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Original article

Ixodes inopinatus – Occurring also outside the Mediterranean region

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ABSTRACT

We report the presence of *Ixodes inopinatus* and its sympatric occurrence with *Ixodes ricinus* in southeastern Germany, western Austria, and Romania. The identification of *I. inopinatus* was based on morphological and molecular 16S rRNA and 12S rRNA gene features. We also report the finding of *Rickettsia monacensis* and *Rickettsia helvetica* in *I. inopinatus* collected from a fox and a sheep in Romania. Although the vector competence of *I. inopinatus* for these pathogens remains to be proven, there is evidence of transstadial persistence, an important prerequisite for acting as a vector.

1. Introduction

Ixodes ricinus (Linnaeus, 1758), an important vector tick of *Borrelia burgdorferi* s. l. and tick-borne encephalitis (TBE) virus, was thought to occur not only in Europe but also in northern Africa until the molecular studies by de Meeûs et al. (2002) and Noureddine et al. (2011) showed that there is a significant genetic difference between the Eurasian and North-African populations. Estrada-Peña et al. (2014) described a new species, *Ixodes (Ixodes) inopinatus* (Estrada-Peña, 2014), and these authors claimed that this new species might have been historically confused with and erroneously reported as *I. ricinus* in parts of Spain, Portugal, and northern Africa. However, original specimens of *I. ricinus* from those regions could not be examined in that study. The hitherto known distribution of *I. inopinatus* has been restricted to parts of Spain, Portugal, Morocco and Tunisia, together with 3 specimens found in Rhineland-Palatinate, Germany (Estrada-Peña et al., 2014). Data regarding the life cycle of *I. inopinatus*, its seasonal activity, and the potential role as a vector of pathogens are unknown, and the list of its hosts is restricted to those recorded in the original description. The adult and immature stages of *I. inopinatus* share many morphological features with several other species of the *I. ricinus* complex, including *I. ricinus*, *Ixodes gibbosus* Nuttall, 1916, *Ixodes persulcatus* Schulze, 1930, *Ixodes kazakstani* Olenev and Sorokoumov, 1934, Ixodes nipponensis Kitaoka and Saito, 1967, *Ixodes pavlovskyi* Pomerantzev, 1946, *Ixodes eldaricus* Djaparidze, 1950, *Ixodes laguri* Olenev, 1929, *Ixodes festai* Tonnelli-Rondelli, 1926, and *Ixodes ventalloi* Gil-Collado, 1936. The morphologically most similar species is *I. ricinus*, from which *I. inopinatus* can be separated by some morphological characters in both adults and immature stages and genetically by its 16S rDNA sequence (Estrada-Peña et al., 2014).

The data of the present study add new information about the distribution of *I. inopinatus* in central and southeastern Europe and its sympatry with *I. ricinus*. Furthermore, we investigated the collected specimens for carrying TBE virus and rickettsiae of the spotted fever group. We report the collection of questing nymphs, females, and males of *I. inopinatus* during two consecutive years in southeastern Germany. Furthermore, we present data on the first detection of *I. inopinatus* in Romania and Austria. We further provide new morphological features that can be used for the differential diagnosis between *I. inopinatus* and *I. ricinus* together with new 16S rDNA sequences.

2. Materials and methods

2.1. Tick collecting

Ticks were collected in known TBE natural foci, Immenstetten, Heselbach, and Haselmühl in southeastern Germany, and in Wald, Austria (Table 1). Flagging was always carried out at the ecotone of mixed deciduous-coniferous forests (mainly with beech trees, oaks, pines, and spruce) with forest meadows.

In Romania, the ticks were collected from a fox that was shot in the course of a rabies vaccination program in southern Romania and from a

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Table 1
Data of *Ixodes inopinatus* ticks investigated in the present study (N = negative; P = positive; *tick-borne encephalitis; **positive in panRick PCR, could not be sequenced due to low DNA content; ***not tested).

No.	Date	Locality (country)	No. of *Ixodes* ticks collected (nymphs/females/males in brackets: *Ixodes inopinatus*)	Flagging/host	TBE virus*	*Rickettsia* (*R.*) species
1	28th June 2015	Immenstetten (Germany)	341(1)/31/43	Flagging	N	P**
2.	11th July 2015	Immenstetten (Germany)	100/10(1)/7	Flagging	N	N
3	18th July 2015	Immenstetten (Germany)	78/10(1)/2	Flagging	N	N
4	19th March 2016	Heselbach (Germany)	55/5(1)/10(1)	Flagging	N	N
5	06th August 2016	Immenstetten (Germany)	82(8)/31(1)/16(5)	Flagging	N	N
6	25th September.2016	Immenstetten (Germany)	34(12)/24(7)/16(6)	Flagging	N	N
7	30th October 2016	Immenstetten (Germany)	10(5)/14(5)/8(3)	Flagging	N	N
8	28th March 2016	Haselmühl (Germany)	389/22/52(1)	Flagging	N	N
9	28th September 2016	Haselmühl (Germany)	44/7(2)/7(1)	Flagging	N	N
10	30th October 2016	Haselmühl (Germany)	29/1/4(3)	Flagging	N	N
11	21st September 2015	Wald (Austria)	83/20(1)/17	Flagging	N	N
12	25th February 2014	Corbeanca (Romania)	0/26(1)/0	*Vulpes vulpes*	NT***	*R. monacencis*
13	October 2014	Suceava (Romania)	14(1)/4/0	*Ovis aries*	NT***	*R. helvetica*

sheep in northern Romania.

2.2. Tick identification

Ticks were identified to the species level using the morphological characters according to Feider (1965), Filippova (1977), and Estrada-Peña et al. (2014). For documentation, a Keyence VHX–900 F Microscope was used with a tiltable stand of upper light together with polarized light for focus stacking.

2.3. RNA/DNA extraction

Total nucleic acid was extracted from the 64 *I. inopinatus* ticks (18 females, 20 males, 26 nymphs), individually, using the LC RNA/DNA Kit (Roche, Mannheim, Germany) in a MagNA Pure LC instrument (Roche) according to the instructions of the manufacturer. The extracted total nucleic acid was stored at −80 °C until use.

2.4. RNA/DNA amplification and sequence analysis

The 16S rRNA gene was amplified using a previously described polymerase chain reaction (PCR) protocol (Mangold et al., 1998). Phylogenetic analyses of ca. 400-bp sequences of a 16S rRNA gene fragment were performed using the Neighbor-Joining distance (NJ) and the Maximum-Likelihood (ML) methods. To construct the ML tree, the best-fitting substitution model (GTR) was determined with the Akaike information criterion using the ML model test implemented in Mega 5 (Tamura et al., 2011). Gaps were excluded in the pairwise comparison, and support for the topology was tested by bootstrapping over 1000 replications. The analyses were carried out by using Mega 5.0 (Tamura et al., 2011).

Ticks were individually tested for TBE virus (Schwaiger and Cassinotti, 2003) and screened for rickettsiae using a panRickettsia real-time PCR (Wölfel et al., 2008). Whenever ticks tested positive for rickettsiae, identification down to *Rickettsia* species level was conducted by analyzing the 23S-5S intergenic spacer region. For this purpose, primers 23S for (5′-„GAT„A„G„G„T„C„G„G„G„G„T„G„T„G„G„A„A„G„CAC-„3′) and 23S rev (5′-„GGG„A„T„G„G„G„A„T„C„G„T„-G„T„G„T„T„T„CAC-„3′) and the thermoprofile of a previously published method (Jado et al., 2006) were modified to achieve optimum sensitivity. Briefly, 5 µl DNA, 0.5 µM Primer 23S for and 23S rev, 1 U Platinum® *Taq* DNA Polymerase High Fidelity (Invitrogen), 1 × reaction buffer, and a final concentration of 4 mM $MgSO_4$ were added to a final volume of 50 µl per reaction. Initial denaturation at 95 °C for 2 min was followed by 45 cycles at 95 °C for 30 s, 30 s at 58 °C, and 30 s at 68 °C and a final extension at 68 °C for 10 min.

For all PCR methods, standard procedures for PCR testing (three room concept, inclusion of positive and negative controls, extraction controls) were included in each run. The obtained RNA/DNA amplicons were identified by size in gel electrophoresis and sequenced by Sanger sequencing (GATC Biotech, Konstanz, Germany).

3. Results

Ticks were collected in three localities, Immenstetten, Heselbach and Haselmühl, in southeastern Germany, in Wald (western Austria), and near Corbeanca and Suceava (southern and northern Romania, respectively). Details on collecting locations, seasons of collecting, and the collected number of ticks from each location are given in Table 1.

3.1. Morphological comparisons

Ixodes inopinatus adults and nymphs collected in Germany can be

Fig. 2. (a) *Ixodes inopinatus* male, anal plate with anterior margin rounded and widely divergent lateral margins; spiracular plates smaller. (b) *Ixodes ricinus* male, anal plate with anterior margin almost straight and almost parallel lateral margins; spiracular plates larger.

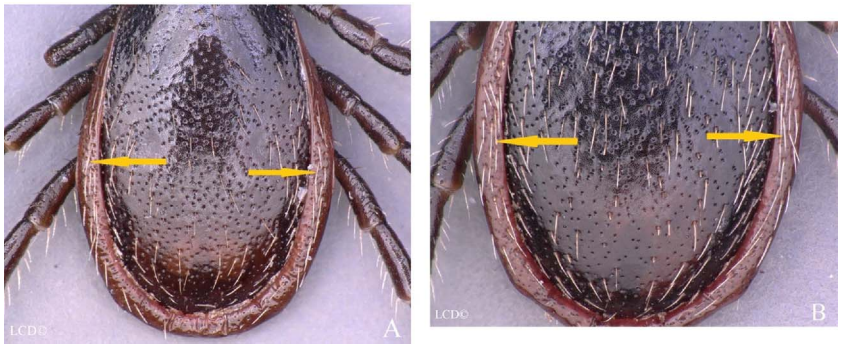

Fig. 1. (a) *Ixodes inopinatus* male, one row of setae in the lateral margins of the conscutum. (b) *Ixodes ricinus* male, several rows of setae in the lateral margins of the conscutum.

separated from the most similar species, *I. ricinus,* by a combination of important characters. The most prominent features to allow the separation of *I. inopinatus* from *I. ricinus* are as follows: Punctations on the dorsal scutum are larger and conscutal setae are longer in the male of *I. inopinatus* than in *I. ricinus*. Pre-genital and median plates are with largest and deepest punctuations (Fig. 2A). In *I. ricinus* there are several rows of lateral conscutal setae between the lateral margin of the idiosoma and the marginal groove, while there are only one or two rows (the second row can be observed only in the central part of idiosoma) in *I. inopinatus* (Fig. 1A and B, arrows). Ventrally, the males of both species can be differentiated by the anal groove, with parallel margins in *I. ricinus* and going divergent in *I. inopinatus* (Fig. 2A and 2B).

The females of *I. inopinatus* have deep and large punctations in the central field of the scutum, which are less numerous and almost inconspicuous in *I. ricinus*. The internal spur on coxa I has a long tapering pointed internal spur reaching coxa II in *I. inopinatus*, but is longer and curved, touching coxa II in *I. ricinus* (Fig. 3A and B). The internal spur of coxa I is a feature of diagnostic importance only in unengorged specimens. In the female of *I. inopinatus* there are four concentric rows of goblets in the spiracular plate, while there are (5 or 6 rows) in the female of *I. ricinus* (Fig. 4A and B).

The nymphs have a dorsal scutum wider than long in *I. inopinatus*, but longer than wide in nymphs of *I. ricinus*. Coxa I carries an external spur, short and straight, and a prominent, sharply pointed internal spur not reaching the coxa II. The internal spur is slightly longer than the external one in *I. inopinatus* (Fig. 5A). The internal spur in coxa I is about 2 times longer in *I. ricinus* than in *I. inopinatus* (Fig. 5B). The anal groove is slightly divergent posteriorly in *I. inopinatus* and almost parallel in *I. ricinus* (Fig. 5A and B). Spiracular plates are widely rounded with two rows of goblets in *I. inopinatus* and larger and with 3–4 rows of goblets in *I. ricinus* (Fig. 5A and B). The alloscutal setae of *I. inopinatus* nymphs are 9–10 times longer than median scutal setae, as around 4 times longer in *I. ricinus* nymphs.

3.2. Tick collections

Altogether, 64 unfed specimens of *I. inopinatus* were collected by flagging vegetation in Germany (18 females, 20 males, and 26 nymphs) plus one adult female *I. inopinatus* in a mountainous region in Austria in September 2015 (Table 1). In Romania, one feeding female was collected from a fox in February 2014 together with other ixodid ticks (*I. ricinus*: 14 females and 11 males; *Ixodes crenulatus* Koch, 1844: three females and a nymph; *Dermacentor reticulatus* Fabricius, 1794: a male and a female). A feeding *I. inopinatus* nymph was collected from sheep together with *I. ricinus* (13 nymphs and 4 females) in Romania in October 2014 (Table 1).

All the flagged *I. inopinatus* and the ones collected from the sheep and the fox occurred sympatrically with *I. ricinus* (Table 2). In Immenstetten, questing nymphs and adults of *I. inopinatus* were collected on five occasions in June and July 2015 and again in August, September, and October 2016 (Table 1). In Heselbach, *I. inopinatus* was found in March 2016 (one female and one male) and in Haselmühl in March, September, and October 2016 (females and males) (Table 1).

3.3. Molecular analyses

The 16S rDNA sequences of selected specimens showed that the ticks determined as *I. inopinatus* from Germany, Austria, and Romania cluster with 16S rRNA sequences of *I. inopinatus* from Spain and Tunisia (Fig. 6a and b). Although the general topologies of NJ (Fig. 6a) and ML (Fig. 6b) trees were a bit different, the phylogenetic clades with good support were the same.

3.4. Testing for tick-borne pathogens

All flagged *I. inopinatus* were tested for TBE virus RNA with negative results (Table 1). The specimens were also screened for rickettsiae, and three specimens were positive in the panRick PCR, but only two rickettsiae could be identified by sequencing. *Rickettsia monacensis* was detected in a female of *I. inopinatus* removed from a fox in southern Romania. From the same fox, 29 other ticks, 25 *I. ricinus* and 4 *I. crenulatus* (all partially engorged females) were collected. Of these, three *I. ricinus* (1 male, two females) were positive for *R. monacensis* and one *I. ricinus* (female) and one *I. crenulatus* (female) were positive for *R. helvetica*. The partially engorged *I. inopinatus* nymph collected from a

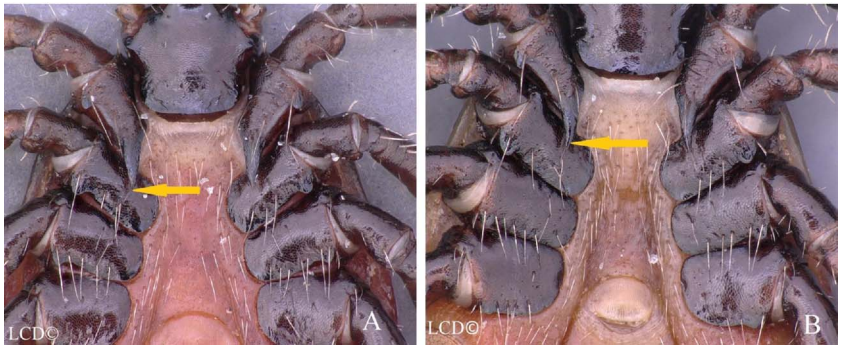

Fig. 3. (a) *Ixodes inopinatus* female, internal spur of coxa I in the female with a wide insertion, essentially straight. (b) *Ixodes ricinus* female, internal spur of coxa I in the female with a narrower insertion, slightly curved (200x).

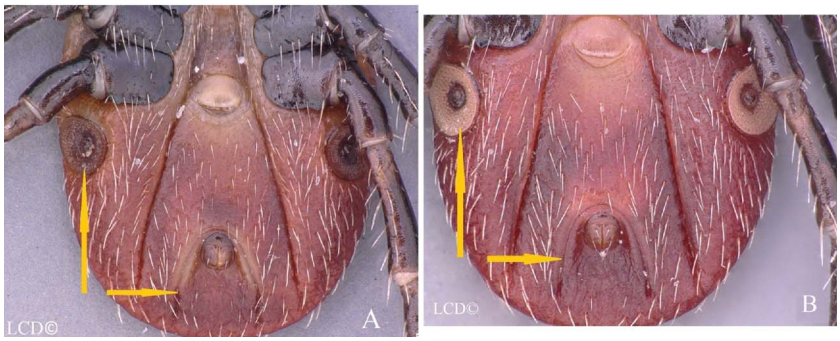

Fig. 4. (a) *Ixodes inopinatus* female, spiracular plate in the female with few goblets, four rows (at least one concentric row less than in *I. ricinus*); anal groove with diverging sides. (b) *Ixodes ricinus* female, spiracular plate in the female with more goblets, 5–6 rows (at least one concentric row more than in *I. inopinatus*); anal groove with almost parallel sides.

sheep in northern Romania was positive for *R. helvetica*. The 17 *I. ricinus* (13 nymphs, 4 females) that were collected from the same sheep tested negative for *Rickettsia* spp.

The sequence of the 23S-5S amplicon of the female tick from the fox near Corbeanca, Romania, shared 100% identity with *R. monacensis* IrR/Munich (gb|LN794217.1) while the sequence of the 23S-5S amplicon from the nymph collected from a sheep near Suceava, Romania, was 99.8% identical to *R. helvetica* SzPK1-09 (gb|JQ796866.1) (Table 1). The unfed nymph collected near Immenstetten (2015) was found positive for rickettsial DNA by the screening PCR, but identification by 23S-5S PCR was not possible due to a low DNA content.

Table 2
Summary of ratio between the number of *Ixodes inopinatus* versus *Ixodes ricinus* (questing nymphs and adults).

	No. of nymphs/ adults of *Ixodes inopinatus*	Ratio between collected *Ixodes inopinatus* and *Ixodes ricinus* nymphs and adults (*I. inopinatus*/*I. ricinus*)
Immenstetten (Germany)	26/29	26/619 nymphs and 29/183 adults
Heselbach (Germany).	0/2	0/55 nymphs and 2/13 adults
Haselmühl (Germany)	0/7	0/462 nymphs and 7/93 adults
Wald (Austria)	0/1	0/83 nymphs and 1/36 adults
Corbeanca (Romania)	0/1	0/2 nymphs and 1/26 adults
Suceava (Romania)	1/0	1/13 nymphs and 0/4 adults

4. Discussion

Estrada-Peña et al. (2014) described *I. inopinatus* from specimens collected in Spain, Tunisia, Algeria, and Morocco. Three specimens (one male, two females) were collected in Rhineland-Palatinate, Germany (Estrada-Peña et al., 2014). The current study generated additional data on *I. inopinatus* occurrence in central and southeastern Europe. With 26 collected nymphs and 29 collected adults in two years, it seems proven that there is an established *I. inopinatus* population in Immenstetten (Germany), outside the Mediterranean region. However, more evidence is needed to confirm whether there are established populations of *I. inopinatus* in other parts of Germany and in Austria and Romania and other still undetected areas in Europe.

Ixodes inopinatus was described as allopatric with *I. ricinus* in Spain. The most northern detection of *I. inopinatus* in the Spanish province of Guadalajara was some 90–100 km south of one of the most southern known foci of *I. ricinus* in the region of Rioja (Estrada-Peña et al., 2014). Our data in southern Germany show, however, that *I. inopinatus* and *I. ricinus* can occur sympatrically. Bugmyrin et al. (2013) noticed a sympatric zone for two members of the *I. ricinus* complex, *I. ricinus* and *I. persulcatus* in southern Karelia, Russia. At the moment, it is unclear whether any hybridization occurs also between *I. ricinus* and *I. inopinatus*. The so far available data suggest that sheep are common hosts of *I. inopinatus* nymphs and adults and foxes of *I. inopinatus* adults (Estrada-Peña et al., 2014; Petney et al., 2015; this study). We did not find any larvae of *I. inopinatus* during our tick collecting activities. It is unclear whether larvae were not active during our surveys or we were not able to recognize them and to tell the larvae of the two species apart. We have presented additional details on morphological features which may be useful for the routine morphological identification and differentiation of *I. inopinatus* and *I. ricinus* (see Figs. 1A– 5B).

In a phylogenetic analysis performed with 16S rDNA sequences, ticks previously determined as *I. inopinatus* or *I. ricinus* can be grouped in two separate, but closely related, phylogenetic groups. This fact and the morphological and ecological differences described by Estrada-Peña et al. (2014) support the differentiation of these two taxa as different species. However, further biological experiments (cross-mating) and genetic analyses with additional molecular markers should be made to improve the knowledge of the evolutionary relationship between *I.*

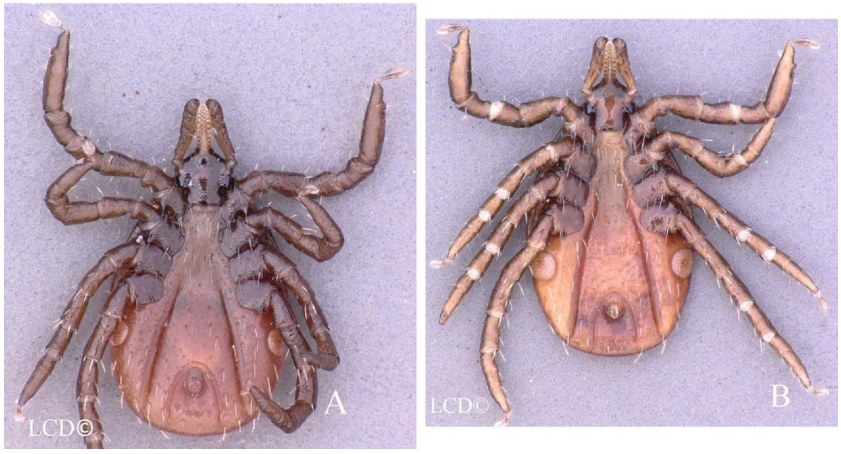

Fig. 5. (a) *Ixodes inopinatus* nymph and (b) *Ixodes ricinus* nymph, auriculae of different size and shape, spiracular plate smaller and with less goblets in *I. inopinatus*, proportions of internal and external spurs in coxa I different.

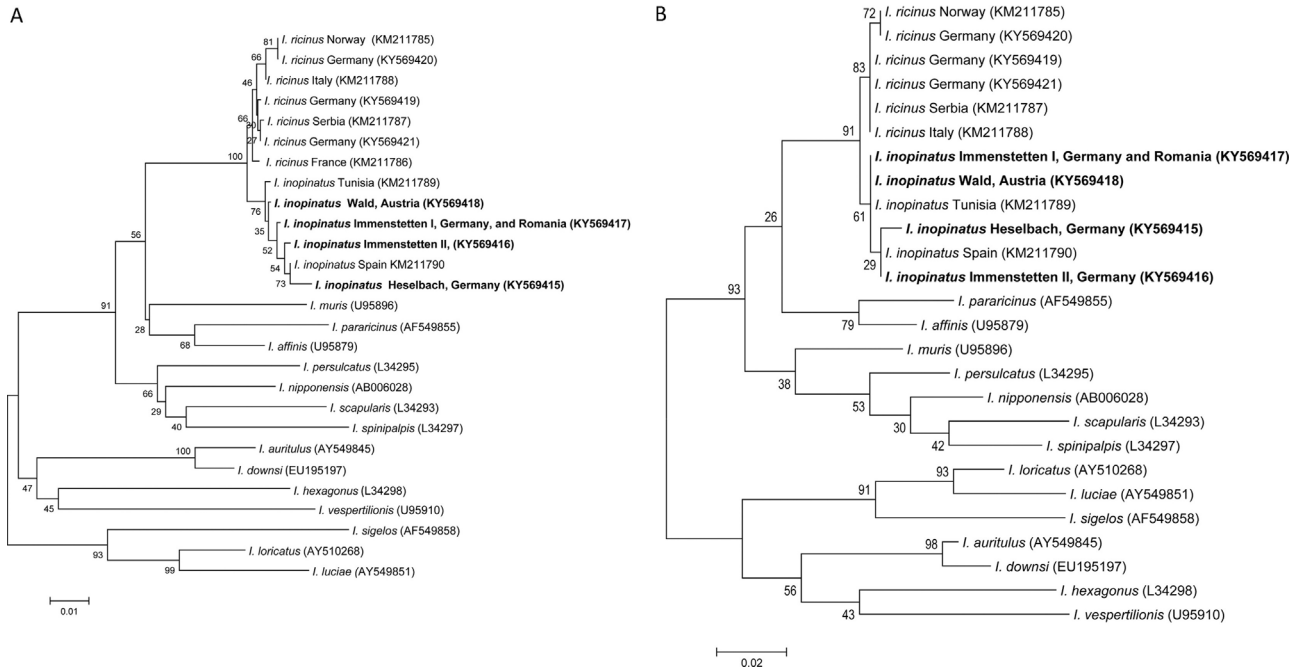

Fig. 6. Phylogenetic trees (a:NJ; b:ML) based on 16S rRNA sequences obtained from specimens of *Ixodes inopinatus* collected in Germany and in Austria and from *I. inopinatus* (KU211790) collected in Spain. GenBank accession numbers of the new sequences of *I. inopinatus* from Germany (KY569416, KY569415), from Germany and Romania (KY569417, the sequences were 100% identical), and *I. inopinatus* from Austria (KY569418). Other sequences include data already available in GenBank, from other reported *Ixodes* species from different countries.

inopinatus and *I. ricinus,* because the genetic differences of 16S rDNA sequences between the two species is low. Furthermore, it would be important to know whether any (natural) hybridization occurs between *I. inopinatus* and *I. ricinus*. This could add further complexity to the molecular and morphological identification of both species. In this sense, natural hybridization was determined between sympatric populations of *I. ricinus* and *I. persulcatus*, and also between *I. persulcatus* and *I. pavlovskyi*, both belonging to the *I. ricinus* complex (Bugmyrin et al., 2016; Kovalev et al., 2015, 2016). But anyway, the low genetic divergence between 16S rRNA gene sequences of *I. inopinatus* and *I. ricinus* warrants the analysis of complementary molecular markers.

It is unclear whether the engorged *I. inopinatus* female had taken up *R. monacensis* with rickettsiaemic fox blood or whether it was already infected with *R. monacensis* before the blood meal. It is unlikely, however, that the *I. inopinatus* nymph taken from the sheep was *R. helvetica*-positive due to rickettsiaemic blood as all other *I. ricinus* taken from the same sheep were tested negative for rickettsiae. Therefore, the detection of the *R. helvetica*-positive *I. inopinatus* nymph favours the hypothesis that *R. helvetica* can survive the moult in *I. inopinatus,* a prerequisite for acting as a vector for this rickettsia. However, the competence of *I. inopinatus* as a vector of rickettsiae remains to be proven in a transmission experiment.

Petney et al. (2015) discussed the potential medical and veterinary significance of *I. inopinatus* using the records on '*I. ricinus*" from northern Africa carrying different pathogens. Also the detection of rickettsial DNA in an unfed nymph (Immenstetten) implies the infection of the tick as a larva (either transovarially or during feeding) and the transstadial persistence of rickettsiae in *I. inopinatus*. The negative results for TBE virus in *I. inopinatus* are not surprising as even in the known TBE foci the prevalence of the virus in *I. ricinus* is usually below 1% in questing nymphs and only up to 4% in questing adults (G. Dobler, unpublished data). The vector competence of *I. inopinatus* for TBE virus has to be investigated by transmission experiments.

Conflict of interest statements

The author declares no conflict of interest.

Acknowledgements

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