates long, falcate, relatively narrow but then broad at the posterior margin, which is pilose and acutely rounded, never truncated, with a width like the two closer festoons (Fig. 1B, with details in Fig. 1E). The inner margin of adanal plates has a deep concavity posterior to the level of the anus *not carrying* an inner spur. Adanal plates with clearly visible deep punctuations of heterogeneous size and number, covering in part the surface of the adanal plates (Fig. 1E); accessory adanal plates conspicuous, pointed, and slightly curved outwards, shorter than adanal plates, posterior end narrower (about 1/2) than the width of adjacent festoon (Fig. 1B, 1E). *Capitulum: basis capituli* dorsally hexagonal, wider than long (Fig. 1C), posterior margin with a deep concavity, width 0.78 ± 0.02 mm, length 0.54 ± 0.04 mm (dorsal view); length from palpal apices to cornua apices 0.77 ± 0.02 mm, cornua broad and triangular; palps short and rounded apically; hypostome short, blunt, dental formula 3/3 in 6–7 rows, apex with corona of fine denticles; palps total length 0.26 ± 0.001 mm (Fig. 1D). *Festoons* quadrangular in shape, almost as wide as long (except the central festoon, which is slightly wider than long, and the first, which is slightly narrower than the rest), with some setiferous punctations (Fig. 1E). *Spiracular plate*: elongate, wide, barely curved, dorsal prolongation clearly wider than the adjacent festoon, with many goblets; distal portions of the spiracular plate plenty of goblets (Figure1F). *Legs*: coxa I with two long spurs, the external clearly narrower than the internal, both spurs almost parallel, not divergent, the internal subtriangular; coxae II-IV each with one single short external spur (barely observed on coxa IV), and one smaller internal spur; those in coxa IV approximately of the same size (Fig. 1B).

Female (Voucher specimen number: Rh2. Fig. 2)

Body: outline broadly oval, broadest at the level of insertion of legs IV, slightly narrower anteriorly, total length 4.99 ± 0.36, length from apices of scapulae to posterior body margin 4.33 ± 0.32, maximum width 2.41 ± 0.17 (Fig. 2A). *Scutum*: inornate, yellowish- or reddish-brown coloration, barely longer than broad, length 1.98 ± 0.11, maximum width 1.91 ± 0.08, length to width ratio 1.03 ± 0.01, posterior margin sinuous, with two clear concavities near the apical part; cervical grooves broad, shallow, diverging posteriorly; cervical fields depressed; punctations on scutum moderate in number, unequal in size but commonly large and deep (Fig. 2A, 2E); eyes flat, located at the point of maximum width of the scutum; scapulae rounded. *Alloscutum* with three vertical linear grooves; setae of the alloscutum inapparent. Ventral surface as illustrated (Fig. 2B) with genital opening showing a wide "V" contour. *Spiracular plate*: broad and almost round with a wide dorsal prolongation visible dorsally (Fig. 2E), covered by goblets on its entire surface and broad as the adjacent festoon. *Capitulum*: basis capituli dorsally hexagonal, clearly wider than long and depresed, basis capituli width 0.89 ± 0.05, basis capituli length 0.59 ± 0.04 (dorsal view), length from palpal apices to cornua apices 1.53 ± 0.01, lateral angles broad, cornua small but well visible, palps short narrowly rounded apically; porose areas small, oval, separated from one another by an interval clearly larger than their diameter (Fig. 2C); hypostome short, blunt, dental formula 3/3 in 6–7 rows, apex with a corona of fine denticles; palps total length 0.66 ± 0.01, segment I length 0.09 ± 0.003, segment II length 0.21 ± 0.005, segment III length 0.31 ± 0.004 (Fig. 2D). *Legs*: coxa I with two long triangular spurs, subparallel, the external narrower than the internal, the internal with a clear inner convexity; coxae II-III with one single short external spur each and one small internal spur; the spur on coxa II may be absent in some specimens; coxae II-IV of similar size. Genital aperture broadly V-shaped, lateral margins diverging anteriorly, located between coxae II (Fig. 2B).

Nymph (Voucher specimens number: #Rh3. Fig. 3)

Body: outline oval, broadest at level of insertion of legs IV, total length 1.19 ± 0.13, length from apices of scapulae to posterior body margin 1.00 ± 0.12, maximum width 0.95 ± 0.13 (Fig. 3A). *Scutum*: inornate, approximately as long as broad, lateral margins nearly straights, posterior margin broadly rounded, length 0.42 ± 0.02, maximum width 0.41 ± 0.03, ratio length to width 1.02 ± 0.07 (0.88–1.03); cervical grooves short, deep anteriorly, shallow posteriorly, sigmoid in shape, extending posteriorly to the level of the eyes; scapulae rounded and short; few and shallow punctations on scutum, barely visible, and few short setae; eyes flat at the level of posterior third of scutum. *Alloscutum* and ventral surface covered by scattered setae (Fig. 3A, 3B). *Capitulum: basis capituli* subtriangular dorsally, *basis capituli* width 0.30 ± 0.01, *basis capituli* length 0.06 ± 0.02, length from palpal apices to posterior margin of *basis capituli* 0.18 ± 0.01, cornua absent, lateral angles very slightly curved, ventral processes present, palps short and acute in its apical portion; hypostome short, blunt, dental formula 2/2 (Figs. 3A, 3B). Legs: coxa I with two triangular spurs, parallel (not diverging), the external slightly longer than the internal; coxa III-IV each with a single, well visible, acute, external spur, subequal in size in coxa III and IV. Coxa II with an external spur, larger than those in coxae III and IV and one small internal spur, almost quadrangular

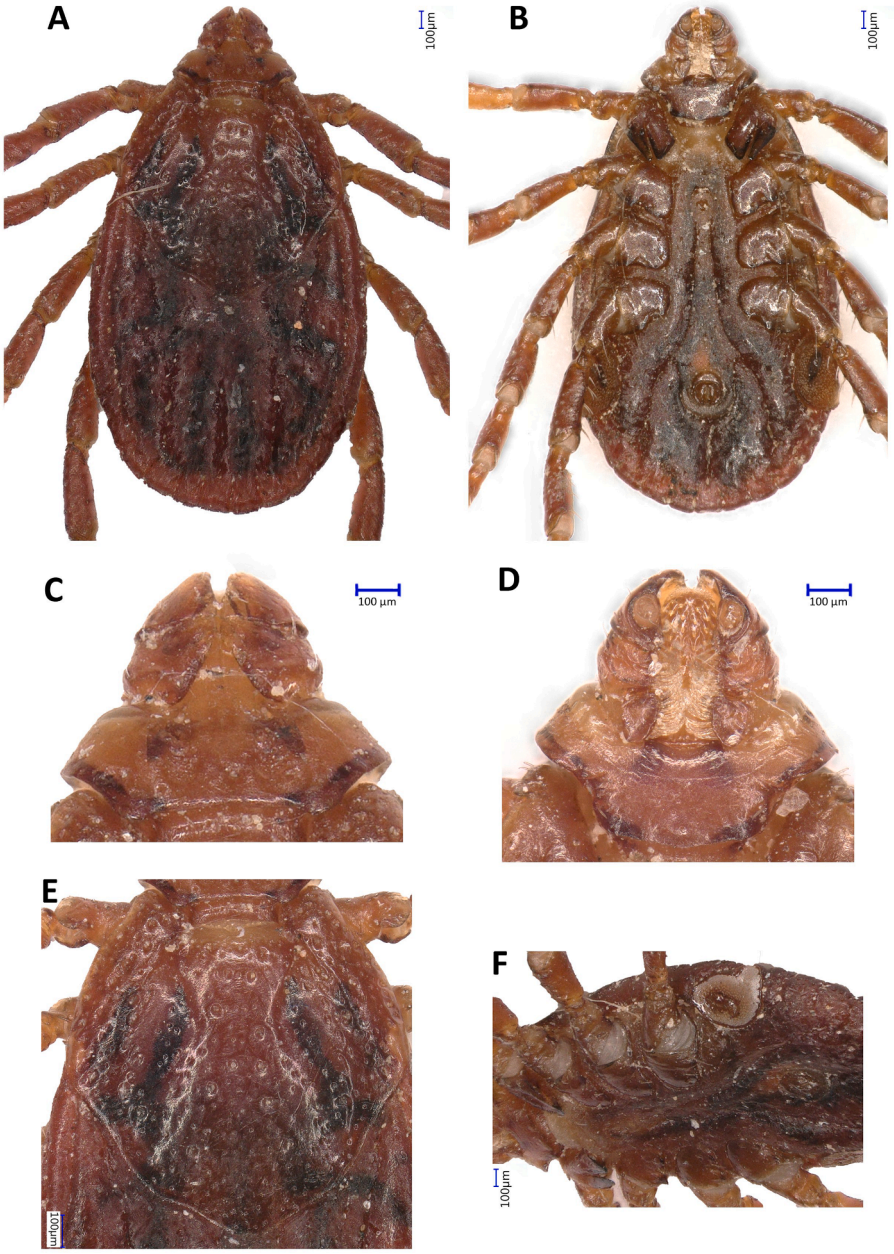

Fig. 2. Female paratype of *Rhipicephalus hibericus* n.sp. A: dorsal aspect. B: ventral aspect. C: capitulum, dorsal view. D: capitulum, ventral view. E: magnification of the scutum, intended to show the large and deep punctations. F: lateral view of the of the body showing the spiracular plate and the festoons (ventral part of the body is downwards).

(Fig. 3B).

Larva (Voucher specimens number: #Rh4; measurements are in micrometres. Fig. 4).

Body: outline sub-oval, broadest at the level of insertion of legs III, total length including *capitulum* 546.67 ± 50.96, length from apices of scapulae to posterior body margin 465.20 ± 54.13, maximum width 413.54 ± 25.40 (382–470) (Fig. 4A). *Scutum*: broader than long, length 218.82 ± 12.61 (202–245), maximum width 370.00 ± 9.86 (353–392), ratio width to length 1.69 ± 0.09 (1.53–1.83); posterior margin slightly sinuous; cervical grooves shallow, ending at the level of eyes; scapulae rounded; presence of few and very shallow punctations, barely visible; 3 pairs of short scutal setae (*Sc*): Sc1 18.40 ± 2.21, Sc2 12.50 ± 1.72, Sc3 12.40 ± 1.13; eyes flat at the level of the widest part of the scutum (Fig. 4A). Ten pairs of dorsal body setae, which can be summarized as 2 pairs of central dorsal (Cd): Cd1 18.23 ± 2.99, Cd2 16.41 ± 1.21, and 8 marginal dorsal (Md): Md1 16.40 ± 1.13, Md2 17.51 ± 2.23, Md3 17.20 ± 1.70, Md4 14.41 ± 1.69, Md5 15.13 ± 1.03, Md 6 15.59 ± 1.46, Md7 14.03 ± 1.64, Md8 14.39 ± 1.45 (Fig. 4A). Fifteen pairs of ventral setae, that include 3 pairs of sternal setae (St): St1 22 ± 1.07, St2 22.51 ± 1.01, St3 29.01 ± 0.91; 2 pre-anal pairs (Pa): Pa1 13 ± 1.89, Pa2 15.12 ± 1.99; 4 premarginal pairs (Pm): Pm1 13.64 ± 1.41, Pm2 16.70 ± 1.61, Pm3 12.91 ± 2.01, Pm4 10.63 ± 2.07; 5 marginal ventral pairs (Mv): Mv1 16.69 ± 1.25, Mv2 14.69 ± 1.21, Mv3 14.33 ± 1.61, Mv4 14.13 ± 1.52, Mv5 16.18 ± 1.41 and 1 pair on anal valves (A): A 16.19 ± 0.89 (Fig. 4B). *Capitulum: basis capituli* about two times broader than long, *basis capituli* width 153 ± 4.51 (146–158), length from posterior margin to posthypostomal setae (Ph) 81.11 ± 7.70 (68–95), length from palpal apices to posterior margin of *basis capituli* 141 ± 7.10 (130–153), lateral angles relatively long and almost straight, posterior margin slightly convex; palps short, length 83 ± 2.77 (78–88), and apically acute (Fig. 4C); hypostome short, length from Ph to apex 55.21 ± 1.51

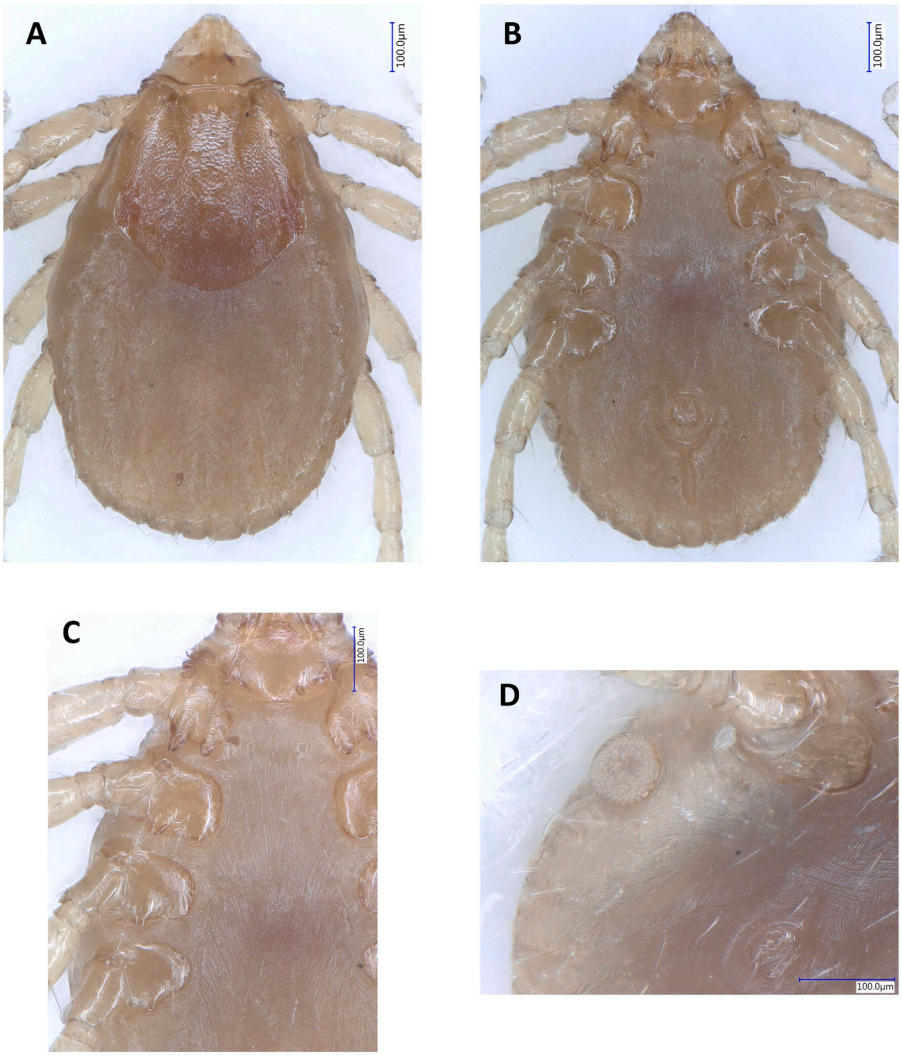

Fig. 3. The nymph of *Rhipicephalus hibericus* n.sp. A. dorsal aspect. B. ventral aspect. C. details of the spurs on coxae I-IV, together with the large auriculae in the ventral side of the capitulum. D. position, size and aspect of the spiracular plate.

(53.6–58.5), width 33.77 ± 3.10 (29–39), blunt, dental formula 2/2 with four to five denticles per file (Figs. 4C, 4D). One relatively long coxa in the inner margin of plapal segment II, over a conspicusous chitinous prominence. *Legs*: coxa I-III each with a single, short, rounded, external spur, decreasing in size from coxa I to coxa III (Fig. 4B). Tarsus I long, length 188 ± 5.40 (180–195).

3.2. Molecular characterization

***CoxI* gene**

Eight readable sequences were obtained belonging to seven different ntST, showing between 93.92 % and 99.81 % identity among them and between 98.81 % and 100 % identity with other sequences published in GenBank with different specific names (Table 1). Both Bayesian (Fig. 5) and Maximum Likelihood (Fig. 6) phylogenetic reconstructions of the *coxI* gene classified six of our seven ntST in a clade including previously published sequences of *R. sanguineus* s.l. from Portugal, separated of the rest of species of the *R. sanguineus* group, with bootstrap support of 99.5 % for the best tree. The newly described species is in a sister clade of *R. sanguineus* s.s. One of the *coxI* sequences of *R. hibericus* clustered in a separate branch of *R. sanguineus* s.s. After morphological re-examination of the carcass of the tick (specimen #41) stored after DNA extraction, it was concluded that the initial morphological identification was consistent with the morphological traits defining *R. hibericus*; considering the results of the interspecific breeding with *R. sanguineus* s.s. (see below) we consider this to be a hybrid specimen. Both phylogenetic reconstructions recognized several clades other than the outgroups, divided into two main branches. One of them included the recently reinstated *R. rutilus* and *R. linnaei, R. camicasi*, as well as the *R. secundus/R. turanicus* lineage. The other branch groups the *R. sanguineus* s.s. samples and the representatives of *R. hibericus*.

***12S rRNA* gene**

Thirteen readable sequences were obtained belonging to eight different ntST, showing between 95.95 % and 99.71 % identity among them and between 99.42 % and 99.71 % identity with sequences published in GenBank© (Table 1). Both the Bayesian and the ML phylogenetic analysis grouped four of these ntST sequences, with 99 % bootstrap support, in a branch including other sequences of *R. sanguineus* s.l. from Portugal, but also sequences identified as *R. sanguineus* worldwide (Supplementary File 1). This branch was sister with the *R. sanguineus* s.s. clade (99 % bootstrap), where one of our sequences (ntST-4, from the same specimen #41) was included, as occurred with the *coxI*-derived phylogenetic tree, supporting its status as a probable hybrid specimen.

***16S rRNA* gene**

Sequencing yielded eleven sequences belonging to six ntST, showing between 98.70 % and 99.74 % identity among them and between 99.48 % and 100 % identity with sequences published in GenBank© (Table 1). Both phylogenetic analyses grouped five of these ntST with diverse

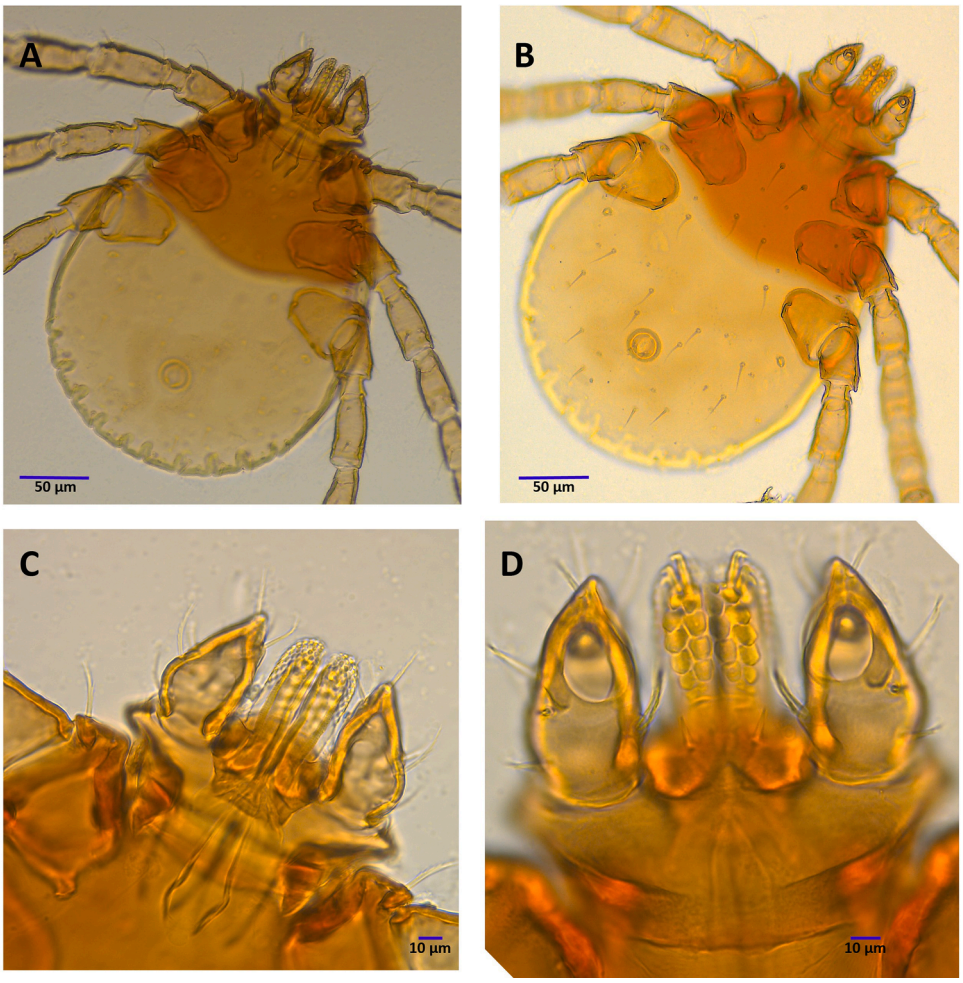

Fig. 4. The larva of *Rhipicephalus hibericus* n.sp. A. dorsal aspect. B. ventral aspect. C. capituum, dorsal. D. capitulum, ventral.

Table 1
Nucleotide sequence types (ntST) obtained from each tick specimen for each gene fragment studied.

	Specimens	% identity	Accession number (closest sequence)	Origin
***cox*1**				
ntST-1	#27	100 %	MF425998	Dog, Portugal
ntST-2	#31	98.81 %	MF426008	Dog, Portugal
ntST-3	#37	99.62 %	MF425998	Dog, Portugal
ntST-4	#39	99.43 %	MF426008	Dog, Portugal
ntST-5	#41	99.24 %	KX757903	Dog, Italy
ntST-6	#54	99.43 %	MF426008	Dog, Portugal
ntST-7	#112, #67	99.81 %	MF426008	Dog, Portugal
12S rDNA				
ntST-1	#25, #31, #37, #117	99.71 %	MF425945	Dog, Portugal
ntST-2	#27	99.43 %	MF425945	Dog, Portugal
ntST-3	#39, #112	99.42 %	MF425935	Dog, Portugal
ntST-4	#41	98.01 %	KU255849	Dog, France
ntST-5	#44	99.43 %	JX304713	Dog, France
ntST-6	#46	99.42 %	MF425945	Dog, Portugal
ntST-7	#50	98.85 %	MF425945	Dog, Portugal
ntST-8	#54, #67	99.71 %	MF425935	Dog, Portugal
16S rDNA				
ntST-1	#25, #27, #37, #46, #50, #117	99.74 %	OP326205	Dog, Spain
ntST-2	#31	100 %	MG855662	Hedgehog, Malta
ntST-3	#54, #39	100 %	MW202408	Dog, Argentina
ntST-4	#41	99.48 %	MH018845	Unknown, California (USA)
ntST-5	#44	99.48 %	OP326205	Dog, Spain
ntST-6	#112	99.74 %	OP326206	Dog, Spain

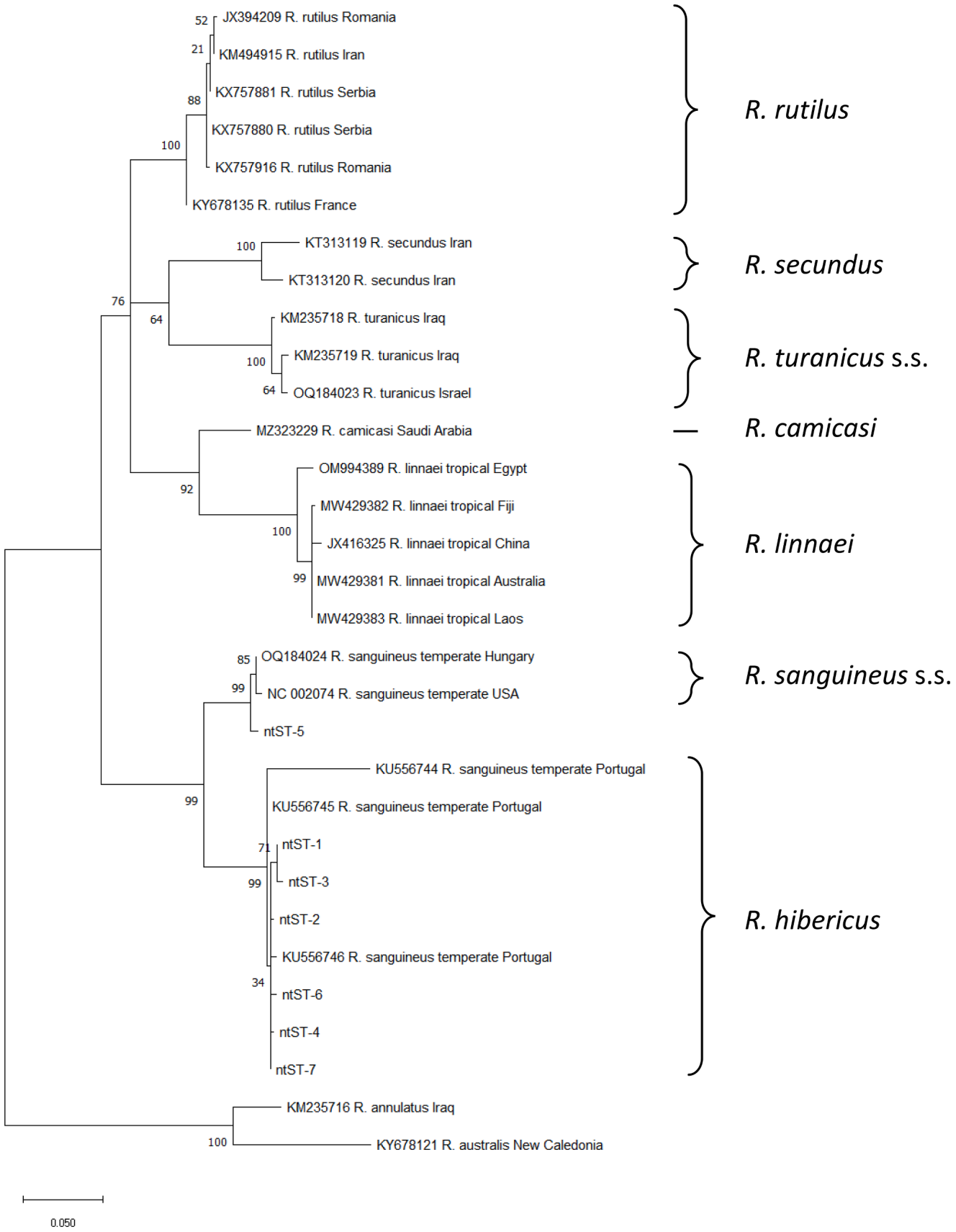

Fig. 5. Maximum-likelihood tree based on partial *coxI* gene sequences of tick species of the genus *Rhipicephalus*. Numbers represent bootstrap support generated from 1000 replications. GenBank© accession numbers are indicated in brackets. The tree was constructed with the General Time Reversible model (GTR+*G*).

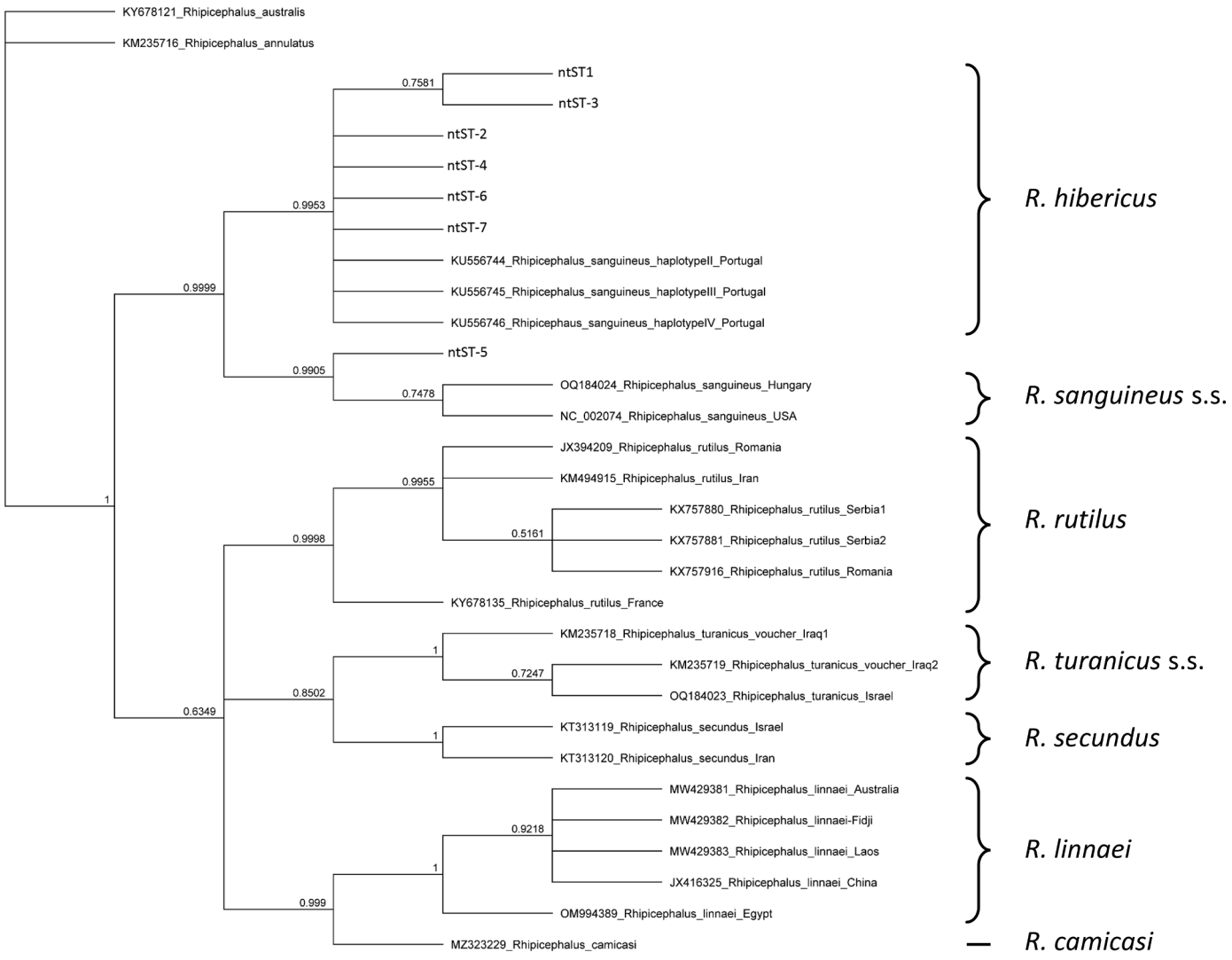

Fig. 6. Bayesian reconstruction of several lineages of both *R. sanguineus* s.l. and *R. turanicus* s.l. using a fragment of the *coxI* gene. Included are samples of *R. hibericus* n.sp., *R. sanguineus* s.s. (from the colony used for the re-description of the species), *R. rutilus* (as re-instated by Šlapeta et al., 2023), *R. turanicus* s.s., *R. secundus* (as re-described by Mumcuoglu et al., 2022), *R. camicasi*, and *R. linnaei* (as re-instated by Šlapeta et al., 2022).

sequences published under the names *R. sanguineus* s.l., *R. sanguineus* "temperate lineage" (that may be *R. sanguineus* s.s. or any of the lineages of the tick present in the Mediterranean region), *R. turanicus*, or *R. sanguineus* group (without further indications) from around the world (Supplementary File 1). Again, the sequence from specimen #41 (ntST-4 in this case) was classified in a sister branch with *bona fide* R. sanguineus s.s., but also with a "*R. turanicus* s.l." sequence from Switzerland. These two clades were placed in a branch with 100 % bootstrap support.

3.3. Taxonomy and basic information

Taxonomic summary

Order Ixodida Leach, 1815

Family Ixodidae Koch, 1844

Genus *Rhipicephalus* Koch, 1844

Species *Rhipicephalus hibericus* Millán, Rodríguez-Pastor and Estrada-Peña

Etymology: *hibericus*, adj., Iberian (of or pertaining to Iberia in southwestern Europe)

Type-host(s). The species has a three-host life cycle. Adult specimens on which the type description is based were collected by flagging, although adults commonly parasitize domestic sheep, dogs, wild carnivores, and wild ungulates (e.g. *Sus scrofa*) in the type locality. Preliminary data indicates that immatures parasitize micromammals such as wood mouse (*Apodemus sylvaticus*), Algerian mouse (*Mus spretus*), and greater white-toothed shrew (*Crocidura russula*). Apparently, only the adults of this tick are exophilic, as suggested by the lack of collections of immatures by flagging in the type locality during the years 2001–2008 and 2020–2023, in the same months in which immatures are common on the mentioned hosts.

Type-locality. La Cartuja Baja (41°36′16″N 0°49′21″W), Zaragoza province, Spain.

Known distribution, biogeography, and phenology. The species seems to be present in southwestern Europe (Spain, Portugal, south of France, as per available references), together with *Rhipicephalus pusillus, R. sanguineus* s.s. and perhaps other yet undescribed taxa of the *R. sanguineus* group. Adult specimens collected in sites of Spain are morphologically and molecularly similar to specimens reported from Portugal and parts of southern France (molecular data for comparison available in GenBank© under a variety of names, such as *R. turanicus*). *Rhipicephalus hibericus* prefers a Mediterranean-type vegetation, with warm and dry summers and mild winters; however, it has been found on medium-range mountains, about 600 m a.s.l. Adult ticks of the species are active mainly in spring and the beginning of summer. Adults may be active all year long in southern and southeastern Spain (collections on file). No ticks have been collected by standard flagging in the type

locality before March or after the end of July, but a few adults were observed questing as late as August and sporadically observed on sheep in early September. Immatures are active in summer but are probably endophilic and parasites of micromammals; they have been collected only on hosts. The life cycle suggests either a diapause of the larval-nymph molt to synchronize the nymphs that molt in the vegetation at the beginning of the spring or most probably a diapause of the newly molted adults in the vegetation. Both hypotheses remain untested. The complete details of the life cycle of *R. hibericus* as related to environmental features and complete data about prevalence and incidence on hosts are under preparation.

Vectorial role. *Rhipicephalus hibericus* could be involved in the transmission of *Anaplasma ovis* (Lacasta et al., 2021; mentioned as *R. turanicus*). The findings were based on field observations, but no laboratory analysis were developed to demonstrate such vectorial role. Other tick-borne pathogens were recorded in *R. hibericus* ticks collected while feeding and therefore cannot support a vectorial role. Interestingly, *Rickettsia massiliae* was isolated and described from "*R. turanicus*" collected in France (Beati and Raoult, 1993) although the tick was later mentioned as *R. sanguineus* (Parola et al., 2013). *Rickettsia massiliae* has been reported in *R. sanguineus* s.l. collected feeding on hosts in northern Spain (Fernández-Soto et al., 2006; Portillo et al., 2015; Millán et al., 2016). A line of transmission restricting *Rickettsia conorii* to *R. sanguineus* s.s. and *R. massiliae* to *R. hibericus* has not yet been established, but both bacteria belong to different rickettsial groups; we strongly believe that such a study could shed light on the separation of both clades of ticks and the tick-symbiont behavior of these bacteria.

Gene sequences:

The nucleotide sequence data used in this study were deposited in GenBank© (NCBI) under the following accession numbers: **OR965519, OR965523, PP234669–70, PP236415–16, PP261623** (*coxI*); **OR946402-OR946409** (*12S*); and **OR946396-OR946401** (*16S*). Additionally, one sequence of the *16S* gene of a *Rhipicephalus* sp. nymph from an *Apodemus sylvaticus* previously collected in NW Spain (Cevidanes et al., 2016) was deposited in GenBank© under the accession number **OR946410**.

3.4. Experimental crosses with Rhipicephalus sanguineus s.s.

It is necessary to note that when these experiments were carried out, we were unaware of the existence of *R. hibericus*; morphological traits were not evaluated on the resulting crossed specimens. They were performed only to obtain a pure colony of *R. sanguineus* s.s. allowing its redescription (Nava et al., 2018), and to test if sympatric exophilic ticks resembling *R. sanguineus* s.s. could produce fertile progeny when crossed with endophilic specimens; the alternative hypothesis was that the production of hybrid progeny was immediately associated to at least two sympatric and morphological close species. Adults of both exophilic and endophilic "strains" were collected, respectively, questing and from the wall holes of a kennel, at a distance of no more than 150 m, and distributed randomly among the laboratory rabbits. Approximately 3000–5000 eggs with a hatchability of about 95 % were recorded from the F1 of the mixed colony of *R. sanguineus* s.s. and *R. hibericus*. No differences in fertility were found in crosses of males and females of each species. The F1 larvae were smaller than those of *R. sanguineus* s.s. and fed normally on rabbits; an adequate number of nymphs and adults were obtained (molting success of about 96 % in every stage at the conditions mentioned in Methods). After the feeding and mating of the adults, the oviposition began late (~20 days), and all the eggs were dry and exhibited a dark color. No larvae hatched, indicating that specimens were of different species producing a hybrid and sterile F1 generation, resulting in the lack of an F2 generation. In parallel, specimens of *R. sanguineus* s.s. (resulting only from the endophilic adults collected in the wall holes of the kennel) have been reproducing in the colony since the year 2015 without apparent losses of fertility.

4. Discussion

Rhipicephalus hibericus n. sp. has long been confused with either *R. turanicus* or *R. sanguineus* s.s. in reports about *Rhipicephalus* ticks collected in Portugal and Spain (Almeida et al., 2017; Coimbra-Dores et al., 2018; Moraes-Filho et al., 2011; Silva, 2017) and in France (Gilot et al., 1977, 1990; Gilot and Pautou, 1981). The new species is known to be present in southern France, but the previously reported specimens could not be re-examined to provide a complete list of localities.

The name *R. turanicus* has been applied in the Iberian Peninsula to specimens with a deeply punctuated body surface and a wide tail of the spiracular plates of males and females; the character of a wide tail of the spiracular plate of males was the main clue used for almost the last 40 years, females being identified by the mating with males (on hosts) with these features. Nymphs and larvae have been seldom considered for the identification of *Rhipicephalus* species at least in the Mediterranean region. This is of special interest for *R. hibericus* n. sp., probably because the immature stages were never captured by flagging and because of its absence from pets, livestock, or large wildlife. The re-description of *R. sanguineus* s.s. and the separation of other species of the *R. sanguineus* group was carried out by Nava et al. (2018); similar studies were addressed by Mumcuoglu et al. (2022) reinstating *R. secundus*. The south-eastern lineage of *R. sanguineus* s.l. was reinstated as *R. rutilus* by Šlapeta et al. (2023). We encourage a strategy based on laboratory colonies for crosses and back-crosses of several species of ticks of the group (i.e. *R. camicasi, R. guilhoni*) to improve these studies. In any case, the fact is that both *R. sanguineus* s.s. and *R. hibericus* are sympatric and could be found even on the same individual dog (personal observation by AEP in Montpellier, France, June 2015) and simultaneously feeding on wild carnivores (unpublished data).

While some morphological features of *R. hibericus* n. sp. could superficially remind those of *R. turanicus*, critical characters are completely different. Males have falcate adanal plates relatively short and narrow, without internal spurs, and with an inner small concavity; they are deeply punctuated, which overlaps well with handmade illustrations and photographs of the ticks named *R. turanicus* in southwestern Europe. Further on this, the tail of the spiracular plate is larger than the adjacent festoon, a character which is typical of several species but that has been never observed in *R. sanguineus* s.s. This is probably one of the reasons behind the extended use of the name *R. turanicus* for this species, which was said to parasitize goats and dogs (Encinas Grandes, 1986; Gago et al., 2022; Gilot et al., 1990; Monerris Mascaró and Colom Noguera, 2020). Other morphological details can separate the new species, such as the spiracular plate of males, and the V-shaped genital opening, which is different from the U-shaped one of *R. sanguineus* s.s. Moreover, *R. hibericus* n. sp. is a heavily punctate tick, in opposition to *R. sanguineus* s.s. The adanal plates of the male are a clear example of large punctations, absent in *R. sanguineus* s.s.

The immatures collected on hosts were selected to represent the correct measures of the species, mounting, and measuring only those with a minimal degree of engorgement, as observed by the overall size, and the gut contents. However, it is not possible to ascertain if the beginning of feeding could affect the measurements of the body features. However, it must be noted that the immatures *R. hibericus* are clearly smaller than those of *R. sanguineus* s.s., larvae being almost half of the length of the nominal species of the group. On the other hand, some critical structures will remain unaffected, like the length of body setae (all of them were measured for the larva), the dimensions of the scutum, or the relative proportions of the capitulum. All these features are critical for the comparison of the immatures of both species. All the stages of *R. sanguineus* s.s. are endophilic, and adults can be collected only when they abandon their shelters in broken walls of kennels, gardens, etc. However, the adults of *R. hibericus* are exophilic and can be easily collected by flagging.

Partial molecular characterization was performed based on the sequencing of three mitochondrial gene fragments. The most coherent

results were obtained for the *coxI* gene, for which both the Bayesian and the ML phylogenetic trees provided a clear separation of clades, with high support, separating *R. hibericus* n. sp. from *R. sanguineus* s.s. and other species in the group. The phylogeny of the *16S* and *12S* gene fragments, however, were less elucidative, in part due to the existence in GenBank© of several sequences of wrongly identified sequences. Special mention deserves one specimen of the current study (#41) that, despite having unmistakably morphological characteristics of *R. hibericus*, was clustered with *R. sanguineus* s.s. in the three gene fragments phylogeny. We consider this specimen to be a *R. sanguineus* s.s. x *R. hibericus* hybrid, demonstrating that the true specific status of a field-caught specimen may be difficult to establish. As already mentioned, hybrids were obtained using an approach based on laboratory-maintained colonies and specimens of both species have been found feeding on the same host, on shepherd dogs that live in a kennel and move with the local flocks of sheep. We cannot discard the existence of hybrids in nature. As far as we know, there is no information about the morphological or molecular variability of hybrids in the genus *Rhipicephalus*. The specimens observed from the colony prepared to demonstrate the hybridisation did not show any previously unobserved features in the F1, while the F2 did not hatch.

Rhipicephalus hibericus became separated from the main clade of *R. sanguineus* s.s. most likely by an ecological isolation. In this case, the exophilic features of the adults of *R. hibericus* and the endophilic ones observed in *R. sanguineus* generate a clear separation between both species. The approximate date of separation of both species is yet to be determined. In any case, the old paradigm of the presence of *R. turanicus* in southwestern Europe must be abandoned, and old collections re-examined to establish the actual status in the light of the reports regarding the extending lineages of the *R. sanguineus* group in the large areas it colonizes.

5. Conclusions

In this study, the type and paratypes of *R. hibericus* n. sp. are described and designated by considering the rules of the International Code of Zoological Nomenclature. All parasitic stages of *R. hibericus* were morphologically described and DNA sequences of different molecular markers of this taxon are now available. We conclude that, among the three genes examined, only *coxI* can separate without ambiguities the new species of the *R. sanguineus* group. The definition of *R. hibericus*, as stated herein, should constitute a benchmark for further taxonomic investigations to fully characterize the different species of the *R. sanguineus* complex present in Europe and northern Africa.

CRediT authorship contribution statement

Javier Millán: Writing – review & editing, Writing – original draft, Project administration. **Ruth Rodríguez-Pastor:** Writing – review & editing, Methodology, Data curation. **Agustín Estrada-Peña:** Writing – review & editing, Writing – original draft, Data curation, Conceptualization.

Declaration of competing interest

Authors declare no conflict of interests.

Data availability

Data will be made available on request.

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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.2024.102340.

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