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Prevalence of *Ixodes ricinus* and possible hybrids of *I. ricinus* and *I. inopinatus* on the edible dormouse in a Central European woodland

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Abstract: *Ixodes inopinatus* Estrada-Peña, Nava et Petney, 2014 was described in 2014 from the Iberian Peninsula and later reported from Algeria, Morocco, Tunisia, Germany, Austria and Romania. However, recent studies raised serious doubts about the presence of *I. inopinatus* in Central Europe and reported hybridisation between the *Ixodes ricinus* (Linnaeus, 1758) and *I. inopinatus*. In this study, we selected a locally common rodent host, the edible dormouse *Glis glis* (Linnaeus) (Rodentia: Gliridae), to study the prevalence of these two tick species and their hybrids in a Central European woodland. The *TROSPA* nuclear gene and the *COI* mitochondrial gene were used for tick identification. Overall, 581 dormice were screened and 383 *I. ricinus*, 17 *I. ricinus/inopinatus* hybrids and no *I. inopinatus* were found. Co-infection of *I. ricinus* and hybrids was found on 11 dormice with the overall prevalence of *I. ricinus* 28.8% and hybrids 2.5%. Seasonal occurrence of *I. ricinus* and hybrids reached a peak in August. Edible dormouse males were more frequently infected than females and larvae of both tick taxa greatly outnumbered the nymphs. Detection of a large number of hybrid larvae on this mammal host demonstrates that tick hybridisation likely occurs further north and outside the originally described distribution range of *I. inopinatus.*

Keywords: *Glis glis*, hybridisation, infestation, rodent, tick-host interactions

This article contains supporting files (Table S1, Figure S1) online at http://folia.paru.cas.cz/suppl/2025-72-022.pdf

Ixodes inopinatus Estrada-Peña, Nava et Petney, 2014 was described by Estrada-Peña et al. (2014) and later reported from Algeria, Tunisia, Morocco, Portugal, Germany, Austria and Turkey (Chitimia-Dobler et al. 2018, Hauck et al. 2019, Bursali et al. 2020, Hekimoğlu 2022). It has a sympatric occurrence with *Ixodes ricinus* (Linnaeus, 1758) in North Africa and it is allopatric with *I. ricinus* in Spain and Portugal (Petney et al. 2015, Younsi et al. 2020). However, the geographical range of this species is not well understood. Furthermore, details on *I. inopinatus* ecology and seasonal dynamics are lacking (Estrada-Peña 2017).

The first description of *I. inopinatus* was based on tick morphology and sequencing of the 16S rRNA gene (Estrada-Peña et al. 2014). However, since then, several studies demonstrated that morphological differences and 16S rDNA are not reliable enough to differentiate *I. inopinatus* from *I. ricinus* and questioned the presence of *I. inopinatus* in Central Europe (Plantard et al. 2022, Hrazdilová et al. 2023, Rollins et al. 2023, Velez et al. 2023). The recent studies showed that a combination of nuclear (*TROSPA*) and mitochondrial (*COI*) markers offers a reliable method to identify *I. inopinatus*, and it can also be used for the detection of possible hybrids of *I. ricinus* and *I. inopinatus* (Hrazdilová et al. 2023, Daněk et al. 2024).

It was also proposed that the dispersal of *I. inopinatus* is maintained by migratory birds returning every spring from

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North Africa to Europe (Toma et al. 2021, Hrazdilová et al. 2023) and it was shown that the Italian Peninsula might serve as the major hybridisation site between *I. ricinus* and *I. inopinatus* lineages (Daněk et al. 2024). However, theoretically, there are also two other alternative explanations: an isolation by distance with a gradual change of allelic frequencies across large geographical distances within a single species, or alternatively, a historical introgression from one lineage to another, with a slow dispersal of the introgressed allele.

Only a detailed multilocus analysis could distinguish between these scenarios. No matter that, there is a clear indication of either current or historical admixture between the two species lineages, and for the purpose of this study, we will call the individuals bearing different *TROSPA* alleles as hybrids.

In this study, we present the results of a field survey of ticks found attached to a long-lived arboreal nocturnal mammal, the edible dormouse *Glis glis* (Linnaeus) from the Czech Republic. This small mammal can be very abundant in suitable deciduous woodlands (Adamík et al. 2019), which makes it a suitable model species to study tick-host interactions (Matuschka et al. 1994, Fietz et al. 2014, 2016).

MATERIALS AND METHODS

Data acquisition

Ticks were collected from dormice at a study site in a mixed deciduous woodland near Dlouhá Loučka, Czech Republic (49.825 N, 17.212 E). At this site, wooden nest boxes are used for regular monitoring of dormice populations (Holcová Gazárková and Adamík 2016). The dormice use the nest boxes as an alternative den site to natural tree cavities. Approximately twice a month from early June until the end of October in 2019, all captured dormice were checked for the presence of ticks. Each individual dormouse was marked with a PIT tag. Dormice were classified according to age categories as juveniles (J – young-of-the-year), yearlings (SY – after the first hibernation), and adults (ASY – after the second hibernation). For two individuals, we did not record their sex or age. The entire dormouse body was screened for ticks. Ticks were removed with forceps and placed in 96% ethanol. As we checked the dormice once every fortnight, which is well beyond the blood meal feeding of larvae and nymphs, we consider each sampling period as an independent tick count (Fietz et al. 2016).

Tick identification

All collected ticks were first screened using a stereomicroscope according to the morphological features using Estrada-Peña et al. (2017) as reference. Out of 437 ticks collected a subset of 400 undamaged samples (some tick bodies were damaged in the field during removal from the host body) was selected for identification. Total DNA was isolated from whole larvae or longitudinal halves of nymphs using the NucleoSpin Tissue XS kit (Macherey-Nagel, Düren, Germany). The nuclear *TROSPA* and mitochondrial *COI* genes were used as markers to distinguish *Ixodes ricinus* and *Ixodes inopinatus* (see Noureddine et al. 2011, Hrazdilová et al. 2023). Purified PCR products only those with two bands in the on-gel of the multiplex PCR were sequenced by the Macrogen Capillary Sequencing Services (Macrogen Europe, Amsterdam, the Netherlands). Obtained sequences were processed and aligned using Geneious Prime (Kearse et al. 2012). Ticks were identified as hybrids if double peaks were observed in all 23 determining positions in partial *TROSPA* following the protocol of Hrazdilová et al. (2023).

Representative sequences identified as hybrids were cloned using pGEM®-T Easy Vector Systems (Promega Corporation, Madison, Wisconsin, USA), plasmid purification was done using the GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich, Burlington, Massachusetts, USA), and sequencing was conducted by the Macrogen capillary sequencing services (Macrogen Europe) using universal T7/SP6 primers.

Phylogenetic analysis

The phylogenetic analysis of *TROSPA* was constructed using representative sequences of available *Ixodes* species obtained from the GenBank database, originating from different studies and geographical regions. From this study, molecular clones of representative samples (two representative sequences per sample) were used. The details of all the phylogenetic analyses (number of used sequences, algorithm, length of the final alignments and chosen evolution models) are described in Figure 1.

The alignment for the phylogeny was done by MAFFT alignment in Geneious Prime 2024.0.5 (Kearse et al. 2012). The phylogeny was constructed in IQ-TREE version 1.6.12 (Nguyen et al. 2015), and the best-fit evolution model was selected based on the Bayesian information criterion (BIC) computed by implemented ModelFinder (Kalyaanamoorthy et al. 2017). Branch supports were assessed by the ultrafast bootstrap (UFBoot) approximation (Minh et al. 2013) and by the Shimodaira-Hasegawa-like approximate likelihood ratio test (SH-aLRT) (Guindon and Gascuel 2003). Trees were visualised and edited in FigTree v1.4.4 and Inkscape 1.3.

Statistics

For characteristics of tick burden on the host, we used the terminology as in Reiczigel et al. (2019) and test statistics were calculated in QPweb version 1.0.15. Data on tick prevalence in dormice hosts are provided with an exact 95% confidence interval (CI) using Sterne's method and 95% CI for mean intensity was estimated with the bias-corrected bootstrap using QPweb. Confidence intervals for the proportions were estimated with the *binom.test* function in R version 4.3.2. For comparing the prevalence between the samples of host categories, we used the Fisher's Exact Test (FET).

RESULTS

In 2019, 581 dormice on 614 occasions were captured, and 438 ticks were collected. In total 30.6% (95% CI 27.0–34.4) of all captured dormice were infected with at least one tick (188/614). Infected dormice most commonly hosted one tick with a maximum of 14 ticks (mean intensity ± 95% CI = 2.33 (2.03–2.72), median intensity = 1, mean abundance = 0.71 (0.59–0.85), variance/mean ratio: 3.91).

From the 438 collected ticks, a subset of 400 (from 172 dormice on 598 occasions) was selected for molecular identification. Out of these, 383 (95.8%) were *Ixodes ricinus*, and 17 (4.3%) were hybrids of *I. ricinus/Ixodes inopinatus* based

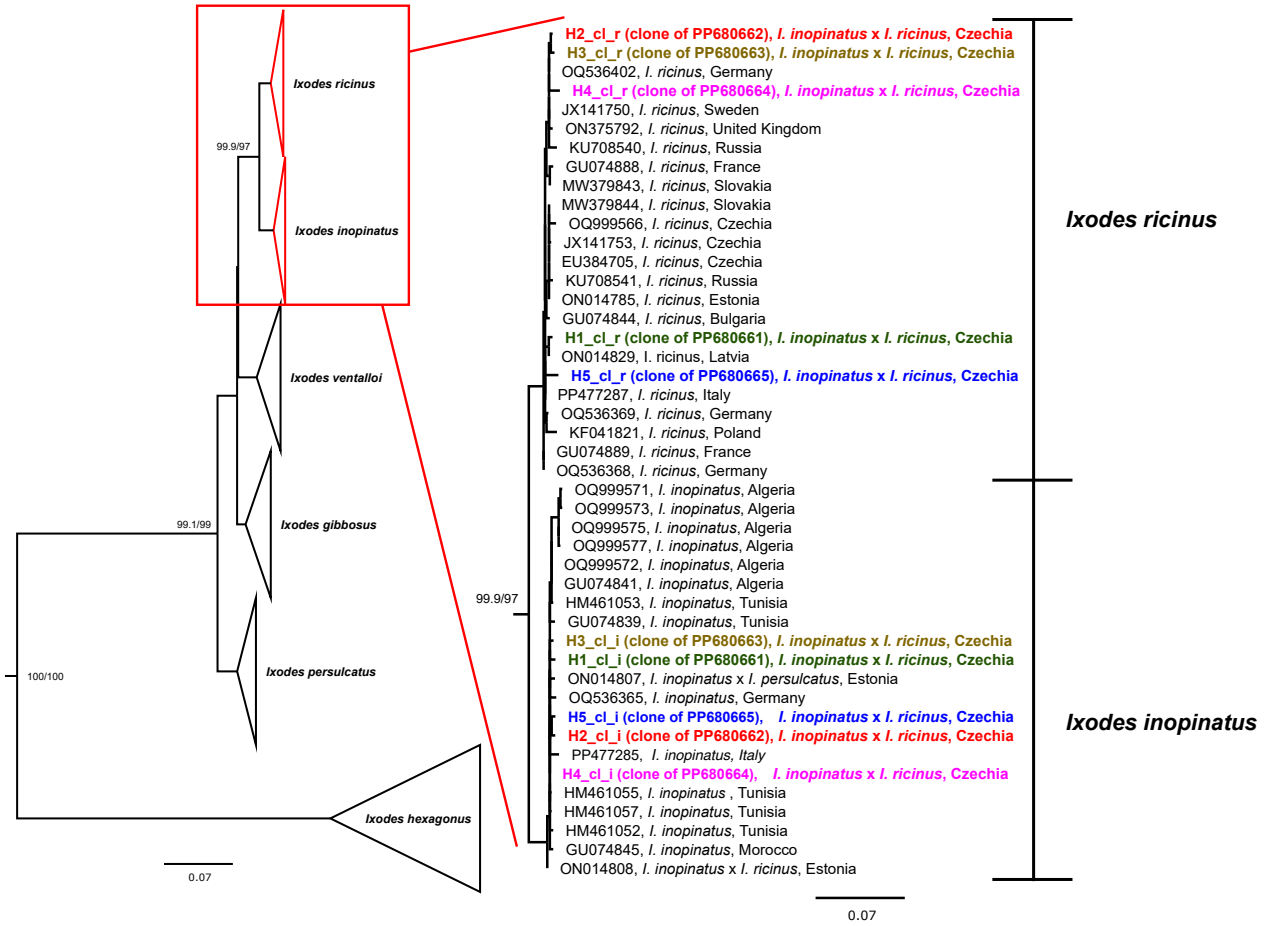

Fig. 1. Schematic representation of the maximum likelihood phylogenetic tree based on the *TROSPA* gene sequences of available *Ixodes* spp. The tree was constructed using 74 sequences (10 generated in this study). The final length of the alignment was 1,101 bp and the tree was built using the evolution model TPM2u + F + G4. Three sequences of *I. frontalis* used as an outgroup are not displayed. The clades of *I. ricinus* and *I. inopinatus* are shown in detail. Sequences from this study are marked in bold with matching colours for clones originating from the same tick. The scale bars indicate the number of nucleotide substitutions per site. The bootstrap values (SH-aLRT/UFB) above the 80/95 threshold are displayed. Sequences are labelled by accession number, species and country of origin (if available).

on a combination of multiplex PCR targeting the *TROSPA* gene and *COI* sequence analysis (Table 1; Supplementary online material 1). All obtained *COI* sequences corresponded to the *I. ricinus* variant of the gene. Three ticks (all identified as *I. ricinus* based on *TROSPA*) did not have *COI* amplified and sequenced due to an insufficient amount of DNA.

Corresponding sequences from representative samples were deposited to the GenBank database (PP680661–PP680665 for *TROSPA* and PP663828–PP663832 for *COI*). *TROSPA* of the same samples was also cloned. After aligning the obtained clones with the reference sequences of *I. ricinus* (GU074844) and *I. inopinatus* (GU074845), one representative sequence per sample and variant was chosen for phylogenetic analysis. The resulting phylogenetic tree depicts well-defined clades of *I. ricinus* and *I. inopinatus* and clearly separates the cloned samples into the respective clades of both species. All other species of *Ixodes* used in the phylogeny formed distinct clades (Fig. 1, Fig. S1).

Table 1. Identification of ticks from edible dormice (*Glis glis* [Linaeus]) based on the *TROSPA* and *COI* genes.

Gene	Tick age category	Adults	Larvae	Nymphs	Total
	Sample size	1	255	144	400
TROSPA	*I. ricinus/inopinatus* hybrids	0	14	3	17
	I. inopinatus	0	0	0	0
	I. ricinus	1	241	141	383
COI	*I. inopinatus*	0	0	0	0
	I. ricinus	1	252	144	397
	not specified (genetically)	0	3*	0	3*

*Due to insufficient DNA, three samples were not processed for the *COI* gene.

Dormouse males were slightly more frequently infected with *I. ricinus* (prevalence 31.9%, 95% CI: 27.0–37.1, 111/348) than females (23.0%, 95% CI: 17.9–28.7, 57/248; FET, P = 0.075). The sex-bias pattern in prevalence was also found for *I. ricinus*/*I. inopinatus* hybrids where we detected males more often as tick hosts than females (males: 3.7%, 95% CI: 2.0–6.3, 13/348 and females: 0.8%, 95% CI: 0.1–2.9, 2/248; FET, P = 0.032). In males, 11 instances of a single hybrid tick and two cases of two hybrids on the host were detected. Two females had a single larva of *I. ricinus/I. inopinatus* hybrid. Eleven male dormice had co-infection of *I. ricinus* and *I. ricinus/I. inopinatus* hybrids.

There was not a significant effect of age on the infection rate in either *I. ricinus* (juveniles: 23.5% 39/166, SY: 28.2% 77/273, ASY: 33.1% 52/157; FET, P = 0.348) or hybrid ticks (J: 2.4% 4/166, SY: 2.2% 6/273, ASY: 3.2% 5/157; FET, P = 0.753).

Table 2. Seasonal counts of ticks and their developmental stages collected from edible dormice.

Month	*Ixodes ricinus*	*I. ricinus/ inopinatus* hybrids	Total set of ticks	No. screened dormice	No. dormice with ticks
	larvae/ nymphs/ adults	larvae/ nymphs/ adults	255/144/1	-	-
June	3/10/0	0/0/0	13	32	8
July	11/41/0	1/1/0	54	115	31
August	142/59/1	9/2/0	213	268	90
September	67/29/0	3/0/0	99	162	38
October	18/2/0	1/0/0	21	21	5
Total numbers	241/141/1	14/3/0	400	598	172

Overall, larvae (63.8%, 95% CI: 58.8–68.5%, 255/400) were found feeding on dormice more frequently than nymphs (36%, 95% CI: 31.3–40.9%, 144/400). We found only one adult tick of *I. ricinus* (Table 1). Also, hybrid larvae were detected more often (82.4%, 95% CI: 56.6–96.2, 14/17) than hybrid nymphs (17.6%, 95% CI: 3.8–43.4%, 3/17). The overall prevalence of hybrid ticks parasitising dormice was 2.5% (15/598, 95% CI: 1.4–4.1, mean intensity ± 95% CI = 1.13 (1–1.27), mean abundance = 0.028 (0.015–0.045), variance/mean ratio: 1.21). The overall prevalence of *I. ricinus* on dormice was 28.8% (172/598, 95% CI: 25.2–32.6, mean intensity = 2.21 (1.95–2.56), mean abundance = 0.64 (0.53–0.77), variance/mean ratio: 3.52).

The number of ticks peaked in August for larvae and nymphs (Table 2). *Ixodes ricinus* was most often found on dormice in August (32.5% prevalence, 87/268) and in the remaining months the prevalence was between 23.5% (38/162) in September to 26.1% (30/115) in July, and overall there were no significant differences in prevalence among months (FET, P = 0.638). Hybrid numbers were also high in August (3.4%, 9/268) and the overall monthly prevalence ranged from 0% in June (0/32) to 4.8% in October (1/21). In July and September, the prevalence was 1.7 (2/115) and 1.9% (3/162), respectively. There was no significant difference in prevalence among months (P = 0.639). For *I. ricinus*, there was a clear difference in seasonal peak between larvae and nymphs. Larval numbers peaked later, with a sudden increase in August, while nymph numbers had already started high in July and continued high until August. Hybrid nymphs were detected only in July and August (Table 2).

DISCUSSION

Our study presents the first data on the sympatric occurrence of *Ixodes ricinus* and *I. ricinus/inopinatus* hybrids feeding on a small mammal in Central Europe. If we omit those studies that relied only on the morphology and/or the 16S rRNA gene for tick identification, our results are in line with the two recent studies of Rollins et al. (2023) and Hrazdilová et al. (2023), suggesting that *I. inopinatus* has not been detected in Central Europe so far.

Rodents play a key role as hosts of ticks from the family Ixodidae (Durden 2006). To date, a few studies that brought data on *I. ricinus* infection rates on dormice (family Gliridae, Rodentia) show that these rodents are among the most utilised hosts (Matuschka et al. 1991, 1994, 1999, Richter et al. 2004, Fietz et al. 2016). In contrast, data on hosts of *I. inopinatus* are still largely missing, as majority of reports used unreliable methods for differentiation of *I. inopinatus* from *I. ricinus*. So far, *I. inopinatus* was recorded feeding on cattle and migratory birds (based on *TROSPA* or multiple markers) and previously also on foxes, sheep and lizards (based on 16S and/or morphology) with no record of feeding on rodents (Noureddine et al. 2011, Estrada-Peña et al. 2014, Chitimia-Dobler et al. 2018, Bursali et al. 2020, Toma et al. 2021, Mancuso et al. 2023).

Previous studies showed signs of hybridisation between *I. ricinus* and *I. inopinatus* (Hrazdilová et al. 2023, Daněk et al. 2024). While this methodology is not suitable for showing the level of hybridisation, the analysis of sequence chromatograms supported by cloning and phylogenetic analysis demonstrates the presence of DNA of both species, *I. ricinus* and *I. inopinatus*. This is also supported by the discrepancies between the nuclear and mitochondrial genomes observed in Italy (Daněk et al. 2024). Although this cannot differentiate between hybridisation and gene introgression (Harrison and Larson 2014), it shows that the gene pools of *I. inopinatus* and *I. ricinus* are mixed.

Similar cases of hybridisation of two related tick species have been described in the USA and Finland, using the amplified fragment length polymorphisms (AFLP) and double digest restriction-site associated DNA (ddRADseq), respectively (Araya-Anchetta et al. 2013, Alale et al. 2024). The multiplex PCR developed in our previous study (Hrazdilová et al. 2023) supported by phylogeny based on partial *TROSPA* of cloned sequences with mixed chromatogram signals indicates hybridisation between *I. ricinus* and *I. inopinatus*.

In our dataset, 4.3% of ticks were *I. ricinus/inopinatus* hybrids, and we did not detect any *I. inopinatus*. For comparison, in a recent study from three study sites in the Czech Republic (Hrazdilová et al. 2023), where adult ticks were flagged, the reported occurrence of *I. ricinus/inopinatus* hybrids among all sampled adult ticks was 3.1% (1.8 to 3.9% per site). The slightly higher prevalence of hybrids in our dataset might be due to mortality across developmental stages, as almost all ticks in our sample were juveniles. By developmental stage, hybrid larvae were more common than nymphs. The dominance of larvae among the detected hybrids may indicate a new generation of ticks suggesting *in situ* hybridisation.

However, based on the available data, we cannot state whether this represents F1, further generations or even backcrosses. Similary to the previous studies, all hybrids showed the *COI* variant corresponding to *I. ricinus* (Hrazdilová et al. 2023, Daněk et al. 2024). This phenomenon could be related to the survival rate of hybrids of different parent pairs or suggests lower reproductive success of offspring of some pair combinations. Further studies are necessary to map and understand the potential hybridisation or introgression of *I. inopinatus* and *I. ricinus* and ecological differences between both species and their hybrids.

We found 30.6% of the dormice to have at least one tick, and the monthly prevalence rate ranged from 23.5 % to 32.5 %, with the highest peak in August. Within the season of dormice activity from June to October, most *I. ricinus* were found in August and September. We recorded a similar pattern for the hybrid ticks, although our sample size was much smaller (in August 9/268, in September 3/162).

In a study from southern Germany, the highest prevalence of *I. ricinus* was recorded on edible dormice in August (27%) and in September (40%, Fietz et al. 2016). Our overall infection rate of ticks feeding on the edible dormouse population is slightly higher than that in the study from southern Germany (26%, Fietz et al. 2016). However, in our study, we checked the entire body of the host while in the latter one, only the ears of the host were examined.

In contrast, another field study from south-western Germany found a 100% infection rate by larvae and a 91% infection rate by nymphs (n = 11 screened dormice, Matuschka et al. 1994). High infection rates were also found in the Garden dormouse (*Eliomys quercinus* [Linnaeus]) population in north-eastern France (95% infection by larvae and 70% of nymphs, Matuschka et al. 1999). In contrast, much smaller infection rates (19%), based on the full body check, was detected from several sites in the Hazel dormouse (*Muscardinus avellanarius* [Linnaeus]) population in Germany (Lang et al. 2018).

Exploration of phenology and host sex preferences showed very similar patterns for *I. ricinus* and the hybrids of *I. ricinus/ inopinatus.* We found edible dormouse males to be more frequently infected than females, and this holds for both *I. ricinus* and the hybrid ticks. This sex-biased infection pattern in ectoparasite load is often reported from small mammal hosts (Krasnov et al. 2005). A higher level of infection of edible dormouse males was also recorded of dormice in German populations (Fietz et al. 2016). Overall, it seems that *I. ricinus* and the hybrid ticks have similar occurrence patterns on the studied rodent host. Further studies from other host systems would be desirable to confirm this finding.

We still know very little about the distribution of *I. inopinatus*. Some authors proposed that a passive dispersal of *I. inopinatus* further north might be due to migratory birds that transfer ticks from Africa to Europe when they return in spring from their African non-breeding grounds (Hasle 2013, Toma et al. 2021, Hrazdilová et al. 2023). Many migratory birds stop for several days to refuel in North Africa. After this stopover, they frequently cross the Mediterranean in single non-stop flights, covering hundreds of kilometres (Adamík et al. 2016). These flights are very fast, sometimes only tens of hours, enabling attached ticks to be carried hundreds of kilometres. These ticks may be carried to Spain, Italy and southern France, where they might hybridise with *I. ricinus*. We speculate that the hybrids could be further carried to the north by other migratory birds. This fits into the observations of a recent study that demonstrated that the Italian Peninsula is an important site for hybridisation between *I. ricinus* and *I. inopinatus* (Daněk et al. 2024).

In conclusion, our data show that DNA of both species, *I. ricinus* and *I. inopinatus* occurs in some individual ticks in our study area. While we cannot differentiate between hybridisation and gene introgression of these two species, we show that the gene pools of some *I. inopinatus* and *I. ricinus* are mixed. We propose that further studies using the identification based on *TROSPA* in combination with *COI* are needed to investigate the distribution of *I. inopinatus* and hybrids along a North-South gradient across Europe.

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Availability of data and materials. Data on individual hosts and collected ticks are available in the Supplementary online table.

Author's contributions. KS: conceptualisation, methodology, formal analysis, investigation, writing – original draft, LZ: methodology, resources, supervision, funding acquisition, writing – review and editing, PP: data curation, investigation, EN: investigation, OD: investigation, writing – review and editing, formal analysis, methodology, DM: conceptualisation, resources, supervision, methodology, IM: data curation, PA: conceptualisation, methodology, resources, formal analysis, supervision, funding acquisition, writing – original draft, writing – review and editing.

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