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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 et al., 1967nipponensis 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 MagNApure 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 16 s 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′-GATAGGTCGGGTGTGGAAGCAC-3′) and 23S rev (5′-GGGATGGGATCGTGTGTTTCAC-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 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

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.