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Single-tube real-time PCR assay for differentiation of *Ixodes affinis* and *Ixodes scapularis*

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Abstract

Ixodes affinis Neumann (1899) and *Ixodes scapularis* Say (1821) are tick vectors of the etiologic agent of Lyme disease, *Borrelia burgdorferi* sensu stricto. *Ixodes affinis* and *I. scapularis* are morphologically very similar, and as they are sympatric in the mid- and south-Atlantic U.S. coastal states, their accurate identification is crucial to studies of disease and vector ecology in this area. This work describes a rapid, single-tube SYBR® Green-based real-time PCR assay for differentiation of *I. affinis* and *I. scapularis* at all life stages. The assay employs 2 pairs of species-specific primers directed against the internal transcribed spacer 2 (ITS2) region of the nuclear rRNA operon. Amplification products for these primer pairs differ in size and may be differentiated with a melt curve analysis. This tool is intended as a supplement to morphological methods for accurate identification of these ticks.

Keywords

Ixodes affinis; *Ixodes scapularis*; Real-time PCR; Identification

Introduction

Ixodes affinis Neumann (1899) and *Ixodes scapularis* Say (1821) are hard-bodied (ixodid) ticks, both of which are vectors of *Borrelia burgdorferi* sensu stricto, the agent of Lyme disease (Oliver et al., 2003). *Ixodes scapularis*, the primary vector of *B. burgdorferi* s.s. to humans in the eastern United States, has a wide distribution ranging from Florida to Nova Scotia, Canada, and west to North and South Dakota and Mexico (Keirans and Clifford, 1978). *Ixodes affinis* is more narrowly distributed, with reports of established populations from Florida, Georgia, South Carolina, North Carolina, and Virginia (Clark et al., 1998; Harrison et al., 2010; Nadolny et al., 2011), however its range appears to be expanding (Nadolny et al., 2011). Although *I. affinis* is rarely known to bite humans (Oliver, 1996), it has a role in the ecological dynamics of Lyme disease in that it shares many of the same hosts as *I. scapularis* and may thus contribute to the amplification of *B. burgdorferi* s.s. In the southeastern U.S., *I. affinis* appears to be more important in the enzootic cycle of *B. burgdorferi* s.s. than *I. scapularis* (Oliver, 1996; Oliver et al., 2003; Harrison et al., 2010; Maggi et al., 2010).

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Because of the overlapping distribution of *I. affinis* and *I. scapularis* in the southeastern U.S., it is necessary to have an accurate method of differentiating these 2 species at any life stage. *Ixodes affinis* and *I. scapularis* can be distinguished morphologically (Keirans and Clifford, 1978; Oliver et al., 1987; Durden and Keirans, 1996). Morphological features, however, can be variable and difficult to determine in engorged and damaged specimens, especially nymphs and larvae. Although the seasonal variation in active questing times of *I. affinis* and *I. scapularis* can serve as an indication of species identity in some areas (Harrison et al., 2010), in other localities both species quest continually throughout the summer months (Nadolny et al., 2011), further complicating the ability to accurately distinguish between the two. Supplemental methods for accurate identification of these 2 species are necessary for retroanalysis of previously examined ticks and reclassification of incorrectly identified specimens, a vitally important task in areas where *I. affinis* is invading.

In this work, we describe a multiplex real-time PCR (qPCR) assay that supplements morphological identification of *I. affinis* and *I. scapularis*. Whereas traditional PCR and sequencing can also be used to accomplish this goal, these procedures can take several days and cost >10 USD per sample to accomplish at minimum sequence coverage. In contrast, the assay described here can be performed rapidly (1 h) in a single tube, at a cost approximately tenfold less than sequencing. Amplification and restriction digestion (PCR-RFLP) has also been used successfully to differentiate *I. affinis*, *I. scapularis*, and other *Ixodes* spp. (Poucher et al., 1999). PCR-RFLP thus provides a viable alternative to sequencing or qPCR methods, but is more time- and labor-intensive than the latter, as well as potentially more sensitive to single-nucleotide polymorphisms. The qPCR assay presented here is effective for all life stages of *I. affinis* and *I. scapularis* and can also be used to differentiate *I. affinis* and *I. scapularis* from other *Ixodes* spp. This assay provides a means to accurately verify morphological identifications and will greatly improve the ability to rapidly and economically identify nymphal and larval *Ixodes* ticks to species level.

Materials and methods

Tick collection and morphological identification

Ticks, including adults, nymphs, and larvae, were collected from several geographic locations in southeastern Virginia in 2010 (Nadolny et al., 2011) and 2011. Questing ticks were collected by dragging white denim cloth flags through areas of vegetation. Engorged *I. scapularis* were collected from white-tailed deer (*Odocoileus virginianus*) at various hunt stations in southeastern Virginia in October of 2010. Adult and nymphal *Ixodes* spp. ticks were identified using morphological features (Keirans and Clifford, 1978). Field-collected ticks were kept at −80°C until their DNA was extracted. Questing *I. affinis* from Beaufort County, North Carolina (n=30) were collected on flags as described above, and *I. affinis* from Bulloch County, Georgia (n=5), were collected either from vegetation or from a domestic dog (*Canis lupus familiaris*). Ten engorged *I. scapularis* nymphs were acquired from a colony (Wikel strain) located at Old Dominion University. This colony was originally established at the University of Connecticut Health Center (UCHC) using ticks collected in Connecticut as described by Bouchard and Wikel (2005). The *I. scapularis* (Wikel strain) colony is the reference strain for the *Ixodes* Genome Project (described in Pagel Van Zee et al., 2007). A single *I. scapularis* specimen collected from a deer in southeastern Virginia was determined via 16S rRNA gene sequencing to belong to the southern clade of the species (J. Brinkerhoff, pers. communication). *Ixodes cookei*, *I. dentatus*, and *Ixodes texanus* specimens were provided by the Centers for Disease Control and Prevention (CDC) and originally collected from Vermont, New York, and an unknown location, respectively. *Ixodes muris* specimens were provided by the Maine Medical Center

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Disease Institute. The *Ixodes pacificus* specimen was collected in California from a domestic dog.

DNA extraction

DNA from individual *Ixodes* spp. adults and nymphs was extracted in an area separate from PCR setup. Adult ticks were cut in half longitudinally with a sterile blade prior to DNA extraction. One half of each adult was extracted and the other half stored at −80°C. The adult halves and whole nymphs were each disrupted using 1.0-mm glass beads and a Mini-Beadbeater-1 (BioSpec Products, Inc., Bartlesville, OK, USA). DNA extraction was performed using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) following the manufacturer's protocol. DNA was eluted in a final volume of 125 μL of buffer AE. DNA samples were stored at −20°C until processing.

Primer design

Primers specific to *I. affinis* and *I. scapularis* (Table 1) were designed within the internal transcribed spacer 2 (ITS2) region of the nuclear rDNA transcriptional unit. These primers amplify fragments of 75 and 142–144 bp for *I. affinis* and *I. scapularis*, respectively, and are designed in such a manner that the reverse *I. affinis*-specific primer overlaps the forward *I. scapularis*-specific primer by 15 bp. *Ixodes scapularis* sequence for ITS2 was obtained from GenBank, whereas the ITS2 sequence for *I. affinis* was obtained by amplification and Sanger sequencing using primers IxSeq5.8SF and 28S R1/1 (Fukunaga et al., 2000; Table 1). ITS2 sequence for *I. affinis* has been deposited under GenBank #JX982149. Briefly, ITS2 (and flanking 5.8S and 28S sequence) was PCR-amplified in a 15-μL reaction volume containing 1X buffer (containing 1.5 mM $MgCl_2$), 1X Coral Load, 1X Q-solution, 0.2 mM dNTPs, 1 μM forward and reverse primer, and 1.25 U *Taq* polymerase (all TopTaq, Qiagen, Valencia, CA). Reaction products were verified on 1.5% agarose gels stained with SYBR® Safe (Invitrogen, Grand Island, NY) and purified with ExoSAP-IT® (USB/Affymetrix, Santa Clara, CA) according to manufacturer's directions. Purified products were directly sequenced using amplification primers and BigDye® v3.1 reagents (ABI, Carlsbad, CA) followed by analysis on an ABI 3130xl capillary sequencer. Sequence alignment and editing was performed with Geneious v.5 software (Drummond et al., 2010), and primers were designed using Beacon Designer v.7 (PREMIER Biosoft Intl., Palo Alto, CA).

Standard PCR

A 454-bp fragment of the tick 16S mitochondrial rRNA gene was amplified using primers 16S+1 and 16S–1. Amplifications were performed in 25-μL reaction volumes containing EconoTaq® PLUS 2X Master Mix (Lucigen, Middleton, WI), 0.4 μM of each primer, and 2 μL of target DNA. Reaction conditions were an initial denaturation at 95°C for 4 min, followed by 35 cycles of 95°C for 1 min, 50°C for 1 min and 68°C for 1 min, and a final extension at 68°C for 10 min. Amplified PCR products were electrophoresed on a 2% agarose gel stained with ethidium bromide.

Real-time PCR

Amplifications were performed in duplicate in 15-μL reaction volumes containing 1X Bio-Rad iQ™ SYBR® Green supermix, 0.5 μM of each primer (aff_f8, aff_r8, scap_f2.2, scap_r2.2), and 2 μL target DNA. Reaction conditions were an initial denaturation at 95°C for 3 min, followed by 40 cycles of 95°C for 15 s, 62°C for 30 s, and 72°C for 30 s, with a plate read after the 72°C (extension) step. A melt curve analysis was performed after amplification by cooling samples to 65°C and raising the temperature at 0.5°C intervals to 95°C for 5 s/interval and a plate read at each step. Real-time PCR reactions were performed on a CFX96™ thermocycler (Bio-Rad, Hercules, CA).

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Statistical analysis

Student's t-tests were performed using Systat 10 statistical software (Chicago, IL).

Results

Single-tube, multiplex PCR of specimens identified morphologically as *I. affinis* (n=122) or *I. scapularis* (n=89) returned strong (Ct<20) amplification in greater than 90% of samples for both species (Fig. 1). Three *I. affinis* and one *I. scapularis* either did not amplify or returned mean replicate Ct values of >30. One tick identified morphologically as *I. affinis* and with mean replicate Ct of >30 was subsequently identified via 16S mitochondrial rRNA gene sequencing as *Ixodes minor* (98.5% similarity with *I. minor*, GenBank #AF549841). Two clearly separated ranges of melt peaks were characteristic of *I. affinis* (81.5–82.5°C) and *I. scapularis* (84.0–85.5°C) (Fig. 2). Melt peaks of 84.0°C were observed in only 2 specimens from the Connecticut-originating *I. scapularis* colony. In a single adult specimen morphologically identified and sequence-confirmed as *I. scapularis*, replicate melt peaks of 83.5°C were returned (Table 2). Sequence data from ITS2 in this individual revealed a G-to-T transversion at position 84 of the amplicon relative to all other individuals sequenced at this locus in the study; it is unknown whether this single base change is sufficient to account for the observed melt peak anomaly. Mean Ct values among samples with Ct<30 did not significantly differ (Student's t-test; p=0.89) with respect to adult (15.93 ± 3.00 [mean ± S.D.]) or nymphal (16.02 ± 3.00) life stage, and a single larval *I. affinis* was amplified with a mean Ct value of 14.77.

A subset of samples was sequenced at the 16S mitochondrial rRNA gene and/or ITS2 loci for validation of the qPCR assay. Concordance between qPCR results and sequence data for both loci was 100% for both *I. affinis* and *I. scapularis*, regardless of life stage. The entire panel of ticks for which both sequence (16S and/or ITS2) and qPCR data were obtained included 53 individuals identified by molecular means as *I. affinis*, 18 individuals identified as *I. scapularis*, and one nymphal tick that failed to amplify by the qPCR assay and that was subsequently identified as *I. minor* based on 16S mitochondrial rRNA gene sequencing. The sequenced group comprised 58 adults, 13 nymphs, and 1 larva, with adult and nymphal specimens represented for both *I. affinis* and *I. scapularis.* The larval specimen was provisionally morphologically identified as *I. scapularis* based on the presence of black legs; qPCR and 16S sequence data indicated this individual was *I. affinis*.

Ixodes spp. other than *I. affinis* and *I. scapularis* amplified weakly (>30) or not at all by the qPCR assay. Two *Ixodes brunneus* individuals amplified with mean Ct values of 33.05 and 34.86. Melt peaks for these individuals were inconsistent, with one showing peaks at 82.0°C and 84.0°C for the replicate reactions, and another showing one peak at 82.0°C with the replicate peak undetectable. The single *I. dentatus* specimen amplified with mean Ct of 33.83 and melt peaks of 74.0°C and 84°C for the duplicates. No amplification (Ct<35) was observed for 2 *I. muris*, one *I. pacificus*, or one *I. texanus* specimen. No Ct values of less than 32 were observed in non-*Ixodes* ticks, with the exception of 2 *Dermacentor variabilis*, which amplified at mean Ct values of 24.21 and 28.24. Melt curves for these specimens were inconsistent, with the first at 85.0°C in both replicates and the second at 81.5°C and 82.0°C. No amplification (Ct<35) was observed in no-template control reactions accompanying all experimental runs, and melt curves of 74.0–75.0°C were occasionally observed, likely representing primer dimers.

Engorged adult (identified morphologically) and nymphal (Wikel colony) *I. scapularis* were assayed by qPCR. Mean Ct for this group was 12.88 ± 3.47, as compared to unfed *I. scapularis* adults (16.93 ± 2.09). All ticks in this group demonstrated melt curves consistent

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with *I. scapularis*. These results indicate engorgement status had no negative effect on the qPCR assay performance.

Discussion

The multiplex single-tube qPCR assay described in this paper provides rapid, reliable, and accurate means to differentiate between *I. affinis* and *I. scapularis.* This will provide a tool for verification of morphological identifications and has potential to refine ongoing studies of the biology, phylogeography, and disease ecology of these important vectors of *B. burgdorferi* s.s. *Ixodes affinis* appears to be expanding its range northward (Nadolny et al., 2011) and has recently been identified (as larvae and a nymph) on migratory songbirds in Canada (Scott et al., 2012). Given the close morphological similarity between *I. scapularis* and *I. affinis*, this rapid molecular method will be useful in confirming new range expansions, as well as accurately categorizing individuals from field collections. PCR-RFLP-based procedures for differentiation of *I. affinis*, *I. scapularis*, and other *Ixodes* spp. have been described previously (Poucher et al., 1999), however the assay presented here provides an alternative method allowing suitably equipped laboratories to take advantage of reagent and labor savings inherent to qPCR, relative to older amplification and digestion procedures.

Results from the panel of ticks used demonstrated the potential for misidentification of *I. affinis* and *I. scapularis* by morphological means alone. We found a total of 5 *I. scapularis* misidentified morphologically as *I. affinis* (4.1% of all ticks identified as *I. affinis*) and 6 cases in which the reciprocal misidentification was made (8.7% of ticks identified morphologically as *I. scapularis*, excluding Wikel colony specimens). Of 5 nymphs identified morphologically as *I. affinis*, 4 were found to be *I. scapularis*, and one was shown by sequence data to be *I. minor* after non-amplification with the qPCR assay. Incidentally, this represents the first report from Virginia for *I. minor*, which has previously been reported in Florida, Georgia, and South Carolina (Keirans and Clifford, 1978; Clark et al., 2001). All nymphs identified morphologically as *I. scapularis* were confirmed via qPCR, although 1 larva and 5 adults identified as *I. scapularis* were misidentified *I. affinis*.

Based on the performance of this assay, the following recommendations need to be considered for its use. First, despite a general lack of reactivity with non-target *Ixodes* and non-*Ixodes* spp., the assay did show sporadic cross-reactivity with *Dermacentor variabilis*, and melt peaks, although inconsistent, did overlap with those of *I. affinis* and *I. scapularis.* Further, although *I. affinis* and *I. scapularis* could be readily differentiated with this assay, it was not designed to positively identify other species. Therefore, it is important that this assay be used as a supplement to morphological identification, rather than as a replacement. Differentiation of *Ixodes* from non-*Ixodes* is straightforward even at larval and nymphal stages (Keirans et al., 1996) as is differentiation of most *Ixodes* adults and nymphs from *I. affinis* and *I. scapularis*. Larval identification among *Ixodes* spp. can, however, be somewhat more challenging, and further multiplexing variants of this assay may be useful for specific research projects. Second, we noted no amplification of non-target *Ixodes* spp. at Ct values of less than 32 and therefore recommend that Ct=30 be considered a conservative cutoff for determining validity of qPCR-based identification of *I. affinis* or *I. scapularis*. We have taken such an approach in Table 2 and note that if a much more conservative approach of setting the cutoff at Ct=20 were used, this would only remove 12 additional individuals from the Ct<30 *Ixodes* data set (n=207). As a compromise, we suggest that amplifications with Ct>30 be considered invalid, and those >20 be treated with caution, possibly requiring re-extraction using the other tick half and assay or sequence confirmation. Melt peaks for individual samples should fall within the ranges of 81.5–82.5°C and 84.0–85.5°C for *I. affinis* and *I. scapularis*, respectively. We note here that this assay is not quantitative in

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nature, and Ct value is in no way intended to be used for differentiating between *I. affinis* and *I. scapularis*. This is particularly the case as gDNA concentration was not standardized between individuals. Rather, Ct values are presented as a reference to potential users of this assay and are intended to be used as a quality control measure.

Internal transcribed spacer regions of rDNA transcriptional units are generally much more variable than ribosomal subunits, as they are not subject to the high selective pressure of maintaining specific ribosomal structure. As such, polymorphism in ITS2 sites may conceivably compromise the performance of this assay in genetically divergent populations through the ranges of *I. affinis* and *I. scapularis*. This possibility was tested by inclusion in the data set of 35 *I. affinis* from North Carolina (30 ticks) and Georgia (5 ticks), and 10 *I. scapularis* of Connecticut origin (Wikel colony). All of these specimens demonstrated amplification (Ct<25) and expected melt peaks for the respective species. The existence of a distinct southern population of *I. scapularis* is well known (Norris et al., 1996; Humphrey et al., 2010), and we performed a search of GenBank for ITS2 sequences from *I. scapularis* in this region. Unfortunately, several deposited sequences (Rich et al., 1997) of southern (Florida) *I. scapularis* do not include our primer binding sites in the sequenced range. However, 4 ITS2 sequences of ticks from North Carolina (L22276), and Georgia (L22273, L22274, L22275) were analyzed in silico and were found to be compatible with the *I. scapularis*-specific primers used in this study, producing predicted amplicons of 143–144 bp. We also tested an *I. scapularis* individual collected in Virginia, but determined via 16S sequencing to belong to the southern clade for this species, and it returned results consistent with other *I. scapularis* in the study (mean Ct = 14.07; melt peak = 85.5). The G-to-T transversion noted at position 84 of the *I. scapularis* amplicons was also noted in GenBank sequences JQ868566 and L22272 representing *I. scapularis* from Illinois and New York, respectively (Wesson et al., 1993; Hamer et al., 2012). It is possible that this substitution may lead to lower melt temperatures (83.5°C) for certain *I. scapularis* individuals, however the assay should be robust to this possibility as melt ranges for *I. scapularis* and *I. affinis* would continue to be separated by a full degree Celsius. This indicates that the qPCR assay presented here should be useful for *I. affinis* and *I. scapularis* differentiation at least along the U.S. Atlantic coast. Given the caveats about the ITS2 locus described above, however, it would be advisable for qPCR results from ticks outside the geographic ranges described here to be confirmed by limited sequencing before large-scale assay deployment.

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Scott JD, Anderson JF, Durden LA. Widespread dispersal of *Borrelia burgdorferi*-infected ticks collected from songbirds across Canada. J. Parasitol. 2012; 98:49–59. [PubMed: 21864130]

Wesson DM, McLain DK, Oliver JH, Piesman J, Collins FH. Investigation of the validity of species status of *Ixodes dammini* (Acari: Ixodidae) using rDNA. Proceedings of the National Academy of Sciences. 1993; 21:10221–10225.

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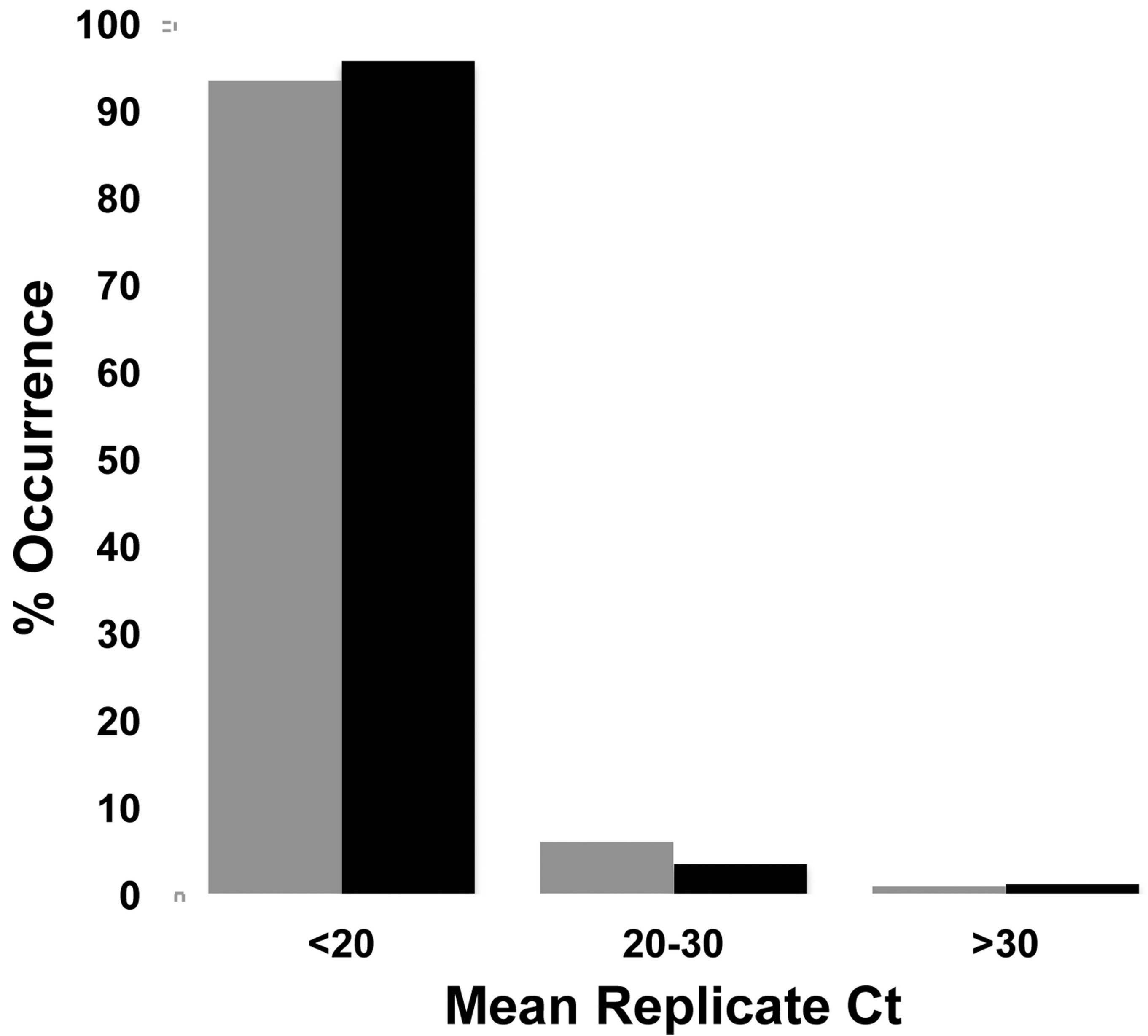

Fig. 1.
Frequency histogram of mean replicate Ct values for ticks identified morphologically as *I. affinis* (grey bars) or *I. scapularis* (black bars).

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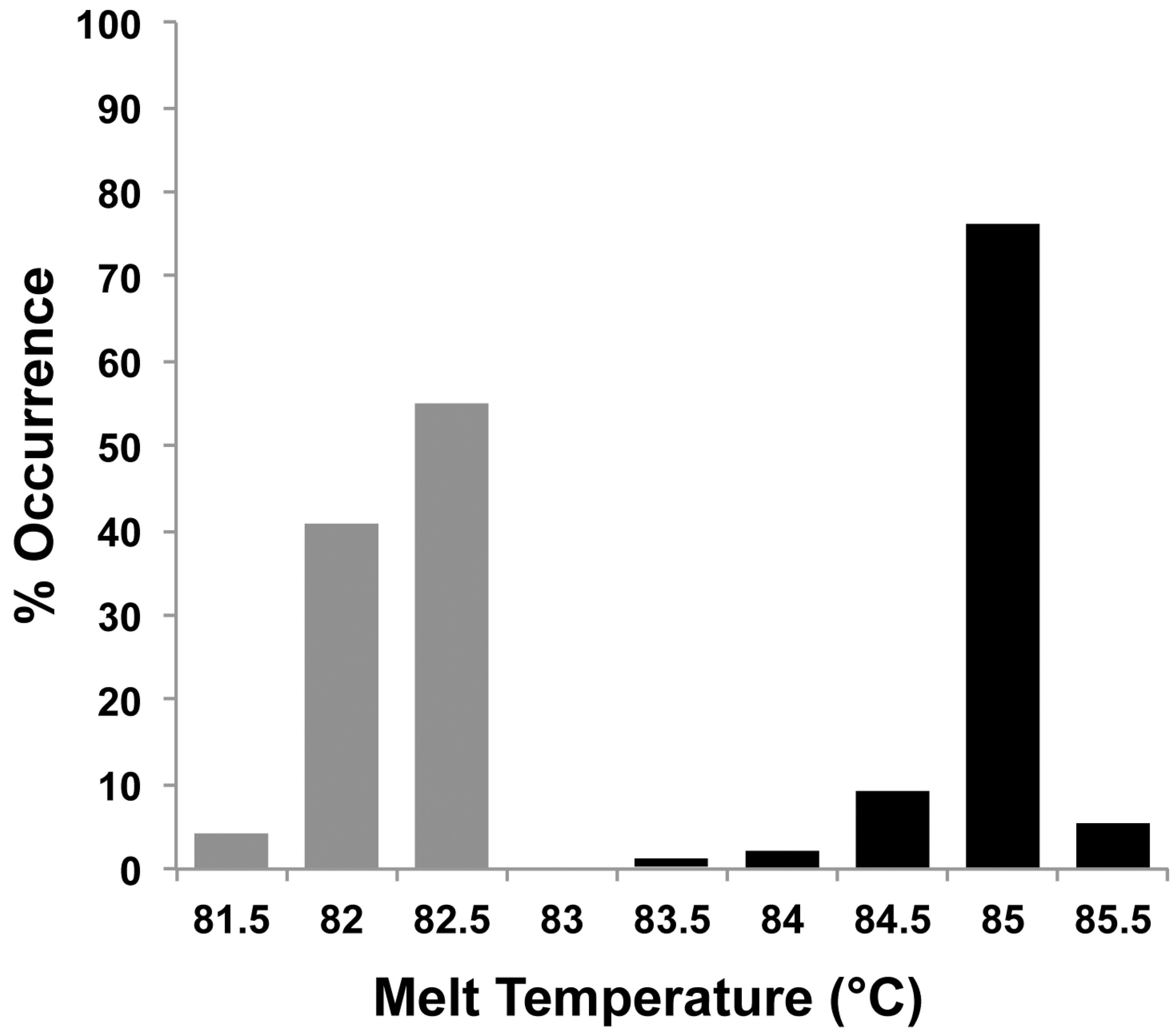

Fig. 2.
Frequency histogram of observed melt temperature range for *I. affinis* (grey bars) and *I. scapularis* (black bars).

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Table 1

Oligonucleotide primers used in this study.

Primer name	Primer sequence	Locus (position)	Expected size (bp)	Reference
IxSeq-5.8SF	5'-TCGATGAAGAACGCAGCCAG-3'	5.8S rRNA gene[a] (31–50)	846	Modified from (Fukunaga et al., 2000)
28S R1/1	5'-TTCTATGCTTAAATTCAGGGGGTTGTC-	28S rRNA gene (20–46)[b]		(Fukunaga et al., 2000)
16S-1	5'-GTCTGAACTCAGATCAAGT-3'	16S mito. rRNA gene (183–201)[c]	451	(Macaluso et al., 2003)
16S+1	5'-CTGCTCAATGATTTTTTAAATTGCTGT-3'	16S mito. rRNA gene (609–634)[c]		(Nadolny et al., 2011)
aff_f8	5'-TGGAAATCCCGCAAATCT-3'	ITS2 (214–231)[a]	75[d]	This study
aff_r8	5'-CCGTTCCAATCTCCGTTTA-3'	ITS2 (277–296)[a]		This study
Scap_f2.2	5'-GCGTTAGAAACGGAGATTTGA-3'	ITS2 (271–291)[a]	142–144[e]	This study
Scap_r2.2	5'-CCACGAGATTTACATTTGCC-3'	ITS2 (394–413)[a]		This study

[a]GenBank #L22266, *Ixodes scapularis*.

[b]GenBank #D88878, *Ixodes ricinus*.

[c]GenBank #AB105167, *Ixodes acutitarsus*.

[d]in *I. affinis*.

[e]in *I. scapularis*.

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Table 2

Summary of real-time PCR (qPCR) and sequencing results for ticks used in this study. Ticks are categorized by initial morphological identification in column 1. qPCR results are separated by column, and numbers in parentheses represent number of animals returning sequence-based identification consistent with qPCR divided by total number of animals sequenced at either ITS2 or 16S mitochondrial rRNA gene loci. Some individuals were sequenced at both loci and are represented in both denominators within a given set of parentheses.

Species (morphological ID)	Life stage	N	Ct < 30	Ct > 30	qPCR results, sequencing results: (ITS2; 16S)	
					I. affinis	*I. scapularis*
Ixodes affinis	Adult	117	115	2	115, (6/6; 48/48)	1, (1/1; 1/1)
	Nymph	5	4	1[a]	0	4, (3/3; 2/2)
Ixodes scapularis	Adult	59	58	1	5, (4/4; 0/0)	53[b], (4/4; 5/5)
	Nymph	29	29	0	0	29, (5/5; 8/8)
	Larva	1	1	0	1 (0/0; 1/1)	0
Ixodes brunneus	Adult	2	0	2	0	0
Ixodes cookei	Adult	1	0	1	0	0
Ixodes dentatus	Adult	1	0	1	0	0
Ixodes muris	Adult	2	0	2	0	0
Ixodes pacificus	Adult	1	0	1	0	0
Ixodes texanus	Adult	1	0	1	0	0
Amblyomma americanum	Nymph	2	0	2	0	0
Amblyomma maculatum	Adult	2	0	2	0	0
Dermacentor albipictus	Adult	1	0	1	0	0
Dermacentor variabilis	Adult	11	2	9	1	1
Haemaphysalis leporispalustris	Nymph	2	0	2	0	0
	Larva	1	0	1	0	0

[a]One nymph identified morphologically as *I. affinis* did not amplify by the qPCR assay and was subsequently identified as *Ixodes minor* via 16S mitochondrial rRNA gene sequencing (98.5% pairwise identity with GenBank #AF549841, *I. minor*).

[b]One additional specimen returned a melt curve not consistent with either *I. affinis* or *I. scapularis* (83.5°C); this specimen was determined to be *I. scapularis* by 16S and ITS sequencing. This specimen returned Ct values >20 in repeated assays.