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Redescription, molecular features, and neotype deposition of *Rhipicephalus pusillus* Gil Collado and *Ixodes ventalloi* Gil Collado (Acari, Ixodidae)

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Abstract

Two species of ticks, *Rhipicephalus pusillus* Gil-Collado and *Ixodes ventalloi* Gil-Collado are redescribed, their molecular features (16S rDNA) compared with near species, and neotypes named and deposited. The male of *R. pusillus* is characterized by a smooth dorsal surface, with relatively short marginal grooves, not reaching the eyes and reaching the first festoon, longer than wide adanal plates, internally concave and widely rounded posteriorly, without internal spurs, accessory adanal plates not projecting over the cuticle, and a narrow, long and straight process on the spiracular plate. The female has very small porose areas, separated by 2.5-3 diameters, and a large triangular spur on the ventro-internal side of the palpal article 1. The nymph has lateral processes curved anteriorly in the ventral aspect of the basis capituli, long auriculae, and internal spurs on the four coxae. The larva of *R. pusillus* is separated by the presence of spurs on the ventral surface of palpal segment I, together with the rounded apices of the palpi. Features of 16S rDNA cluster this species near the *R. sanguineus* s.l. complex of species. The distinctive feature of the female of *I. ventalloi* is the long and curved auriculae, which are absent in every other species of *Ixodes* reported in the Western Palaearctic. The male is characterized by relatively long conscutal setae and deep and well defined cervical fields. The nymph is unique by having slightly apparent lateral carinae and small cornua, very abundant and long alloscutal setae, about six times longer than those on the scutum and internal spur of coxa I reaching coxa II. The larva of *I. ventalloi* has Md1-Md3 setae of the same size as the rest of the conscutal setae, which are only slightly longer than the scutal setae, cornua present, and anal groove clearly divergent. In addition to the unique combination of morphological features, the molecular 16rDNA sequence clearly separates *I. ventalloi* from other species of the *I. ricinus* group. We named and deposited in an internationally accessible collection the neotypes of both species, since the holotypes were lost.

Key words: *Rhipicephalus pusillus*, *Ixodes ventalloi*, redescription, neotypes, 16rDNA

Introduction

Ectoparasites on the European rabbit, *Oryctolagus cuniculus*, are represented by an interesting community of fleas, a louse, mites and ticks, restricted to this host or, in some cases, to other mammals and birds (Frank *et al*., 2013). These ectoparasite assemblages seem to be part of the evolution of the European rabbit, linked to the last glaciation refugia (Biju-Duval *et al*., 1991). There are several species of ticks which are commonly reported on this host, namely *Haemaphysalis hispanica* Gil Collado, 1938, *Ixodes ventalloi* Gil Collado, 1936, and *Rhipicephalus pusillus* Gil Collado, 1936. Of these, only *H. hispanica*, which is restricted to the most south-western range of the Mediterranean distribution of *O. cuniculus*, has been adequately characterized (Hoogstraal and Morel, 1970). Both *R. pusillus* and *I. ventalloi* have been scarcely reported in Europe and northern Africa. For some reports, there is speculation about the reliability of identification for these two species, generating uncertainty in the knowledge of the distribution of ticks in the Western Palaearctic.

The topic is of special interest because of the background of existing confusion within the possible species of the *Rhipicephalus sanguineus* group (Nava *et al*., 2015). It has been reported that a complete reappraisal in its main area of distribution is necessary, and *R. pusillus* is a poorly known species which could superficially resemble *R.*

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sanguineus s.l. Since *R. pusillus* has been recorded on wild carnivores, which are also common hosts for *R. sanguineus* s.l., reliable identification of this species is necessary. Further on this, and regarding the genus *Ixodes* in the Western Palearctic, it is necessary to provide an adequate morphological background to separate several species which may share hosts and environment, and thus overlap in distributional ranges. It is extremely important to separate adequately the species of *Ixodes* before further epidemiological results are generated, which could be based on erroneous identification of these ticks.

The study of ticks in the Western Palaearctic region has become more focused on species with epidemiological significance, relegating poorly reported species to the category of neglected. There is a need to generate knowledge on the correct distribution of the taxa, something which can be done only after an adequate morphological and/or molecular complete characterization of these neglected species. This paper aims to morphologically re-describe two poorly known species of ticks commonly found on the European rabbit, namely *R. pusillus* and *I. ventalloi*, provide molecular details, and separate them from similar species. In addition to the morphological description, comparison with similar species, molecular features, and phylogenetic relationships of these two species of ticks are investigated, and neotypes are named and deposited for further studies.

Material and methods

The material used in this study was collected either in dens of the European rabbit or while feeding on the rabbits, in the period 1990–2010. Engorged larvae were always collected on *O. cuniculus*. Flat nymphs were obtained by allowing engorged larvae to molt in conditions of 24ºC and 80% relative humidity in complete darkness. Larvae were obtained from engorged females collected on *O. cuniculus*, allowed to oviposit at 24ºC and 80% of relative humidity. Larvae and nymphs were always mounted on slides in Hoyer's medium for measurements. Observations and measurements of immatures were done with a Nikon "Eclipse Ci-E" using the software NIS Elements. Adults were examined with a Nikon SMZ1270, using the same software for measurements. The total number of specimens examined and/or measured is indicated under each species and stage. Measurements are expressed in millimeters as minimum-maximum (mean).

The 16S rDNA sequences for *R. pusillus* and *I. ventalloi* were obtained following the methods and primers described by Mangold *et al.* (1998 a,b). The sequence of *R. pusillus* was obtained from adult specimens collected in a rabbit den in Pina de Ebro (Spain), 41º29'50''N, 0º29'12''W in May, 1990 (J.J. Osácar coll.). The sequence is deposited in GenBank under accession number KY231932. The sequence of *I. ventalloi* was obtained from adult specimens collected in a rabbit den in Almudévar (Spain) 42º03'41''N, 0º34'55''W (J.J. Osácar coll.). The sequence is deposited in GenBank under the accession number KY231931. Phylogenetic relationships were assessed using the maximum likelihood method (ML). Best fitting substitution models were determined with the Bayesian Information Criterion using the ML model test implemented in MEGA 5 (Tamura *et al*., 2011). The best substitution model for the phylogenetic tree constructed with 16S sequences was Tamura 3-parameter and a discrete Gamma-distribution (+G).

Rhipicephalus pusillus Gil Collado, 1936. Redescription

Male. (Figure 1, A–G). Ten specimens measured. Length from scapular apices to posterior idiosomal margin 1.48–1.74 (1.52) long, breadth 0.88–1.03 (0.92); ratio length/width 1.61–1.69 (1.65) (Fig. 1A). Eyes at a level slightly behind coxa II. Dorsal punctations small to medium, few and scattered over central portions of conscutum; more abundant in posterior part of conscutum, over posteromedian and posterolateral grooves. Cervical fields shallow but present. Marginal lines long, shallow and marked by some punctations, beginning behind eye level, and delimiting first festoon. Actual posterior limit of marginal lines usually diffuse and difficult to determine; occasionally enclosing one festoon on one side and two festoons on the other in the same specimen. Scapular areas and areas lateral to marginal lines relatively free of punctations. Posteromedian groove wide and longer than posterolateral grooves, which appear as irregular depressions with an oval or subcircular shape. Adanal plates two times longer than broad (Fig. 1B), curved inwards medially, without pointed projections in medial margin, with posterior margin widely convex. Accessory adanal plates slightly visible, with posterior margin not separated from

cuticle. Spiracular plates elongate (Fig. 1C), caudal prolongation narrow and straight, slightly visible from dorsal view, like small virgule, clearly smaller than size of each festoon. Perforations on spiracular plate very small, slightly denser in margins of plate, with narrow peripheral portion of plate which is devoid of perforations. A few slightly larger goblets present in central portions of spiracular plate, surrounding spiracle and extending over caudal prolongation.

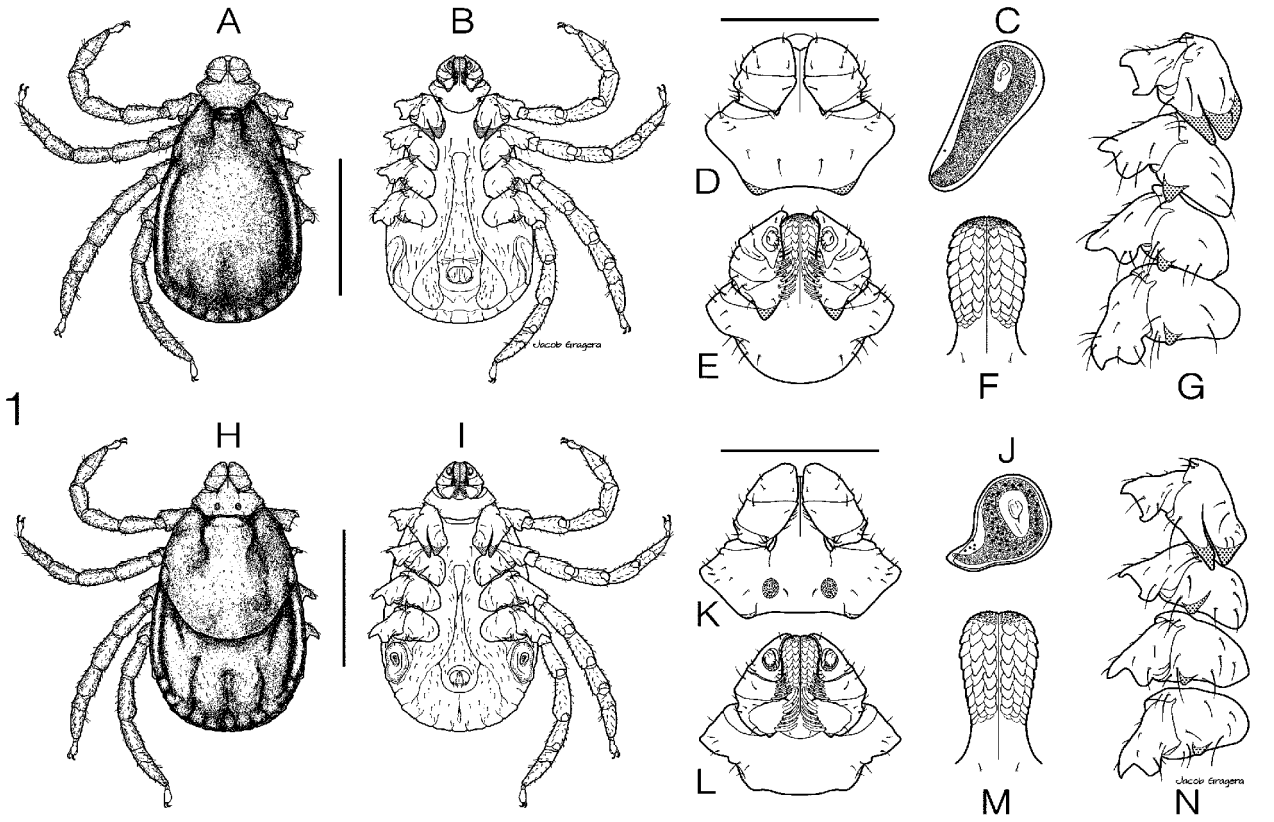


FIGURE 1. Adults of *R. pusillus*. A, male dorsal; B, male ventral. C, male, spiracular plate. D, male, gnathosoma, dorsal view. Bar length for figures A, B, H, and I: 1 mm. E, male, gnathosoma, ventral view. F, male, hypostome. Bar length for D and E: 0.4 mm. G, male, coxae and trochanters I to IV. H, female dorsal; I, female ventral. J, female, spiracular plate. K, female, gnathosoma, dorsal view; L, female, gnathosoma, ventral view. Bar length for K and L: 0.5 mm. M, female, hypostome. N, female, coxae and trochanters I to IV.

Gnathosoma dorsally (Fig 1D) slightly broader than long, but as long as broad in a few specimens. Dorsally, length from palpal apices to posterior margin of basis capituli 0.36–0.43 (0.41), breadth 0.42–0.45 (0.44). Ratio of gnathosomal length/width 0.92–0.95 (0.93). Basis capituli 0.19–0.22 (0.21) long. Basis capituli dorsally hexagonal. Basis capituli dorsally with postero-lateral margins concave, but in some specimens very slightly curved or even essentially straight. Cornua very small, 0.013 long. Ventrally, basis capituli with posterior margin broadly convex, with two large lateral processes that in some specimens may overlap the coxa I. Palpi 0.21–0.23 (0.21) long, 0.33–0.34 (0.34) broad, rounded apically. Ratio of palpal combined length/width 0.61–0.64 (0.63). Ventrally, palpal segment I with a triangular process posteriorly directed (Fig. 1E), covering most of ventral surface of first palpal segment. In most specimens, this palpal triangular process is slightly directed posterolaterally. Palpal segment III ventrally with short, very pointed, and essentially straight chitinous spur, slightly curved in some specimens, directed backward. Hypostome (Fig. 1F) 0.29–0.31 (0.29) long, 0.09–0.1 (0.1) broad. Ratio of hypostomal length/width 2.8–2.9 (2.9). Dental formula 3/3 with 8–10 rows of hypostomal teeth and a small apical corona.

Legs moderately short. Coxae I to IV of similar size (Fig. 1G). Coxa I with two large spurs, subparallel, internal spur almost two times wider and slightly shorter than external. Coxae II to IV with only external spurs, of slightly decreasing size from coxae II to IV. Trochanter I with very small spur, posteriorly directed.

Female. (Figure 1, H–N). Nine unengorged specimens measured. Length from scapular apices to posterior

idiosomal margin 1.52–1.89 (1.67), breadth 0.92–1.05 (1.01). Ratio of total length/width 1.61–1.69 (1.65) (Fig. 1H). Length of the scutum 0.90–0.95 (0.91), breadth 0.90–0.94 (0.92). Ratio of the scutal length/width 0.98–0.99 (0.98). Posterior margin of scutum widely rounded. In some specimens, posterior margin slightly sinuous. Eyes mildly convex, placed on widest part of scutum, enclosed by few very small punctations, mostly posteriorly. Cervical fields scalpel-shaped, narrowly elongate but disappearing posterior to eye level. Scapular areas smooth, without punctations; numerous small punctations centrally and posteriorly on scutum. Genital aperture small and U shaped, at level of coxa II. Spiracular plate widely rounded (Fig. 1J), with curved, dorsal process, only slightly visible dorsally. Perforations in spiracular plate very small, with few, larger goblets randomly placed around spiracular plate.

Gnathosoma clearly broader than long. Dorsally, length from palpal apices to posterior margin of basis capituli 0.53–0.57 (0.56), breadth 0.60–0.65 (0.61) broad. Ratio of gnathosomal length/width 0.91–0.92 (0.91). Basis capituli dorsally 0.23–0.29 (0.27) long (Fig. 1K). Basis capituli clearly hexagonal, with posterior and lateral margins straight. Basis capituli with lateral projections at about mid-length, acute. Porose areas very small, about 2.5–3 times their own diameter apart. Cornua short, length 0.02. Palpi short, 0.31–0.32 (0.31) long, 0.42–0.44 (0.42) broad, rounded apically. Ratio of combined palpal length/width 0.73–0.74 (0.73). Ventrally palpal segment I (Fig. 1L) with very broad triangular process posteriorly directed, covering most of ventral surface of palpal first segment, and carrying 6–8 barbed setae. Hypostome (Fig. 1M) 0.23 long, 0.12 broad, with dental formula of 3/3 with 8–10 rows of hypostomal teeth and small apical corona.

Legs moderately short. Coxae I to IV of similar size (Fig. 1N). Coxa I with two large spurs, subparallel, internal spur two times wider and slightly shorter than external spur. Coxae II to IV only with external spurs, of slightly decreasing size from coxae II to IV.

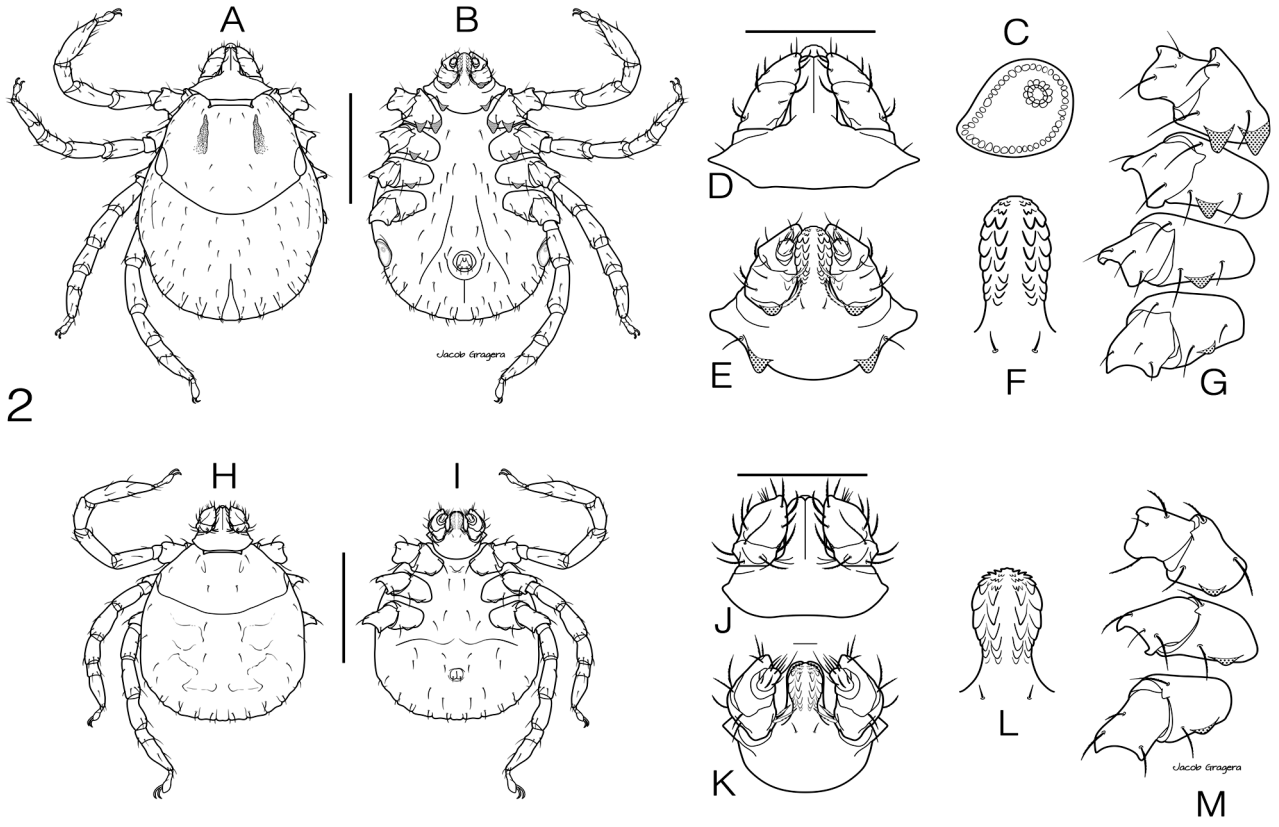


FIGURE 2. Immatures of *R. pusillus*. A, nymph dorsal; B, nymph ventral. Bar length for A and B: 0.4 mm. C, nymph, spiracular plate. D, nymph, gnathosoma, dorsal view; E, nymph, gnathosoma, ventral view. F, nymph, hypostome. Bar length for D and E: 0.2 mm. G, nymph, coxae and trochanters I to IV. H, larva dorsal; I, larva ventral. Bar length for H and I: 0.2 mm. J, larva, gnathosoma, dorsal view; K, larva, gnathosoma, ventral view. Bar length for J and K: 0.1 mm. L, larva, hypostome. M, larva, coxae and trochanters I to IV.

Nymph. (Figure 2, A–G). Twenty unengorged specimens measured, molted in the laboratory from fed larvae collected on *O. cuniculus*. Length from scapular apices to posterior idiosomal margin 0.84–0.93 (0.91), breadth 0.62–0.75 (0.71) (Fig. 2A). Ratio of total length/width 1.24–1.31 (1.28). Scutum 0.39–0.45 (0.40) long, 0.44–0.49 (0.46) broad. Ratio of scutal length/width 0.83–0.89 (0.86). Posterior margin of the scutum widely rounded. Eyes mildly convex, placed on widest part of conscutum, exactly on lateral angles of widely convex posterior conscutal margin. Cervical fields relatively long and narrow, depressed, reaching posteriorly to eye level. Ventral surface as illustrated (Fig. 2B). Spiracular plate widely rounded (Fig. 2C) with small and slightly pointed dorsal process, not visible dorsally. Spiracular plate with only one row of goblets located in periphery, with a few, larger goblets over spiracular plate.

Gnathosoma clearly broader than long, dorsally, length from palpal apices to posterior margin of basis capituli 0.23–0.26 (0.25), breadth 0.29–0.34 (0.33) (Fig. 2D). Ratio of gnathosomal length/width 0.72–0.75 (0.73). Basis capituli 0.10–0.11 (0.10) long. Basis capituli clearly hexagonal, posterior and lateral margins straight or slightly concave. Basis capituli with lateral projections at about mid-length, acute. Cornua absent. Palpi short, 0.14–0.16 (0.14) long, 0.22–0.25 (0.23) broad, tapered apically. Ratio of combined palpal length/width 0.59–0.60 (0.60). Ventrally (Fig. 2E) auriculae long, triangular and straight. Palpal segment I with broad and short triangular process posteriorly directed. Palpal segment III with chitinous ridge. Hypostome (Fig. 2F) 0.10 long, 0.06 broad (ratio 1.66); dental formula of 2/2 with 7–8 hypostomal teeth and an apical corona.

Legs moderately short. Coxae I to IV of similar size (Fig. 2G). Coxa I with two large spurs, separated, not parallel, internal spur almost same length and breadth as external spur, or slightly larger. Coxae II to IV only with external spurs, of decreasing size from coxae II to IV.

Larva. (Figure 2, H–M). All larvae were obtained from eggs laid by two engorged females collected on *O. cuniculus*. Measurements are based on 15 specimens. Because of the small variability in setal measurements, well below the fourth decimal figure, we refer only to the average of these measurements. Denominations of setae follows Clifford and Anastos (1960) and Clifford *et al.* (1973). Length from scapular apices to posterior idiosomal margin 0.30–0.35 (0.32), breadth 0.24–0.27 (0.26) (Fig. 2H). Ratio length/width 1.22–1.25 (1.23). Scutum clearly wider than long, 0.10–0.12 (0.11) long, 0.21–0.24 (0.22) broad. Ratio scutal length/width: 0.49–0.50 (0.50) Posterior margin widely rounded in its median portion, with posterolateral margins essentially straight. Eyes small, placed on widest part of conscutum, exactly on lateral angles of posterior conscutal margin. Scutal setae: 3 pairs (Sc1–Sc3), measuring 0.016, 0.015, and 0.018, respectively. Alloscutal setae: 12 pairs. Four pairs of central dorsal setae (Cd1: 0.037, Cd2: 0.034, Cd3: 0.039, Cd4: 0.037), 7 pairs of marginal dorsal setae (Md1: 0.092, Md2: 0.096, Md3: 0.099, Md4: 0.066, Md5: 0.069, Md6: 0.059; Md7: 0.044) and 1 pair of supplementary setae (S: 0.051). Ventral surface as illustrated (Fig. 2I) with 13 pairs of setae plus 1 pair on the anal valves, i.e. 3 sternal setae (St1: 0.02, St2: 0.02, St3: 0.022), with St1 located below level of coxae I, St2 well below of coxae II and St3 between coxae III, 2 pre-anal pairs (Pa1: 0.034, Pa2: 0.034), 4 premarginal pairs (Pm1: 0.034, Pm2: 0.035, Pm3: 0.029, Pm4: 0.031), and 4 marginal ventral pairs (Mv1: 0.03, Mv2: 0.03, Mv3: 0.031, Mv4: 0.029).

Gnathosoma broader than long, dorsally, length from palpal apices to posterior margin of basis capituli 0.10–0.11 (0.10), breadth 0.11–0.12 (0.12) (Fig. 2J). Ratio gnathosomal length/width 0.90. Basis capituli 0.04 long. Basis capituli clearly rectangular, with posterior margin straight or slightly concave, then widely convex to blunt lateral projections at about mid-length. Palpi short, 0.08 long, 0.03 broad, tapered apically. Ventrally (Fig. 2K) palpal segment I with short chitinous ridge. Palpal segment III with faint chitinous ridge. Ventral basis capituli with two pairs of post-hypostomal setae (Ph1-Ph2), distance between Ph1 0.04 and Ph2 0.03; posterior margin of ventral basis capituli widely round. Auriculae absent. Hypostome (Fig. 2L) 0.04 long, 0.02 broad, with dental formula of 2/2 in every row but apical corona, with rounded apex, and all denticles sharply pointed, 8/10 denticles in row I, 7 in row II. Palpal setae: 7 on genu, i.e. 1 paraxial (Gp1), 2 antiaxial (Ga1–Ga2), 3 dorsal (Gd1–Gd3) and 1 ventral (Gv1); 6 on femur, i.e. 1 paraxial (Fp1), 2 antiaxial (Fa1–Fa2), 1 dorsal (Fd1) and 2 ventral (Fv1–Fv2); 10 on tibiotarsus (Ttt1–Ttt10); trochanter 0.

Legs moderately short. Coxae I to IV of similar size (Fig. 2M). Every coxa with one internal spur, coxa I spur largest. Coxa I with 3 setae, 1 anterior (CIa), 1 posterior (CIp) and 1 paraxial (CIpa); coxae II and III each with 1 anterior setae and 1 posterior seta; trochanters without spurs. Tarsus I length 0.20, width 0.09. Setae on tarsus I: group I 2, dorsal; 4 setae in group II – 2 dorsal (dII1, dII2), 1 lateral antiaxial (laII1) and 1 lateral paraxial (lpII1); 4 dorsal in group III (dIII1–4); groups IV and V dorsal setae absent; 2 dorsal in the group VI (dVI1–2). Ventral and lateral setae: 4 setae in group I – 2 ventral (VvI1–2); 2 lateral antiaxial (laI1) and lateral paraxial (lpI1); 4 setae in group II – 2 ventral (VvII1–2); 1 lateral antiaxial (laII2) and 1 lateral paraxial (lpII2); 4 setae in group III – 2

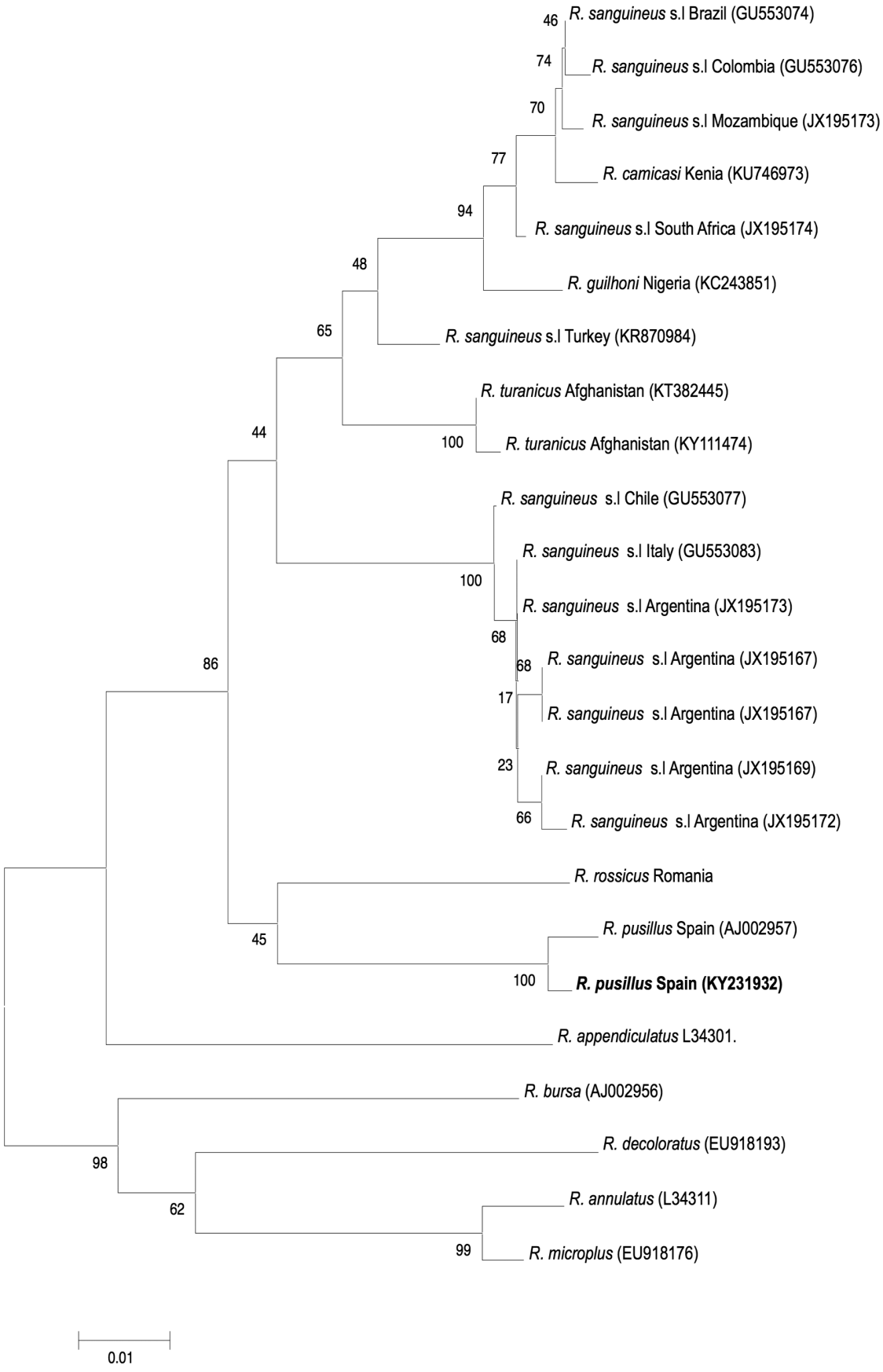
ventral (VvIII1–2); 2 lateral antiaxial (laIII1) and lateral paraxial (lpIII1). Haller's organ with 3 phs setae plus 4 apparent setae in capsule.

Molecular features. The phylogenetic tree obtained from the 16S sequence places this tick in a large cluster that contains the *R. sanguineus* group. It is however separated from *Rhipicephalus bursa* Canestrini & Fanzago, 1878, in which *R. pusillus* was placed after its original description as *R. bursa pusillus*. *Rhipicephalus pusillus* clusters apart from several species of the *R. sanguineus* group, near *Rhipicephalus rossicus* Yakimov and Kohl-Yakimova, 1911.

Hosts and distribution. *Rhipicephalus pusillus* is presumed to be a three-host tick (Walker *et al*., 2000). The European rabbit (*Oryctolagus cuniculus*) is its primary host but the tick has also been reported feeding on other hosts summarized by Walker *et al.* (2000). The original description by Gil Collado (1936) is based on two females collected on *Vulpes vulpes*, which seems either a consequence of predatory habits or the use by the fox of an abandoned rabbit den. The preferences of attachment sites on the rabbit and the seasonal dynamics on *O. cuniculus* have been reported by Márquez and Guiguen (1992). This tick has never been collected by dragging, and the extensive data provided by Osácar (1992) on ectoparasites of rabbits, reported *R. pusillus* as an exclusively endophilous species. *Rhipicephalus pusillus* has been reported to feed extensively on livestock and *C. elaphus* in Spain (Ruiz-Fons *et al*., 2006) and Walker *et al*. (2000) mentioned a female collected "on vegetation". Guglielmone *et al*. (2014) consider birds to be valid but unusual hosts for this tick. The distribution of the tick is related to the original distribution of the European rabbit, including the Iberian Peninsula, southern France and Italy, Morocco and Tunisia (summarized by Walker *et al*., 2000).

Diagnosis and species relationships. Molecular features of 16S rDNA confirm that *Rhipicephalus pusillus* is close to species that cluster into the *R. sanguineus* group. It is however not closer to *R. bursa*, the species for which it was originally described as subspecies, than to other species of the genus. While the specific epithet makes reference to its small size, identification of adults of this species based on size is not advisable, because specimens of other species of the genus may be abnormally small due to poor nymphal feeding period. The male has the dorsal surface with small and scattered dorsal punctations, with relatively short marginal grooves, not reaching the eyes, and reaching the first festoon (in a few specimens, marginal grooves do reach the second festoon). Ventrally, the male of *R. pusillus* has wide adanal plates, two times longer than wide, internally concave and widely rounded posteriorly, without an internal spur, and accessory adanal plates difficult to observe and not projecting over the cuticle. The spiracular plates have a long narrow, straight process, which is slightly visible in a dorsal view in most (but not all) specimens. Coxae II to IV have only external spurs, which is diagnostic. The female has very small porose areas, separated by 2.5–3 diameters, very short cornua, and a large triangular spur on the ventro-internal side of the palpal article I, carrying 6–8 barbed setae, which is diagnostic. The nymph is unique in the ventral aspect of the basis capituli. The large auriculae of the nymphs of *R. pusillus* are also present in nymphs of *R. rossicus* and *R. turanicus* Pomerantzev, 1940. However, *R. pusillus* has two large spurs on coxa I, separated, not parallel, internal spur being slightly larger or of similar size than internal spur. The nymph of *R. rossicus* has external coxal spurs only on coxae I–III. The nymph of *R. turanicus* has the internal spur on coxa I clearly smaller than the external. The larva of *R. pusillus* is more difficult to separate from close species, but has chitinous ridges on the ventral side of palpal segment I (absent in close species), lacks auriculae, and has a chitinous ridge on coxa III (absent in *R. sanguineus* s.l.).

Specimens examined. The type specimens of *R. pusillus* are lost (Sonia Olmeda, Faculty of Veterinary Medicine, Madrid, Spain, pers. comm.) therefore precluding further comparison with other specimens of other species. We therefore proceeded to the naming and deposition of a neotype. The neotype specimen is a male, collected in a burrow of *Oryctolagus cuniculus* in Pina de Ebro, 41º29'50''N, 0º29'12''W (Spain), about 300 km away from the original type locality, by J.J. Osácar in May, 1990 (the original female holotype was collected on a fox, but European rabbit is widely accepted as its typical host). The specimen is deposited in the U.S. National Tick Collection, with accession number USNMENT00862202. The remaining specimens of this collection, 10 females, 8 males, 20 nymphs and 50 larvae were used to complete the redescription. Additionally, 3 females and 12 males (collected from *O. cuniculus*, in Almudévar, Spain, 42º03º41''N, 0º34'55''W, J.J. Osácar coll.) and 19 semi-engorged females and 24 males (collected from *O. cuniculus*, in El Rocío, Spain, 37º07'30''N, 6º28'05''W, R. Segura coll.) were used to complete the redescription. Flat nymphs were obtained from engorged larvae collected with the same data as the neotype. Newly hatched larvae were obtained from several (uncounted) females collected on *O. cuniculus* with the same details as the neotype. Specimens other than the neotype are deposited in the collection of the Faculty of Veterinary Medicine of Zaragoza (Spain) under the accession number FMV1411.



3

FIGURE 3. Phylogenetic tree for *R. pusillus* based on 16S rDNA.

Ixodes ventalloi Gil Collado, 1936. Redescription

Male (Figure 4 A–F). Five specimens measured, collected in a burrow of *Oryctolagus cuniculus*. Idiosoma elongate-oval (Fig. 4A), with broadly rounded posterior margin, widest at level of spiracular plates. Length from scapular apices to posterior idiosomal margin 3.14–3.23 (3.15), breadth 2.22–2.29 (2.25), ratio of idiosomal length/width: 1.4–1.5 (1.4). Punctations numerous and shallow centrally and more numerous laterally along marginal groove. Scapulae short, bluntly rounded. Lateral carinae absent. Eyes absent. Cervical grooves marked, as two divergent lines. Conscutal setae numerous, long 0.20–0.26 (0.25), white. Male with variable number of rows of conscutal setae at lateral margins of body, with most specimens having two rows. Venter (Fig. 4B). Length and breadth of each ventral plate: pregenital 0.39–0.42 (0.37) x 0.11–0.15 (0.13) (ratio, 2.79–2.85, mean: 2.84), median 1.33–1.41 (1.37) x 1.11–1.15 (1.13) (ratio, 1.20–1.22, mean: 1.20), adanal 0.53–0.58 (0.56) x 0.26–0.28 (0.26) (ratio: 2.15), anal 0.35–0.37 (0.35) x 0.31–0.37 (0.33) (ratio: 1.06). Genital aperture at level of coxae III. Spiracular plate (Fig. 4C) broadly oval, 0.42 long, 0.38 wide (ratio length/width: 1.1) with goblets in four concentric rows and one additional row of punctations around margin of plate.

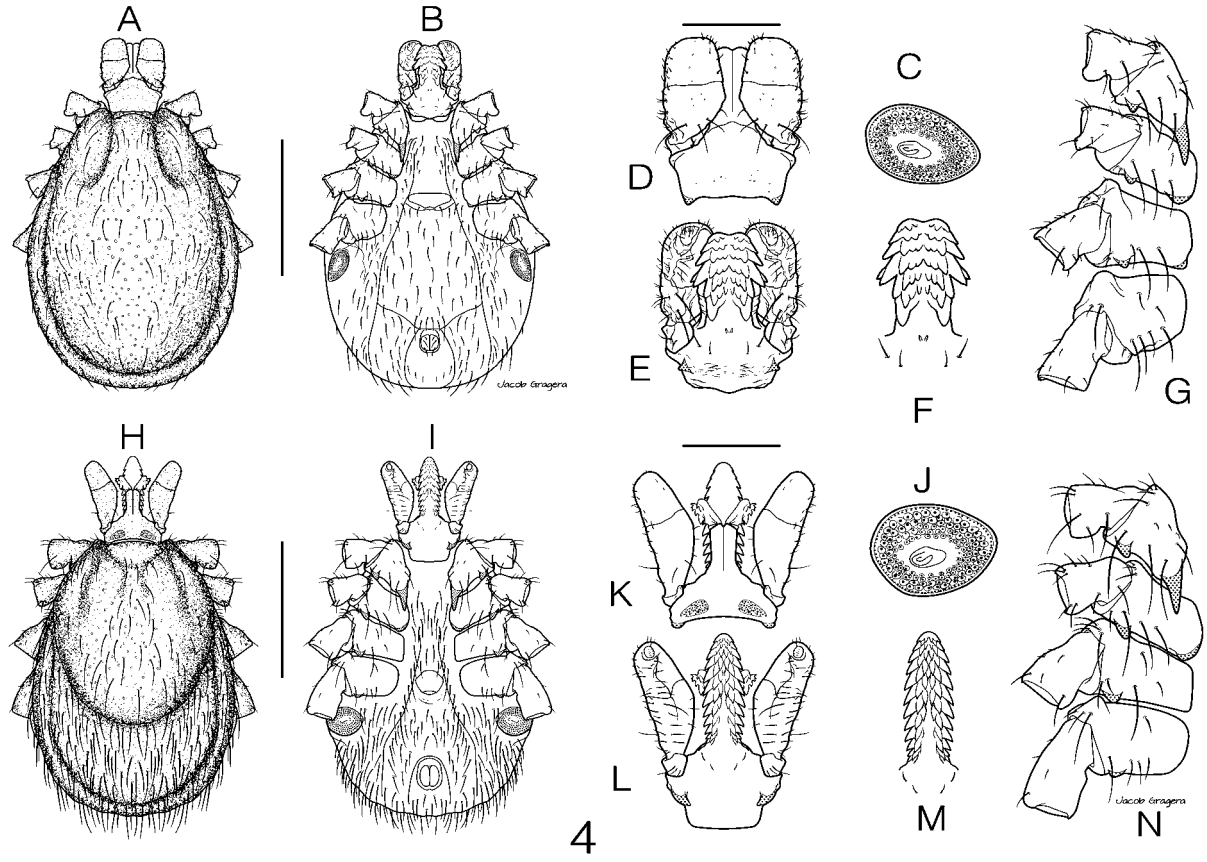


FIGURE 4. Adults of *I. ventalloi*. A, male dorsal; B, male ventral. Bar length for A and B: 1.5 mm. C, male, spiracular plate. D, male, gnathosoma, dorsal view; E, male, gnathosoma, ventral view. Bar length for D and E: 0.25 mm. F, male, hypostome. G, male, coxae and trochanters I to IV. H, female dorsal; I, female ventral. Bar length for A and B: 1.5 mm. J, female, spiracular plate. K, female, gnathosoma, dorsal view; L, female, gnathosoma, ventral view. Bar length for K and L: 0.25 mm. M, female, hypostome. N, female, coxae and trochanters I to IV.

Gnathosoma dorsally (Fig. 4D), length from palpal apices to posterior margin of basis 0.42–0.52 (0.45). Basis capituli 0.24–0.27 (0.25) long, 0.32–0.39 (0.37) broad, ratio length/width: 0.67, posterior margin essentially straight, external margins diverging anteriorly; cornua present but small, triangular, slightly diverging laterally. Ventral outline of gnathosoma as illustrated (Fig. 4E) with small and laterally projected auriculae and central projection produced posteriorly. Palpi length 0.31–0.34 (0.32), breadth 0.14–0.16 (0.15), ratio length/width of palpi: 2.13. Setae as illustrated, with several long setae on dorsal surface of palpal article II. Hypostome (Fig. 4F) 0.15–0.19 (0.16) long, 0.12–0.14 (0.13) broad (ratio length/width 1.23), with a dental formula of 4/4 in most rows,

with 6–8 hypostomal teeth, with irregular rows of median very small crenulations to base, lateral denticles larger and more angular than those in internal rows, basal lateral denticles lobate rather than angular.

Legs moderately long. Coxa I (Fig. 4G) with one long internal and one short external spur. Internal spur on coxa I almost straight reaching over as much as half of coxa II. Coxa II with short external and internal spurs. Coxa III with external and internal spur of subequal length. Coxa IV with small external chitinous ridge, not visible in all specimens.

Female (Fig. 4, H–N). Eight specimens measured. Length from scapular apices to posterior idiosomal margin 4.21–4.91 (4.68), breadth 2.94–3.01 (2.97), ratio idiosomal length/width 1.57. Scutum (Fig. 4H) outline broadly rounded, 2.99–3.57 (3.49) long, 2.72–2.99 (2.81) broad, ratio length/width scutum: 1.19–1.23 (1.24). Cervical grooves distinct, appearing as almost parallel or slightly divergent posteriorly integumental depressions, indicated by more dense punctations. Punctations scattered and more abundant, smaller and deeper in periphery of scutum. Setae abundant, very long, same size as other idiosomal setae, randomly distributed, more abundant as longitudinal rows in central portion of scutum, less numerous on lateral regions. Marginal grooves distinct. Alloscutum with abundant long setae. Setae placed on posterior margin of alloscutum are easily seen from ventral view of specimens. Ventral surface as illustrated (Fig. 4I). Genital aperture at level of coxae IV (Fig. 4I). Spiracular plate (Fig. 4J) sub-circular, 0.39 long, 0.40 wide (ratio length/width: 0.92).

Gnathosoma: Dorsally, length from palpal apices to posterior margin of basis 0.43–0.49 (0.45). Basis capituli dorsally (Fig. 4K) 0.12–0.15 (0.13) broad; ratio gnathosomal length to basis capituli width 3.2–3.5 (3.4). Posterior margin of basis capituli dorsally broadly concave, cornua small. Porose areas irregularly ovoid, anterior margin broadly rounded, posterior margin essentially straight and well delineated, separated by distance slightly larger than breadth of each area. Pores in porose areas irregularly placed. Basis capituli ventrally (Fig. 4L) with posterior margin straight; transverse suture unapparent. Auriculae very large, hook like, internally curved, almost half length of ventral basis capituli. Palpi 0.33–0.39 (0.35) long, 0.05 broad (ratio length/width palpi: 2.33). Hypostome (Fig. 4M) 0.33–0.38 (0.35) long, 0.08 broad, ratio length/width: 4.3. Apex bluntly pointed, corona with few fine denticles, dental formula 3/3 for anterior half, then 2/2 for basal half of hypostome, with 5–11 rows of hypostomal teeth. Medial basal teeth smaller, all denticles sharply pointed.

Legs moderately long, slender, coxae I–IV (Fig. 4N) each with small external spur, that of coxa II longest. Coxa I with long, essentially straight, pointed internal spur, slightly reaching the half of the coxa II. An internal chitinized ring in coxa II. Internal spur or traces of chitinized rings absent on coxae III and IV. Trochanters without spurs.

Nymph (Figure 5, A–F). Eighteen specimens measured. Idiosoma. Dorsally (Fig. 5A) length from scapular apices to posterior idiosomal margin 0.70–0.79 (0.73), width 0.43–0.49 (0.45), ratio length/width 1.62. Scutum broadly pyriform, longer than broad, length 0.57–0.62 (0.59), width 0.54–0.56 (0.55), ratio length/width 1.07; scapulae short, rounded; cervical grooves smooth, widely divergent; lateral carinae slightly visible. Length of median scutal setae: 0.03–0.04 (0.04), length of median alloscutal setae 0.21–0.25 (0.24), length of lateral alloscutal setae 0.12–0.16 (0.14). Alloscutal setae around 5 times longer than median scutal setae. Ventral view as illustrated (Fig. 5B). Anal groove slightly converging posteriorly. Spiracular plates widely rounded, length 0.14–0.17 (0.16), width 0.11–0.12 (0.12), ratio length/width of the spiracular plate: 1.33.

Gnathosoma. Total length (dorsal view) from palpal apices to base of gnathosoma 0.42–0.49 (0.48). Basis capituli triangular (Fig. 5C), width (measured dorsally) 0.29–0.32 (0.31). Ratio gnathosoma length to basis capituli width 1.58. Posterior margin of basis capituli straight; cornua small, narrow, posterolaterally produced, sharply pointed. Lateral margins of base of gnathosoma ventrally convergent from palpal insertions to auriculae (Fig. 5D), then deeply concave, then slightly diverging to posterior margin. Auriculae poorly defined, blunt. Palpi long and internally excavated, combined length 0.33–0.34 (0.33); palpal article I length 0.061; palpal article II length 0.15–0.16 (0.16); palpal article III length 0.13–0.14 (0.13). Combined palpal maximum width 0.15–0.16 (0.15). Article II slightly longer than III. Hypostome (Fig. 5E) length 0.25–0.29 (0.28), width 0.085, (ratio length/width 3.3) with blunt apex; dental formula essentially 3/3 at anterior half of its length, then 2/2 to base, with all denticles sharply pointed.

Legs (Fig. 5F) moderately long. Coxa I with external spur, short and straight, and with wider, sharply pointed, straight internal spur reaching coxa II. Internal spur about twice length of external spur. External spurs of coxae subequal in size, external spur on coxa IV slightly smaller. No spurs on trochanters. Haller's organ with 6 prehalleral setae plus 6 apparent setae in capsule.

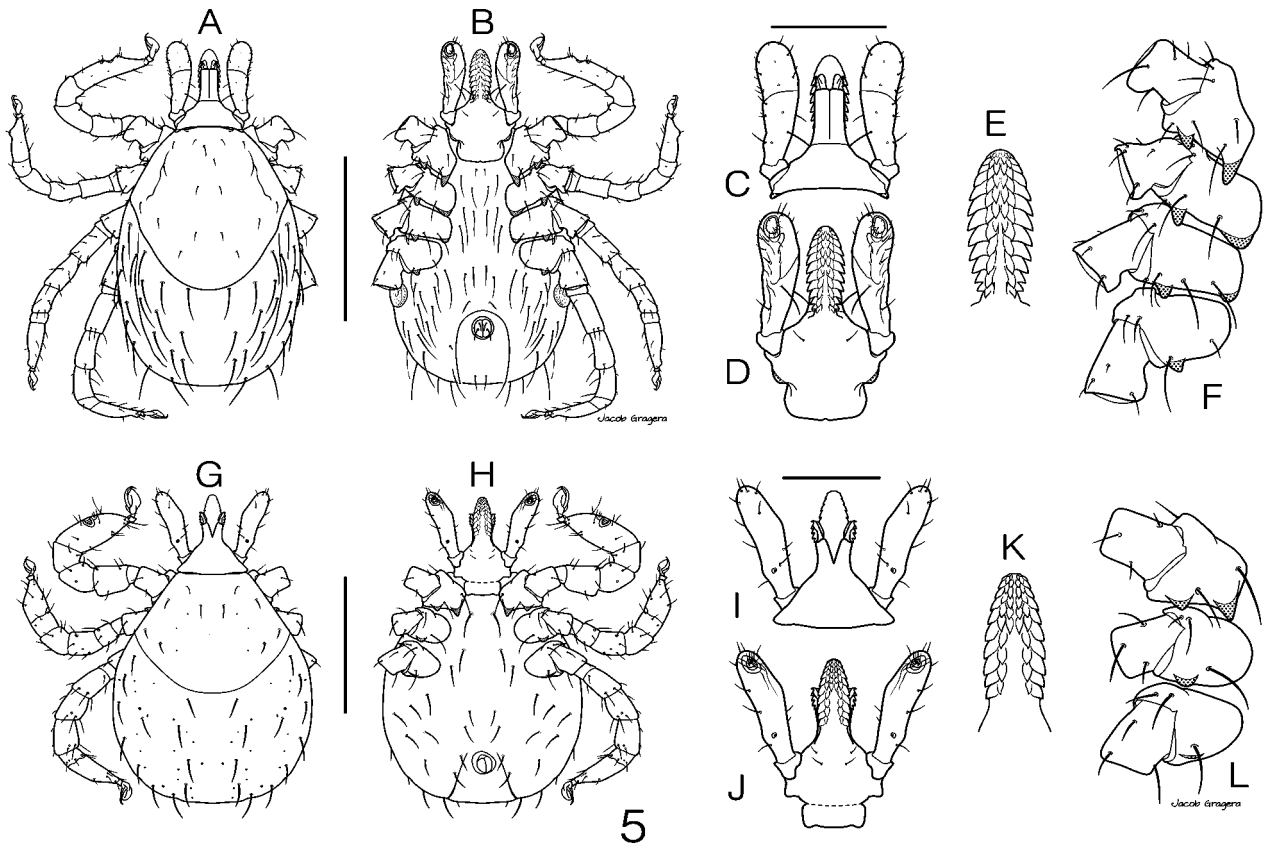


FIGURE 5. Immatures of *I. ventalloi*. A, nymph dorsal; B, nymph ventral. Bar length for A, B: 0.6 mm. C, nymph, spiracular plate. D, nymph, gnathosoma, dorsal view; E, nymph, gnathosoma, ventral view. Bar length for C, D: 0.3 mm. F, nymph, hypostome. G, nymph, coxae and trochanters I to IV. H, larva dorsal; I, larva ventral. Bar length for G and H: 0.4 mm. J, larva, gnathosoma, dorsal view; K, larva, gnathosoma, ventral view. Bar length for I, J: 0.2 mm. L, larva, hypostome. M, larva, coxae and trochanters I to IV.

Larva (Figure 5, G–L). All larvae were obtained from eggs laid by two engorged females. Measurements are based on 14 specimens. Because of the very small variability in setal measurements, well below the fourth decimal figure, we refer only to the average of these measurements. Idiosoma. Dorsal surface oval (Fig. 5G). Sensilla sagittiformia (large wax gland) absent. Scutum broadly rounded, clearly wider than long, length 0.52–0.58 (0.56), width 0.41–0.45 (0.44), ratio length/width: 1.23–1.28 (1.27); integument with irregular hexagonal ornamentation, punctations sparse; eyes absent. Scutal setae: 5 pairs (Sc1–Sc5), measuring 0.017, 0.017, 0.017, 0.019, and 0.014, respectively. Alloscutal setae: 12 pairs. Four pairs of central dorsal setae (Cd1: 0.035, Cd2: 0.031, Cd3: 0.04, Cd4: 0.04), 7 pairs of marginal dorsal setae (Md1: 0.09, Md2: 0.092, Md3: 0.091, Md4: 0.053, Md5: 0.053, Md6: 0.039; Md7: 0.032) and 1 pair of supplementary setae (S: 0.046). Ventral surface (Fig. 5H) with 13 pairs of setae plus 1 pair on anal valves, i.e. 3 sternal setae (St1: 0.03, St2: 0.025, St3: 0.027), with St1 located below level of coxae I, St2 below of coxae II and St3 between coxae III, 2 pre-anal pairs (Pa1: 0.031, Pa2: 0.03), 4 premarginal pairs (Pm1: 0.03, Pm2: 0.021, Pm3: 0.026, Pm4: 0.029), and 4 marginal ventral pairs (Mv1: 0.029, Mv2: 0.03, Mv3: 0.025, Mv4: 0.029). Anal groove divergent (Fig. 5H).

Gnathosoma. Basis capituli (Fig. 6I) triangular, with posterior margin essentially straight and cornua absent, length 0.21–0.24 (0.22), width 0.15 (measured dorsally). Ratio length/width 1.4. Palpi length 0.10–0.13 (0.11), width 0.04, ratio length/width: 2.7) not internally excavated, suture between article II and III inconspicuous, Ventral basis (Fig. 5J) with 2 pairs of posthypostomal setae (Ph1–Ph2), distance between Ph1 0.04 and Ph2 0.03; posterior margin of ventral part of gnathosoma sinuous; auriculae long, acute, projected laterally; hypostome (Fig. 5K) total length from apex to Ph1 setae 0.09, width 0.040 (ratio length/width: 2.25), with rounded apex; dental formula 3/3 to mid-length, then 2/2 to base, with all denticles sharply pointed, 9/10 denticles in row I, 9 in row 2 and 6 in row 3. Palpal setae: 7 on genu, i.e. 1 paraxial (Gp1), 2 antiaxial (Ga1–Ga2), 3 dorsal (Gd1–Gd3) and 1

ventral (Gv1); 6 on femur, i.e. 1 paraxial (Fp1), 2 antiaxial (Fa1–Fa2), 1 dorsal (Fd1) and 2 ventral (Fv1–Fv2); 10 on tibiotarsus (Ttt1–Ttt10); trochanter 0.

Legs. Coxa I with 2 triangular spurs; internal longer (Fig. 5L). Coxa II with single external spur, shorter than homologous spur of coxa I. Coxa III with chitinous ridge. Coxa I with 3 setae, 1 anterior (CIa), 1 posterior (CIp) and 1 paraxial (CIpa); coxae II and III each with 2 anterior setae and 1 posterior seta; trochanters without spurs. Tarsus I length 0.21, width 0.07. Setae on tarsus I: group I absent; 4 setae in group II–2 dorsal (dII1, dII2), 1 lateral antiaxial (laII1) and 1 lateral paraxial (lpII1); 4 dorsal in group III (dIII1–4); groups IV and V dorsal setae absent; 2 dorsal in group VI (dVI1–2). Ventral and lateral setae: 4 setae in group I – 2 ventral (VvI1–2); 2 lateral antiaxial (laI1) and lateral paraxial (lpI1); 4 setae in group II – 2 ventral (VvII1–2); 1 lateral antiaxial (laII2) and 1 lateral paraxial (lpII2); 4 setae in group III – 2 ventral (VvIII1–2); 2 lateral antiaxial (laIII1) and lateral paraxial (lpIII1). Haller's organ with 5 prehalleral setae (phs) plus 3 apparent setae in capsule.

Specimens examined. According to Santos Dias (1985) and Guglielmone *et al.* (2014) the types of *I. ventalloi* were deposited in the collection of the Museo de Ciencias Naturales (Barcelona, Spain). However, a search of the type collection in the museum yielded negative results (Gloria Masò, curator of Arthropods Collection, pers. comm.) therefore precluding further comparison with other collections. We therefore proceed to the naming and deposition of a neotype. The neotype specimen is a female, collected in a burrow of *Oryctolagus cuniculus* in Zuera, 41º88'N, 0.75ºW, Zaragoza (Spain) by J.J. Osácar in April, 1991, about 350 km from the original type locality (the host of the original type was also *O. cuniculus*). The female neotype is deposited in the U.S. National Tick Collection under the accession number USNMENT00862203. The rest of the specimens of this collection, including 18 females, 12 males, 14 nymphs and 114 larvae (the progeny of one engorged female collected on one rabbit in the same burrow) were used to complete the redescription and the illustrations, and obtain additional measurements. These specimens are deposited in the collection of the Faculty of Veterinary Medicine of Zaragoza (Spain) with the accession number FMV1109. Further specimens, including 2 females and 81 nymphs were obtained from the surroundings of Palermo, 38º11'N, 13.36'E (Sicily, Italy) to check for coherence of the morphological details. These specimens are deposited in the collection of the Faculty of Veterinary Medicine of Zaragoza (Spain) with the accession number FMV1317.

Molecular features. As expected, the 16S rDNA sequence of *I. ventalloi* from Spain (Genbank accession number: KY231931) are grouped with those of *I. ventalloi* from Italy (Fig. 6). The 16S rDNA sequence from Spain is closest to genogroup A from Italy (haplotypes 1 and 2) described by Latrofa *et al*. (2016). The 16S sequences of Latrofa *et al*. (2016) available in Genbank (KU178956-KU178963) are 275-bp in length. For that reason, although we obtained a 16S sequence of 410-bp for *I. ventalloi*, the alignment used in this phylogenetic analysis consisted of 275 bp fragments. *Ixodes ventalloi* clustered near other species of the *I. ricinus* group, but seems to be unrelated to these species and in a different clade, which is in concordance with the morphological features described above.

Hosts and zoogeography. *Ixodes ventalloi* is a Palearctic species found in southwest Iberian Mediterranean sclerophyllous and mixed forests, northwest Iberian montane forests, and Iberian conifer forests (Guglielmone *et al*., 2014). It is found in Northern Africa, the Iberian Peninsula, southern France, Cyprus, southern Italy, Portugal, and Spain (Chastel *et al*., 1984; Estrada-Peña *et al*., 1984; Santos Dias and Santos Reis, 1989; Santos Silva *et al*., 2011; Ioannou *et al*., 2009; Mori *et al*., 2015; Latrofa *et al*., 2016) and introduced into Germany (Petney *et al*., 1996) and the United Kingdom (Arthur, 1957, as *I. festai* Tonelli-Rondelli, 1926; Jameson and Medlock, 2011). No additional surveys have been carried out in the last two regions to conclude if they have permanent populations in sites outside of the main distribution range. All developmental stages of *I. ventalloi* have been found on lagomorphs, carnivores and rodents. This species can also be collected on different carnivores such as weasel (*Mustela nivalis*), Iberian lynx (*Lynx pardinus*), red fox (*Vulpes vulpes*), cats, and Egyptian mongoose (*Herpestes ichneumon*) (Santos Dias & Santos Reis, 1989; Petney *et al*., 1996; Millán *et al*., 2007), and on birds such as the red-legged partridge (*Alectoris rufa*) and chukar partridge (*Alectoris chukar*) (Estrada-Peña *et al*. 1984; Ioannou *et al*. 2009). It has also been found on the crested porcupine (*Hystrix cristata*) (Mori *et al*. 2015), hedgehogs (*Erinaceus europaeus*) (Domínguez, 2014) and humans (Gilot and Marjolet, 1982). It is however problematic to ascertain the validity of some of these records, because of the diagnostic uncertainty of *I. ventalloi*, *I. ricinus* and *I. festai* (Gilot and Pérez, 1978) and the impossibility to find and recheck these published records. *Ixodes ventalloi* could be thus considered a species whose distribution overlaps the original distribution of the European rabbit, including the Iberian Peninsula, southern France and Italy, including the island of Sicily.

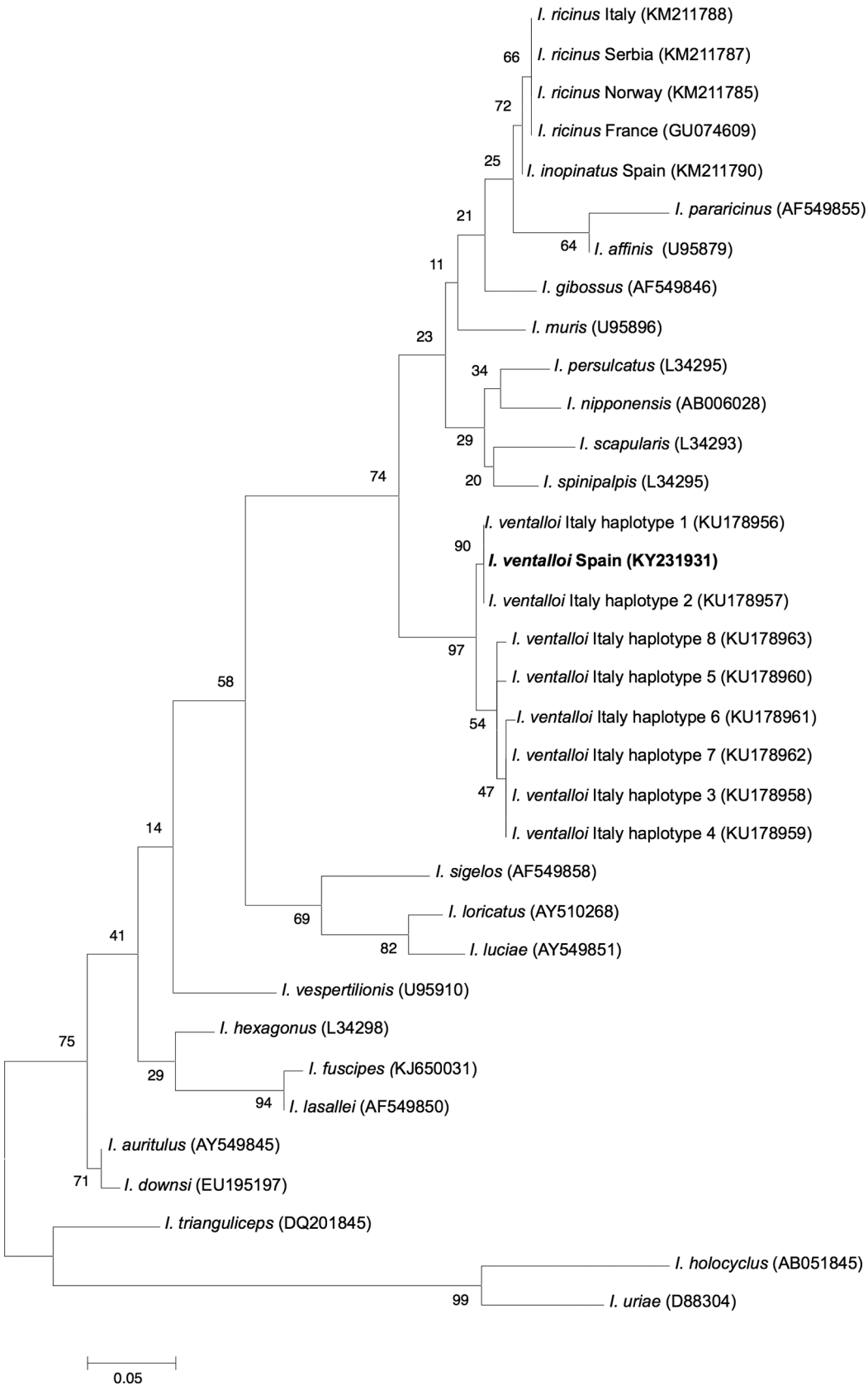


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FIGURE 6. Phylogenetic tree for *I. ventalloi* based on 16S rDNA.

Diagnosis and species relationships. A small sized *Ixodes*. The female is characterized by: elliptical porose areas with irregular disposition of the pores, and relatively superficial and small alloscutal punctations, mainly concentrated in the lateral parts of the scutum; scutal and alloscutal setae long and approximately of similar length; internal spur on coxa I straight and barely reaches over half of coxa II; apparent external spurs on coxae I to IV; marginal groove evident; and dental formula 3/3+2/2. The distinctive feature of the female is the long and curved auriculae, which are absent in every other species of *Ixodes* reported in the Western Palearctic. The original illustration by Gil Collado (1936), shows the ventral view of an engorged female with a curved internal spur on coxa I, which does not reach coxa II (probably because engorgement of the female distorted the proportions). The male is characterized by its relatively long conscutal setae and the presence of deep and distinct cervical fields, which have a few setae. The nymph is characterized by: presence of a broadly pyriform scutum; lateral carinae slightly apparent; cornua small, almost inapparent; hypostome with blunt apex, presenting dental formula 3/3 at more than half of hypostomal length, then 2/2 to base, all denticles sharply pointed; anal groove slightly converging posteriorly; alloscutal setae abundant, long, and about 6 times longer than those on scutum; and internal spur of coxa I reaching coxa II. The larva has: scutum posteriorly rounded; Md1-Md3 setae the same size as the other setae, which are only slightly longer than scutal setae; cornua present; auriculae long; hypostome with apex clearly rounded with dental formula 3/3 to mid-length, then 2/2 to base; and anal groove clearly divergent. In addition to this unique morphology, the molecular features clearly separate *I. ventalloi* from the remaining tick species of the *I. ricinus* group. The morphological features of the specimens collected with the neotype do not differ significantly from the details provided by Latrofa *et al*. (2016) except in the internal spur of coxa I of the female, which is only slightly curved in all the females examined for this redescription.

Considerable confusion exists regarding the identity of *I. ventalloi*, *I. ricinus* (Linnaeus, 1758), *I. festai*, the recently described *I. inopinatus* Estrada-Peña, Nava & Petney, 2016, *I. acuminatus* Neumann, 1901, and *I. bivari* Santos-Dias, 1990. Santos Silva *et al*. (2011) expressed reservation about the validity of the latter species. Few details are known for *I. festai*. This species was described from engorged females collected from several localities in northern Africa on ground-feeding birds. Most of the confusion arose with the illustrations and short re-description of the species by Colas-Belcour and Rageau (1951). The confusion was reinforced by the descriptions by Hoogstraal (1959) without illustrations, and Arthur (1957, 1958, 1961). The texts and illustrations of these studies refer without question to *I. ventalloi*. Illustrations of *I. festai* and *I. ventalloi* in Cringoli *et al*. (2005) represent *I. ventalloi*. The type specimen of *I. festai* is an engorged female, therefore having distorted body proportions, which lacks many critical structures such as the hypostome and parts of the capitulum. The female of *I. festai* was separated from *I. ventalloi* by Gilot and Pérez (1978) based on the shape and relative length of the auriculae, but no clear details are provided for other critical features and no comparison with types was provided. The male of *I. festai* was briefly described by Pérez (2007) and redescribed by Contini *et al.* (2011). Both descriptions lack important details. However, from the illustrations in these reports the male shows several details that are similar to the male of *I. ventalloi*, as redescribed in this study.

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