**German *Ixodes inopinatus* samples may not actually represent this tick species****[☆](https://www.sciencedirect.com/science/article/abs/pii/S0020751923001571" \l "aep-article-footnote-id1)**

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**Highlights**

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German samples previously identified as [Ixodes](https://www.sciencedirect.com/topics/agricultural-and-biological-sciences/ixodes)*inopinatus* represent [Ixodes ricinus](https://www.sciencedirect.com/topics/agricultural-and-biological-sciences/ixodes-ricinus) based on genomic data.

* •

The [mitochondrial genome](https://www.sciencedirect.com/topics/agricultural-and-biological-sciences/mitochondrial-genome) is not sufficient for delineation of *I. inopinatus* and *I. ricinus.*

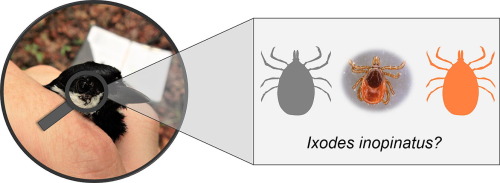
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The German samples most likely do not represent *I. ricinus*/*I. inopinatus* hybrids.

**Abstract**

Ticks are important vectors of human and animal pathogens, but many questions remain unanswered regarding their taxonomy. Molecular sequencing methods have allowed research to start understanding the evolutionary history of even closely related tick species. *Ixodes inopinatus* is considered a sister species and highly similar to *Ixodes ricinus*, an important vector of many tick-borne pathogens in Europe, but identification between these species remains ambiguous with disagreement on the geographic extent of *I. inopinatus*. In 2018–2019, 1583 ticks were collected from breeding great tits (*Parus major*) in southern Germany, of which 45 were later morphologically identified as *I. inopinatus.* We aimed to confirm morphological identification using molecular tools. Utilizing two genetic markers (16S rRNA, TROSPA) and whole genome sequencing of specific ticks (*n* = 8), we were able to determine that German samples, morphologically identified as *I. inopinatus*, genetically represent *I. ricinus* regardless of previous morphological identification, and most likely are not *I. ricinus*/*I. inopinatus* hybrids. Further, our results showed that the entire [mitochondrial genome](https://www.sciencedirect.com/topics/agricultural-and-biological-sciences/mitochondrial-genome), let alone singular mitochondrial genes (i.e., 16S), is unable to distinguish between *I. ricinus* and *I. inopinatus*. Our results suggest that *I. inopinatus* is geographically isolated as a species (northern Africa and potentially southern Spain and Portugal) and brings into question whether *I. inopinatus* exists in central Europe. Our results highlight the probable existence of *I. inopinatus* and the power of utilizing genomic data in answering questions regarding tick taxonomy.

**Graphical abstract**



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**Introduction**

Ticks are parasitic arthropods belonging to the order Acari together with other species of mites and can be found on all continents besides Antarctica (Hillyard, 1996, Estrada-Peña et al., 2004). Additionally, ticks act as major vectors of pathogens to domestic animals and humans worldwide, rivaled only by mosquitos in their medical importance (Hillyard, 1996). Due to their role in human and animal disease, it is of utmost importance to have clear and unambiguous methods for identification of tick species even when closely related species are under study. Even so, many species remain challenging to identify without expert opinion with even this resulting in incorrect identification of specimens in some cases. This highlights the need for methods which identify tick specimens unambiguously to aid in the study of their evolutionary history.

Many tick species have been and still are identified based on morphological characteristics (Hillyard, 1996, Estrada-Peña et al., 2004, Estrada-Peña et al., 2017a, Estrada-Peña et al., 2017b). Progress in molecular sequencing tools and methods/technologies, however, has increased the number of studies using different genetic markers for confirmation of morphological identification (Abouelhassan et al., 2019) and further description of the evolutionary history of various tick species (Xu et al., 2003, Charrier et al., 2019, Wang et al., 2019). Many genetic markers used for tick species identification are located on the mitochondrial genome, which has been widely and preferentially used, due to its overall conservation, ease of amplification, and existing as a haploid sequence (Abouelhassan et al., 2019). On the other hand, more recent studies have started to also utilize genetic data from the nuclear genome, results of which highlight differences that were not apparent based on mitochondrial studies alone (Charrier et al., 2019, Jia et al., 2020, Poli et al., 2020). The tick genome has proved complex for genome assembly due to its large size (>2 Gb) and high variability in chromosome structure, resulting in very few published reference genomes (Gulia-Nuss et al., 2016, Cramaro et al., 2017, Jia et al., 2020, De et al., 2023). Even so, nuclear-based genomic studies have shown the ability of these methods to unravel the evolutionary history of even closely related tick species (Jia et al., 2020, Poli et al., 2020), opening up the possibility of studying the taxonomy and identification of other closely related species such as *Ixodes ricinus* Linnaeus, 1758 and *Ixodes inopinatus* Estrada-Peña, Petney, Nava, 2014.

*Ixodes ricinus* is one of the main vectors in Europe of various tick-borne pathogens such as tick-borne encephalitis virus and *Borrelia burgdorferi* sensu lato (Hillyard, 1996). Based on genetic data, it has been shown that this tick species forms two distinct populations between Europe and northern Africa (Noureddine et al., 2011, Poli et al., 2020). Subsequent research based on morphology and sequencing of the 16S mitochondrial rRNA gene supported that these distinct clusters represented two separate tick species, *Ixodes inopinatus* predominately in northern Africa (with a few European samples) and *I. ricinus* predominately in Europe (Estrada-Peña et al., 2014, Younsi et al., 2020). Many further studies based on morphology and 16S rRNA sequences have supported the presence of *I. inopinatus* in many European countries (Chitimia-Dobler et al., 2018, Hauck et al., 2019, Toma et al., 2021) even though other research based on genomic single nucleotide polymorphism (SNP) data did not support the potential presence of *I. inopinatus* in Europe (Poli et al., 2020). *Ixodes inopinatus* is an exophilic tick of which the immature life stages utilize lizards of several species and adults are most commonly found on red foxes (*Vulpes vulpes*) but rarely on other vertebrates (Estrada-Peña et al., 2014, Estrada-Peña et al., 2017b). Ambiguity in morphological characteristics between *I. ricinus* and *I. inopinatus* requiring expert knowledge to delineate (Estrada-Peña et al., 2014), and challenges to use certain common genetic markers for species determination (Plantard, O., Poli, P., Bouattour, A., Sarih, M., Dib, L., Rispe, C., 2022. *Ixodes inopinatus* cannot be distinguished from *I. ricinus* by the sole use of the 16S ribosomal gene. Tick and Tick-Borne Pathogen Conference, Murighiol, Romania, p. 73), have complicated the unambiguous identification and study of *I. inopinatus*. This fact paired with recent work reporting *I. inopinatus* samples positive for tick-borne pathogens (i.e., *B. burgdorferi* sensu lato) (Hauck et al., 2020, Knoll et al., 2021) and movement on migratory birds (Toma et al., 2021) further underlies the need for a clear methodology to identify *I. inopinatus,* to facilitate research into the overall biology of this tick species.

In the years 2017–2019, 1583 ticks were collected as part of a previous project from breeding great tits (*Parus major*) in various nest-box plots located south of Munich, Germany (Rollins et al., 2021). All ticks belonged to the genus *Ixodes* with 45% of samples identified as *Ixodes* spp, 43.9% as *I. ricinus*, and 8.3% as *I. frontalis* (Rollins et al., 2021). The remaining 2.8% of samples (n = 45), which contained both larvae and nymphs, were morphologically identified as *I. inopinatus* (Rollins et al., 2021). As *I. inopinatus* had previously not been observed on this bird species, in this study we aimed to first determine if the samples that were morphologically identified as *I. inopinatus* truly represented this species. Using genetic markers (16S rRNA, TROSPA) on all tick specimens (n = 37) and whole genome sequencing of specific samples (n = 8), we are able to show here that samples morphologically identified as *I. inopinatus* most likely represent *I. ricinus* regardless of morphological identification. These results, paired with phylogenetic and demographic estimation based on whole genome sequencing, suggests that samples identified as *I. inopinatus* previously in Germany (Chitimia-Dobler et al., 2018, Hauck et al., 2020, Hauck et al., 2019, Knoll et al., 2021, Rollins et al., 2021) and Portugal (Norte et al., 2021) may not actually represent this tick species or potential *I. ricinus/I. inopinatus* hybrids and, further, suggests that *I. inopinatus* is geographically restricted to Northern Africa and, potentially, southern Spain.

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**Section snippets**

**Samples, DNA extraction, and PCR analysis**

For this study, 45 samples morphologically identified as *I. inopinatus* (31 nymphs and 14 larvae) were used from a previous study (Rollins et al., 2021). These ticks were each collected feeding on breeding great tit adults (*P. major*) south of Munich, Germany in the years 2018–2019. Additionally, three German nymphs from the same project, morphologically identified as *I. ricinus*, and two adult, male ticks collected from cattle in the Ain Sandel district (Guelma province) of eastern Algeria and

**Morphological identification of German samples does not match molecular data**

Both high quality 16S rRNA and TROSPA sequences were obtained for 37 out of 45 ticks under study. Samples containing one of the sequences or none were excluded from further analysis. For all *I. ricinus* and Algerian *I. inopinatus* samples included as controls, high quality 16S and TROSPA sequences were obtained. In the 16S rRNA MJN, some separation of *I. ricinus* and *I. inopinatus* samples was observed but overall samples did not definitively cluster based on morphological identification including

**Discussion**

Many tick species act as vectors for various human and animal pathogens (Hillyard, 1996, Guglielmone et al., 2014) but even so, ambiguous identification and unclear taxonomy hamper our ability to unravel the evolutionary history of these important arthropods. This is true in the case of two closely related species, *I. ricinus* and *I. inopinatus,* which potentially are found in sympatry across their northern African and European ranges, although this is contested due to ambiguity in the

**Declaration of Competing Interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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