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Research article

The First Evidence of Nanism in *Ixodes (Ixodes) scapularis* (Acari: Ixodidae), Found Parasitizing a Human Host

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Abstract

Ixodes scapularis Say 1821, the primary vector of several human pathogens in the northeastern and upper Midwestern United States, has considerable genetic and morphological variation throughout its range. Recently, developmental or teratological abnormalities have been observed in this species for the first time, further complicating morphological identification. Here, we report the first evidence of nanism (dwarfism) in *I. scapularis*, found parasitizing a human host. We used molecular methods and scanning electron microscopy to identify the specimen. Morphological identification confirmed that the specimen is substantially smaller, approximately half the size, than a typical *I. scapularis* female. Here we discuss the recent reports of teratological abnormalities in *I. scapularis*, particularly from the Hudson River valley region of the northeastern United States, and highlight the need for additional studies of teratology in this important species and its potential implications in disease transmission.

Key words: *Ixodes scapularis*, teratological abnormality, nanism

The blacklegged tick or deer tick, *Ixodes scapularis* Say 1821, is an important vector of pathogens responsible for Lyme disease, human babesiosis (Spielman et al. 1979), granulocytic anaplasmosis (Pancholi et al. 1995), Powassan encephalitis (Ebel 2010), and others (Nelder et al. 2016). Lyme disease is the most prevalent vector-borne disease in North America and is caused by spirochetes in the *Borrelia burgdorferi* sensu lato species complex (Pritt et al. 2016). An estimated 300,000 human Lyme disease cases occur annually in the United States, the majority from the northeast and upper Midwest (Nelson et al. 2015).

Populations of *I. scapularis* in the United States can be classified into two genetic lineages: a diverse "southern clade" found only in the southeastern United States, and a second lineage, far less genetically diverse, that is found in both the southern and northern range of this species (Norris et al. 1996, Van Zee et al. 2013, Sakamoto et al. 2014). Diversity in morphological (Hutcheson et al. 1995, Keirans et al. 1996) and behavioral characteristics (Arsnoe et al. 2015, Goddard et al. 2015) historically created difficulties in identification of *I. scapularis*. Prior to the widespread establishment of *I. scapularis* in northern and central North America, this morphological variability caused both the misclassification of *I. scapularis* nymphs as *Ixodes muris* (Spielman et al. 1979), and the temporary elevation of some *I. scapularis* populations to the separate species, *Ixodes dammini* (a name later demoted to junior subjective synonym; Spielman et al. 1979, Oliver et al. 1993). However, the redescription of *I. scapularis* in 1996 accounted for variation across the geographic range of this species (Keirans et al. 1996).

Recent studies from Wisconsin (Larson and Paskewitz 2016) and New York (Prusinski et al. 2015) reported morphological abnormalities in field populations of *I. scapularis* for the first time. Abnormalities in tick species have been observed since the late 19th century (Neumann 1899) and are classified into two categories: local, e.g., deformities or absence of specific structures such as legs or mouth parts in an otherwise normal tick; and general, e.g., nanism or dwarfism, gigantism, asymmetry or bifurcation of the idiosoma, and gynandromorphism (Campana-Rouget 1959, Simões et al. 1992). Both categories are thought to be a result of teratological defects and may be caused by genetic factors and changes in temperature and humidity during tick development (Buczek 2000, Kar et al. 2015), feeding on exotic hosts (Nowak-Chmura 2012), or exposure to insecticides or other chemicals (Campana-Rouget 1959, Buczek et al. 2013). Such defects are rare in ticks, with total incidence of

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general or local abnormalities from 0.028% to 0.2% (Tovornik 1987, Kar et al. 2015, Larson and Paskewitz 2016). These abnormalities seem to occur at even lower frequency in *Ixodes* (Nowak-Chmura 2012, Kar et al. 2015, Larson and Paskewitz 2016), and of general abnormalities, only occurrences of gynandromorphism and asymmetry have been reported in this genus (Prusinski et al. 2015, Larson and Paskewitz 2016).

Here, we report the first evidence of nanism in *I. scapularis*, confirming the identity of the specimen using molecular markers and scanning electron microscopy. This specimen was found biting a child and was submitted for screening of tick-associated pathogens to the Tick Testing Laboratory at the Connecticut Agricultural Experiment Station (CAES).

Materials and Methods

Specimen Submission

The specimen was submitted on November 6, 2015 for tick testing at the CAES. The tick had been removed from a child with no out of state travel history in Ridgefield, CT, in early November. Following morphological examination, it was determined the specimen was not engorged and thus was not tested for tick-associated pathogens. The specimen was stored in 75% EtOH for further analysis.

Morphological Identification

Initial morphological identification was attempted following Keirans and Litwak (Keirans and Litwak 1989). Due to the unusually small size and morphological features of the specimen, we used an AXIO Scope.A1 (Zeiss, Göttingen, Germany) with an attached RT3 camera system (SPOT Imaging, Sterling Heights, MI) to capture ventral and dorsal images of the specimen, and then proceeded with scanning electron microscopy (SEM) and molecular identification.

Scanning electron microscopy was used to visualize the morphological characters of the specimen. The specimen was dehydrated in 99.8% EtOH and critical point dried (931GL, Tousimis, Rockville, MD). The dried specimen was mounted on an SEM stub using carbon tape, sputter coated with gold/palladium (E5100, Polaron), and examined with the aid of a Nova NanoSEM 450 (FEI, Hillsboro, OR) scanning electron microscope.

Following SEM, we used a dichotomous key to the adults of *Ixodes* based on scanning electron micrographs (Keirans and Clifford 1978). Morphological characters of the specimen were measured in ImageJ2 (Schindelin et al. 2015), using images from light microscopy or SEM. Morphological structures were defined following Keirans et al. (1996): *breadth* for all features were measured at their broadest point; *idiosomal length* was based on the length from the scapular apices to the posterior body margin; and *coxae length* from the insertion of the anterior most seta adjacent to the trochanter diagonally to the posterior tip of the coxa (or the tip of the internal spur, for coxa I). All measurements are reported in millimeters.

DNA Extraction and Sequencing

For molecular identification, the tarsi and tibiae of the specimen were removed for DNA extraction. DNA was extracted from the removed appendages using DNAzol BD (Molecular Research Center, Cincinnati, OH) according to manufacturer's recommendations with minor modifications. Briefly, the sample was homogenized in a tube containing 400 µl DNAzol BD and two zinc-plated BBs (Daisy Outdoor Products, Rogers, AK) using a TissueLyser Mixer Mill (Qiagen, Valencia, CA). The sample was then incubated at 70 °C for 10 min, then mixed and centrifuged at 14,000 RPM for 10 min. Following the addition of 3 µl Poly Acryl Carrier (Molecular Research Center), DNA was precipitated using 200 µl 100% EtOH. The DNA pellet was washed twice with 750 µl 75% EtOH, air dried briefly, reconstituted in 30 µl dH_20, and stored at −20 °C for further analysis.

The identity of the specimen was confirmed using several molecular markers—the ribosomal internal transcribed spacer 2 (ITS2) region, the 16S mitochondrial ribosomal region, and the nuclear gene coding for the serotonin 4 receptor.

ITS2

The full ITS2 region, along with partial sequences of 5.8S and 28S, was amplified with polymerase chain reaction (PCR). The primers were TITS2F1, 5′-CGA GAC TTG GTG TGA ATT GCA-3′, and TITS2R1, 5′-TCC CAT ACA CCA CAT TTC CCG-3′ (Chitimia et al. 2009) that yielded fragments of ∼900 bp. GoTaq G2 Green Master Mix (Promega, Madison, WI) was used according to the manufacturer's protocols in a 50-µl reaction, containing 0.3 µM of each primer and 2 µl of template DNA. Thermal cycling conditions included an initial denaturation step of 95 °C for 3 min, followed by 35 cycles of 95 °C for 45 s, 60 °C for 1 min, and 72 °C for 75 s.

16S

The mitochondrial 16S region was amplified using primers mt-rrs1, 5′-CTG CTC AAT GAT TTT TTA AAT TGC TGT GG-3′, and mt-rrs2, 5′-CCG GTC TGA ACT CAG ATC AAG TA-3′ (Ushijima et al. 2003) that yielded a fragment size of 456 bp. Reaction conditions for 16S were as described above for the ITS2, except that 1 µl of DNA template was used. Thermal cycling conditions included an initial denaturation step of 95 °C for 2 min, followed by 35 cycles of 95 °C for 30 s, 48 °C for 30 s, and 72 °C for 45 s, with a final extension step of 72 °C for 5 min.

Serotonin 4

The nuclear region serotonin 4 was amplified with primers S4F, 5′-AAC GAA ACC ACG CTC AAG A-3′, and S4R, 5′-GTA GCA GAC AGC GAA CAG CA-3′ (Van Zee et al. 2013), which yielded a fragment size of 650 bp. Reaction mixture included 2 µl of DNA template and 0.5 µM of each primer. Thermal cycling conditions included an initial denaturation of 95 °C for 2 min, followed by 40 cycles of 95 °C for 30 s, 58 °C for 30 s, and 74 °C for 30 s, with a final extension step of 74 °C for 5 min.

Sequence Analysis

All PCR products were purified using QIAquick PCR Purification kit (Qiagen), sequenced in both directions at the Keck DNA Sequencing Center, Yale University (New Haven, CT), and sequences were assembled in Geneious version 9 (Kearse et al. 2012). Geneious was used to query the NCBI's nr database with the megablast program (Camacho et al. 2009), returning the top 50 most similar hits for all sequences. These hits were sorted by "Grade" in Geneious, a weighted combination of pairwise identity, coverage, and E score, and each hit was assigned a rank from 1 to 50, relative to its sorted position. Identity of the specimen was assessed based on the resulting tables.

Results

Specimen Description

Measurements for morphological characters are provided in Table 1, and descriptors of specific regions are given here.

Body

Scutal color brownish, outline oval, light brown opisthosoma.

Capitula

Auriculae broadly rounded. Palpis with suture between article II and III distinct, article II ~1.7 times longer than article III. Hypostome dentition 4/4 apically, 3/3 for most of the visible dentitions, and then 2/2 near base. Cornua small, porose areas semicircular.

Scutum

Carinae absent or not visible, cervical grooves faint, with visible punctations and setae primarily in marginal areas.

Venter

Spiricular plate oval, genital aperture between coxae IV.

Legs

Tarsi and tibiae removed or damaged and were not used in specimen description. Coxa I with internal spur. Syncoxae, if present, not visible. Coxae I–IV with blunt external spur.

Morphological Comparison

Due to the small size of the specimen, identification using morphological characters alone was difficult. The presence of porose areas (not shown in figures), a genital aperture between coxae IV, as well as hypostomal shape and dentition indicated the specimen was an adult female tick despite a size more typical of a nymph (Fig. 1; Supp. Fig. 1 [online only]). Comparisons with SEM images narrowed the species identity based on morphological characters to *I. scapularis*, *Ixodes pacificus* Cooley & Kohls, or *Ixodes jellisoni* Cooley & Kohls. Due to the small size of the internal spur on coxa I, further classification of this specimen was not feasible; however, given the geographic location where the specimen was found, the tick was preliminarily identified as *I. scapularis*.

A comparison of the morphological measurements from the specimen with those reported in the redescription of *I. scapularis* (Keirans et al. 1996) revealed that the specimen in question was approximately half the size of a typical *I. scapularis* female (Table 1, Supp. Fig. 1 [online only]). Furthermore, the dentition of the specimen, 4/4 at the tip of the hypostome, declining to 3/3 and eventually to 2/2, was consistent with that of *I. scapularis*. In addition, the rounded auriculae (Supp. Fig. 2 [online only]) and elongate internal spur of coxa I (Supp. Fig. 3 [online only]) were consistent with an adult female *I. scapularis*.

Sequence Analysis

Sequence analysis of the three genes used in this study indicated the specimen in question is most likely *I. scapularis*. BLAST search results yielded multiple sequences with >96% pairwise identity between the ITS2 sequence from the specimen (GenBank KY985362) and other sequences available on GenBank for *I. scapularis*. Both 16S (GenBank KY985363) and serotonin 4 (GenBank KY985364), with exact matches found on GenBank, unequivocally confirmed the identity of the specimen as *I. scapularis*. Although our ITS2 sequence was not identical to any sequence deposited in GenBank, only *I. scapularis* sequences had "Grades" higher than 91%, with the next nearest taxa sequence belonging to *Ixodes persulcatus* with a rank of 28, a "Grade" of 90.5%, and pairwise identity of 81% (GenBank accession for this *I. persulcatus* sequence, D88872).

Table 1. Measurements of some morphological features of the dwarf *I. scapularis*, compared to the reported range in the species description of *I. scapularis*

Feature	Dwarf *I. scapularis*		Reported range (Keirans et al. 1996)	
	Length	Breadth	Length	Breadth
Idiosomal length[a]	1.47	0.84	2.37–2.7	1.43–1.89
Palpis	0.40	0.13	0.68–0.79	0.17–0.21
Hypostome	0.33	–	0.51–0.59	–
Scutum[a]	0.82	0.69	1.23–1.46	1.02–1.30
Spiricular plate	0.19	–	0.33–0.44	–
Coxa I	0.28	–	0.45–0.56	–
Internal spur (Coxa I)	0.07	0.04	0.11–0.17	–
Coxa II	0.25	–	0.37–0.44	–
Coxa III	0.25	–	0.39–0.46	–
Coxa IV	0.22	–	0.30–0.46	–

All measurements are in millimeters and rounded to the nearest hundredth.
[a] Measurements taken from light microscopy images (not shown).

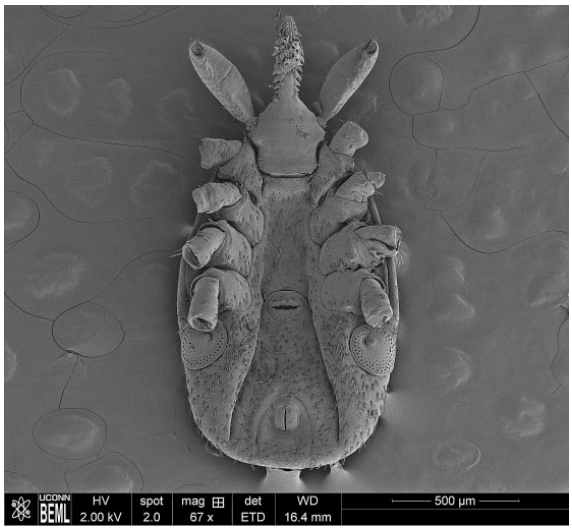

Fig. 1. The ventral scanning electron micrograph of a dwarf *I. scapularis*. Despite its small size, the specimen has features consistent with an adult female, such as the genital aperture visible here.

Discussion

Our analysis of this specimen provides the first evidence of nanism in *I. scapularis*. The specimen possesses the characters of an adult female tick, and because the size of this tick falls far outside the range of previously reported values for female *I. scapularis*, we conclude that this tick is a dwarf *I. scapularis*. Although nanism has been reported in other tick taxa (Campana-Rouget 1959, Simões et al. 1992), to the best of our knowledge, this is the first report of this type of abnormality in *Ixodes*.

General abnormalities such as nanism are extremely rare in *I. scapularis*; of all the ticks examined by investigators throughout

the eastern United States, this is only the seventh reported occurrence of any teratological abnormality in this species. Over the past two decades (1997–2016), the Tick Testing Program at the CAES has received and examined a total of 88,347 ticks, of which 81,475 (92.5%) have been identified as *I. scapularis*; however, this marks the first encounter of an adult *I. scapularis* of abnormally small size. Although it is possible that dwarf ticks may be mischaracterized as nymphs during routine examinations, and thus nanism may occur more commonly, dwarf *I. scapularis* have never been reported previously, suggesting the scarcity of this phenomenon.

It is worth noting that a prior report of general abnormalities in *I. scapularis* detailed two gyandromorphic specimens from the Hudson River Valley region of the State of New York (Prusinski et al. 2015), and the specimen reported here was submitted from the same general region. That three of the general abnormalities reported in *I. scapularis* have been found in the same general geographical region suggests the possibility that certain environmental factors might be involved. Research has shown that teratological defects may be caused by variation in temperature or humidity during tick development (Buczek 2000, Kar et al. 2015). In Europe, the occurrence of morphologically abnormal ticks has reportedly increased (Alekseev and Dubinina 2004, Alekseev et al. 2007) and may be related to increasing levels of pollution (Alekseev and Dubinina 2008). In another Ixodid, *Dermacentor andersoni*, extreme size variation was heritable (de la Fuente et al. 2005), but whether this applies to the teratological defects found in *I. scapularis* is unknown.

Accurate morphological identification of tick specimens is important for understanding of species abundance and diversity, as well as determining which specimens require examination for the evidence of infection. As such, teratological defects complicate identification of tick specimens, but what effect these abnormalities might have on the ability of *I. scapularis* to transmit pathogens is unknown. Studies in *I. persulcatus* suggest ticks with exoskeletal anomalies may have greater infections with *Borrelia* (Alekseev and Dubinina 2000), and may also have more multiple simultaneous pathogen infections, than morphologically normal ticks (Alekseev et al. 2007). Whether these findings can be generalized to include *I. scapularis* with teratological abnormalities is unknown.

Here we report the first evidence of nanism, and and an additional example of a general teratological abnormality, in *I. scapularis*. Given the difficulties associated with species identification of tick specimens with morphological abnormalities and potential implications in pathogen transmission (Alekseev and Dubinina 2000, Alekseev et al. 2007), further investigation of teratologies is needed in *I. scapularis*.

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