Optimization of duplex real-time PCR with melting-curve analysis for detecting the microsporidian parasites *Nosema apis* and *Nosema ceranae* in *Apis mellifera*[1]

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Abstract—Honey bees, *Apis mellifera* (L.) (Hymenoptera: Apidae), are parasitized by the microsporidians *Nosema apis* (Zander) and *Nosema ceranae* (Fries). Molecular techniques are commonly used to differentiate between these parasites because light microscopy is inadequate. Our objectives were to (*i*) adapt the previously published duplex polymerase chain reaction (PCR) targeting the 16S rRNA gene of *N. apis* (321APIS-FOR, 321APIS-REV) and *N. ceranae* (218MITOC-FOR, 218MITOC-REV) using qualitative real-time PCR assay with SYBR® Green I dye (R-T PCR) and DNA melting-curve analysis, and (*ii*) determine whether the two *Nosema* species can be detected simultaneously in honey bees. Total spore counts and purified total genomic DNA were obtained from 37 bee samples (19 individual workers and 18 pooled samples of 15 workers) collected in Nova Scotia, Prince Edward Island, and Newfoundland, Canada. Overall, the prevalence of *Nosema* species was 86.5% (32/37 samples of bee DNA), based on conventional PCR and the optimized R-T PCR assay. The melting-curve analysis showed three groups of curve profiles that could determine the prevalence of *N. apis*, *N. ceranae*, and co-infection (21.9%, 56.2%, and 21.9%, respectively). The duplex R-T PCR assay was efficient, specific, and more sensitive than duplex conventional PCR because co-infection was identified in 5.4% (n = 2) more samples. Sequencing of R-T PCR products confirmed the results of the melting-curve analysis. Duplex R-T PCR with melting-curve analysis is a sensitive and rapid method of detecting *N. apis*, *N. ceranae*, and co-infection in honey bees.

Résumé—Les abeilles domestiques, *Apis mellifera* (L.) (Hymenoptera: Apidae) sont parasitées par les microsporidies *Nosema apis* (Zander) et *N. ceranae* (Fries). Parce que la microscopie optique est inadéquate, on utilise couramment des méthodes moléculaires pour distinguer ces parasites. Nos objectifs sont 1) d'adapter la méthode déjà publiée de la réaction de PCR (amplification en chaîne par polymérase) duplex qui cible le gène 16S de l'ARNr de *N. apis* (321APIS-FOR et 321APIS-REV) et de *N. ceranae* (218MITOC-FOR et 218MITOC-REV) à l'aide d'un test qualitatif au vert de SYBR I en temps réel avec une analyse de la courbe de fusion de l'ADN (R-T PCR) et 2) de voir s'il est possible de détecter simultanément les deux espèces de *Nosema* chez les abeilles. Nous avons obtenu les dénombrements de spores et l'ADN génomique total purifié dans 37 échantillons d'abeilles (19 ouvrières individuelles et 18 échantillons collectifs de 15 ouvrières) récoltés en Nouvelle-Écosse, à l'Île-du-Prince-Édouard

Received 13 November 2009. Accepted 12 February 2010.

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doi: 10.4039/n10-010

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et à Terre-Neuve, Canada. La prévalence globale de *Nosema* est de 86,5 % (32/37 échantillons d'ADN d'abeilles) d'après les analyses de PCR conventionnelle et de R-T PCR optimisée. L'analyse de la courbe de fusion révèle l'existence de trois groupes de profils de courbes qui permettent d'identifier les prévalences de *N. apis*, de *N. ceranae* et de co-infections (respectivement 21,9 %, 56,2 % et 21,9 %). Le test de la R-T PCR duplex est efficace, spécifique et plus sensible que la PCR duplex ordinaire parce que la co-infection a pu être décelée dans 5,4 % (n = 2) plus d'échantillons. Le séquençage des produits de la R-T PCR confirme les résultats de l'analyse de la courbe de fusion. La PCR duplex au vert SYBR I en temps réel avec une analyse de la courbe de fusion est une méthode sensible et rapide de détection de *N. apis*, de *N. ceranae* et des co-infections chez les abeilles.

[Traduit par la Rédaction]

Introduction

Honey bees, *Apis mellifera* (L.) (Hymenoptera: Apidae), are parasitized by two microsporidians, *Nosema apis* (Zander) and *Nosema ceranae* (Fries). Parasitism by *Nosema apis*, the historical microsporidian parasite of honey bees, reduces honey yield, increases winter mortality, and causes poor spring build-up of surviving colonies (Fries 1993). Parasitism by *N. ceranae*, a parasite formerly restricted to Asian honey bees, *Apis cerana* (F.), but now distributed nearly globally in honey bees (*e.g.*, Klee *et al.* 2007; Williams *et al.* 2008*a*; Higes *et al.* 2009), has reduced honey production and increased winter mortality in honey bees in Spain (Higes *et al.* 2006) and was highly pathogenic under laboratory conditions (Higes *et al.* 2007). While numerous methods exist for controlling *N. apis*, including heat treatment or fumigation of comb and equipment, the antibiotic fumagillin dicyclohexylammonium (fumagillin) is most commonly used (Fries 1993). Few data exist, but preliminary results suggest that fumagillin is also effective against *N. ceranae* (Williams *et al.* 2008*b*; Williams *et al.* 2010). To properly assess control methods, a reliable method of quickly and accurately identifying *Nosema* species to determine infection prevalence and intensity is needed.

Nosema species parasitizing honey bees are morphologically similar when viewed using light microscropy but can be differentiated by ultrastructural features (number of polar filaments) and using molecular methods such as the polymerase chain reaction (PCR) (Fries *et al.* 1996). Prior to 2005, because disease in honey bees was attributed solely to *N. apis,* researchers relied on light microscopy to determine prevalence and intensity of *Nosema* infection and did not commonly use molecular techniques. Consequently, *N. ceranae* went undetected in honey bees for 10 years (Huang *et al.* 2007; Klee *et al.* 2007; Paxton *et al.* 2007; Chen *et al.* 2008). Conventional PCR primers initially designed by Higes *et al.* (2007) to amplify a 240 base pair (bp) region of the 16S rRNA gene failed to detect the presence of the two *Nosema* species simultaneously, owing to small size differences (12 bp) between *N. apis* and *N. ceranae* amplicons (*e.g.*, Higes *et al.* 2007; Williams *et al.* 2008*b*). The recent adoption of duplex PCR (Martín-Hernández *et al.* 2007), which employs multiple primers in a single PCR reaction to amplify a region of the 16S rRNA gene for the two species simultaneously, has increased the sensitivity of detection while reducing effort, because size differences (101–102 bp) of amplicons for each species can be observed during gel visualization, thus eliminating the need to sequence every PCR product.

Like previous techniques, qualitative real-time PCR with SYBR® Green I dye (R-T PCR) and DNA melting curve analysis is a technique capable of identifying multiple species in complex samples containing DNA from many species (Berry and Sarre 2007). Species-specific PCR primers are used with R-T PCR to amplify DNA; then melting-curve analysis, which is based on the melting temperatures of different PCR products, can be used to differentiate between species by observing specific curves, thus eliminating the extra step of gel analysis. The R-T PCR method is very sensitive and has been used in pathogen diagnostics (*e.g.*, Yu *et al.* 2005; Burgher-MacLellan *et al.* 2009), including detecting microsporidia in environmental

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samples (Wolk *et al*. 2008). Recently, Chen *et al.* (2009) reported a duplex quantitative R-T PCR protocol (qPCR) using TaqMan probes for quantifying *Nosema* spp. in Asian honey bees; however, using these methods requires the added expense of labeled probes with different dyes that are not required by our single-dye R-T PCR assay. The aim of our study was to optimize and evaluate simplex *versus* duplex R-T PCR for detecting co-infections by the two *Nosema* species in honey bees. We used species-specific primers and DNA melting-curve analysis rather than gel visualization. Our objectives were to (*i*) determine whether previously published species-specific primers for *N. apis* and *N. ceranae* spores could be optimized for the SYBR® Green I R-T PCR method, and (*ii*) use the optimized method and melting-curve analysis to detect *N. apis* and *N. ceranae* in honey bees.

Material and methods

Sample collection

Workers were collected in late summer 2007 from the hive entrances of colonies known to be co-infected by *N. apis* and *N. ceranae* (G.R. Williams, unpublished data), one each in Nova Scotia (NS) and Prince Edward Island (PEI), Canada, and from one colony infected with *N. apis* only in Newfoundland (NF), Canada. In spring 2008, as part of another study, more workers were collected from the hive entrances of >100 colonies from six beekeeping operations in NS. *Nosema* intensity (number of spores per bee) was estimated at the individual-bee and the colony level using light microscopy and a haemacytometer (Cantwell 1970; Rogers and Williams 2007). For individual-bee samples, a single abdomen was crushed using a pellet pestle in 2 mL centrifuge tubes containing 1 mL of distilled water. For colonies (composite-bee samples), 15 worker bees' abdomens collected from the same colony were crushed in 15 mL of distilled water using a mortar and pestle. Samples of the crushed-bee solution were frozen and stored at −20 °C until DNA was extracted.

DNA extraction

Nineteen samples of individual crushed bee solution (9 bees each from one NS colony and one PEI colony and 1 NF bee) and 18 composite crushed bee samples (15 bees from three colonies from each of six NS beekeeping operations) representing a range of spore intensities were selected for molecular analyses (Table 1). For each sample of individual or composite crushed-bee solution, a 250 µL aliquot was pretreated with 10 µL of proteinase K (20 mg/mL) (Sigma-Aldrich Canada, Oakville, Ontario; www.sigmaaldrich.com) for 20 min at 37 °C prior to DNA purification by following a modified protocol (steps 1–3 were omitted) of the Ultra Clean Tissue DNA Extraction Kit (Mo Bio Laboratories, Carlsbad, California, United States of America; www.mobio.com). DNA yields were measured with a Nanodrop 1000 spectrophotometer (Fisher Scientific, Ottawa, Ontario; www.fishersci.ca). DNA samples were stored at −20 °C prior to PCR.

Conventional duplex PCR

Conventional duplex PCR was performed using a Biometra TGradient thermocycler (Montreal Biotech Inc., Dorval, Quebec; www.montreal-biotech.com). The 25 µL reaction mix consisted of 1× ID *Taq* buffer (1.0 U *Taq*, 0.2 mM each dNTP, 1.5 mM $MgCl_2$; ID Labs Inc., London, Ontario; www.idlabs.com), 400 nM each primer (Sigma-Genosys) and 1.0 µL of DNA template (10 ng). Primers 321APIS-FOR and 321APIS-REV for *N. apis* and 218MITOC-FOR and 218MITOC-REV for *N. ceranae* (Martín-Hernández *et al.* 2007), renamed as primer sets NAPIS and NCERANAE, respectively, were used at the concentrations and equimolar amounts used for conventional duplex PCR as described by Martín-Hernández *et al.* (2007). DNA samples from bees previously identified as being co-infected with *N. apis* and *N. ceranae* were used for gradient PCR to determine the annealing-temperature range for primers NAPIS and NCERANAE. PCR was performed using the following parameters: an initial 4-min denaturing period at 94 °C followed by 35 cycles of 15 s denaturing at 94 °C, 30 s annealing at 50–60 °C, 45 s extension at 72 °C, a 7 min final extension period at 72 °C, and finishing at 4 °C. To ensure that there were no PCR inhibitors in the individual-bee DNA samples (n = 18), the

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Table 1. *Nosema* spore intensity estimated using light microscopy, DNA yield (ng/µL), and species detected using conventional duplex PCR for 19 individual worker honey bees (*Apis mellifera*) collected from each of three colonies in Nova Scotia (NS), Prince Edward Island (PEI), and Newfoundland (NF), Canada, and 18 composite worker honey bee samples (15 individuals per sample) collected from six beekeeping operations (NSC-1 to NSC-6) in Nova Scotia.

Sample and type	No. of spores	DNA (ng/µL)	Species
Individual			
PEI-1	50 000	6.17	–
PEI-2	8 650 000	4.42	*N. apis*
PEI-3	38 000 000	13.81	*N. ceranae*
PEI-4	200 000	6.41	–
PEI-5	49 750 000	6.26	*N. ceranae*
PEI-6	15 300 000	5.21	*N. apis*
PEI-7	0	4.51	–
PEI-8	3 500 000	4.68	*N. apis*
PEI-9	33 550 000	7.38	*N. apis* / *N. ceranae*
NS-1	21 300 000	8.91	*N. apis*
NS-2	0	4.86	–
NS-3	4 550 000	8.04	*N. ceranae*
NS-4	250 000	6.25	*N. apis* / *N. ceranae*
NS-5	0	13.32	–
NS-6	150 000	4.85	*N. ceranae*
NS-7	650 000	9.09	*N. apis*
NS-8	11 800 000	5.51	*N. apis*
NS-9	13 500 000	8.54	*N. ceranae*
NF-1	4 650 000	8.08	*N. apis*
Composite			
NSC-1.a	350 000	12.3	*N. ceranae*
NSC-1.b	40 800 000	14	*N. ceranae*
NSC-1.c	3 250 000	9.3	*N. ceranae*
NSC-2.a	400 000	11.3	*N. ceranae*
NSC-2.b	10 500 000	14.4	*N. ceranae*
NSC-2.c	26 800 000	15.8	*N. ceranae*
NSC-3.a	1 600 000	10.1	*N. ceranae*
NSC-3.b	2 350 000	7.1	*N. ceranae*
NSC-3.c	2 650 000	6.4	*N. ceranae*
NSC-4.a	3 800 000	11.1	*N. ceranae*
NSC-4.b	1 600 000	9.6	*N. ceranae*
NSC-4.c	3 750 000	6.6	*N. ceranae*
NSC-5.a	4 650 000	7.9	*N. apis* / *N. ceranae*
NSC-5.b	1 050 000	8.9	*N. ceranae*
NSC-5.c	22 800 000	11	*N. apis* / *N. ceranae*
NSC-6.a	10 250 000	11.8	*N. ceranae*
NSC-6.b	550 000	11.1	*N. apis*
NSC-6.c	6 350 000	11.7	*N. apis* / *N. ceranae*

*A dash indicates that duplex PCR failed to identify *Nosema* species.

universal mitochondrial cytochrome *c* oxidase I (COI) primers LCO1490-F and HCO2198-R (Hebert *et al*. 2003) were used for simplex PCR with the following PCR parameters: an initial 2-min denaturing period at 95 °C, followed by 35 cycles of 15 s denaturing at 95 °C, 30 s annealing at 50 °C, 30 s extension at 72 °C, a 7-min final extension period at 72 °C, and finishing at 4 °C. Bee DNA and other mitochondrial DNA in the multispecies-complex DNA samples were considered to be internal controls because of the absence of a

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mitochondrial genome in *Nosema* spp. (Cavalier-Smith 1987).

All PCR products were visualized by electrophoresis in 1.4% agarose gels stained with SYBR® Safe DNA gel stain (Invitrogen, Burlington, Ontario; www.invitrogen.com). The negative PCR control (no template control (NTC)) and 100-bp DNA ladder (Mandel Scientific Company Inc., Guelph, Ontario; www.mandel.ca) were included on the gel. A subset of amplified PCR products from the individual-bee DNA samples ($n = 6$, 3 samples from each of two colonies in NS and PEI) was purified using a QIAquick Gel Extraction Kit (Qiagen, Mississauga, Ontario; www1.qiagen.com) prior to double-stranded sequencing performed by a gene analyzer with capillary electrophoresis (Applied Biosystems, Foster City, California; www.appliedbiosystems.com) at Florida State University.

R-T PCR assay using SYBR® Green I dye

The *Nosema*-specific primers (NAPIS and NCERANAE) were optimized for R-T PCR using purified DNA (10 ng/reaction) combined from 2 individual-bee DNA samples containing approximately equal amounts of *N. apis* and *N. ceranae* spores (NF-1 and NS-3; Table 1). Real-time assay performance was optimized for both simplex (one primer pair) and duplex (both primer pairs) conditions using primer concentration titrations (50–600 nM) for the forward and reverse primers of each primer pair in simplex and duplex R-T PCR. Optimal forward and reverse primer concentrations were determined by selecting conditions with the lowest threshold cycle (C_t) value, the largest change in fluorescence normalized to the reference dye ROX, and if possible, the lowest primer concentration necessary to decrease duplex primer interactions.

All optimized simplex and duplex R-T PCR assays were performed on the Mx4000 thermocycler (Stratagene, La Jolla, California; www.stratagene.com). The 25 µL R-T PCR reaction mix consisted of a 1× master mix (Stratagene), 50–600 nM of each primer (forward and reverse) for the NAPIS and (or) NCERANAE primer sets, 0.4 µL of ROX (reference dye) diluted 1/500, and 1 µL (approximately 10 ng) of template DNA. Each reaction was repeated in triplicate, included negative controls, and had the following PCR parameters: an initial 10-min denaturing period at 95 °C followed by 40 cycles of 30 s denaturing at 95 °C, 30 s annealing at 60 °C, 30 s extension at 72 °C, and a final 5-min extension period at 72 °C. Data were visualized as amplification plots (fluorescence *vs.* cycle number) showing the C_t value for each sample. Melting-curve analysis was plotted as the derivative change in fluorescence divided by the change in temperature ($-R'(T)$) *versus* temperature (T) to show individual melting curves with peak melting temperatures (T_m). Temperature profiles for melting curves were 1 min at 95 °C and 30 s at 55 °C, followed by 40 successive 30-s increases of 1 °C and a final holding temperature of 4 °C. Samples (10 µL) were loaded in 1.4% agarose gels prestained with SYBR® Safe DNA gel stain (0.1 µL/mL) (Invitrogen) after R-T PCR to verify that data corresponded to expected PCR-product size.

R-T PCR assay performance

To ensure the accuracy of R-T PCR, a standard curve was included to test precision, limit of detection (LOD), linear dynamic range, and efficiency of the primers NAPIS and NCERANAE (simplex and duplex). Composite sample NSC-5.c (Table 1) was chosen for a 10-fold DNA dilution series (11.0 ng/µL – 1.1 pg/µL) over 5 orders of magnitude because it contained both *N. apis* and *N. ceranae*, and when diluted over 5 orders of magnitude, spore intensity extended the range of most samples. All reactions were run in triplicate with simplex and optimized duplex R-T PCR conditions. In addition, a melting-curve analysis was performed to ensure reaction specificity.

Nosema survey using optimized duplex R-T PCR

All DNA samples listed in Table 1 were analyzed in triplicate for *N. apis* and *N. ceranae* infection (10 ng DNA/reaction) using the optimized duplex R-T PCR assay and melting-curve analysis; for confirmation of results, R-T PCR products were visualized by

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electrophoresis in 1.4% agarose gels as previously described. Three R-T PCR products were sequenced to confirm the specificity of the optimized duplex R-T PCR assay. Samples PEI-6 and NS-3 were purified (Ultra Clean PCR DNA purification kit, Mo-Bio Laboratories), and both PCR products (218 and 321 bp) for sample NSC-6.a were extracted from the gel and purified (Quantum Prep Freeze ′N Squeeze DNA Gel Extraction Kit, Bio-Rad, Hercules, California; www.bio-rad.com). Sequence reactions were performed using the ABI BigDye Terminator V3.1 Cycle Sequence Ready Reaction Kit (Applied Biosystems). Both DNA strands were sequenced using the ABI 3100 Sequencer (Applied Biosystems) at the Crops and Oilseeds Development Centre, Agriculture and Agri-Food Canada, Ottawa, Ontario. All sequences from conventional PCR and R-T PCR were edited using Lasergene 7.1 Seqman software (DNASTAR, Madison, Wisconsin; www.dnastar.com). Consensus sequences were retrieved from the GenBank database (National Center for Biotechnology Information; www.ncbi.nlm.nih.gov) using the BLAST server for sequence identification.

Results

Conventional end-point duplex PCR

The total genomic DNA yield from crude suspensions of crushed bee abdomens ranged from 221 ng (4.42 ng/μL) to 720 ng (14.4 ng/μL) for all individual- and composite-bee samples (Table 1). DNA purity measured with 260/280 ratios ranged from 1.7 to 2.1 nm, indicating high-quality genomic DNA. On average, total DNA yields were 30% higher for composite-bee samples than for individual-bee samples; however, on average, total spore counts were 28% higher in individual-bee samples (data not shown). Simplex- and duplex-gradient PCR indicated that 60 °C was the optimal annealing temperature (T_a) because it produced the brightest PCR product band on the agarose gel; this T_a value is compatible with the thermocycle profile for R-T PCR. Conventional duplex PCR yielded products of two sizes, indicating the presence of *N. apis* (321 bp) and *N. ceranae* (218–219 bp) (Martín-Hernández *et al.* 2007). With duplex PCR for individual-bee DNA samples, *Nosema* prevalence was estimated to be 72.2% (13/18 samples); with light microscopy it was estimated to be 83.3% (15/18 samples), although 2 samples had spore counts <200 000, meaning that <4 spores were observed. Among individual-bee DNA samples, 5 with spore counts ≤200 000 failed to yield PCR products. Conversely, 100% (18/18) of composite-bee DNA samples yielded PCR products; however, all had spore counts of >200 000 per sample. Overall, with conventional PCR, *Nosema* prevalence was 86.5% (32/37) and *N. apis* and *N. ceranae* were detected in 25% (8/32) and 59.4% (19/32) of infected bee samples, respectively; co-infection occurred in 15.6% (5/32) of infected samples (individual and composite). Agarose-gel analysis showed that for all DNA samples, PCR products varied in light intensity, and the results of agarose-gel analysis using COI universal primers yielded the expected 658-bp PCR product for all individual-bee DNA samples (n = 18), thus confirming the absence of PCR inhibitors in 5 individual-bee DNA samples that failed to yield PCR products for *Nosema* infection (Fig. 1).

GenBank BLAST searches of sequence data from 6 samples confirmed the presence of both *Nosema* species; data are published in GenBank (accession Nos. FJ527824, FJ527825, and FJ536220 for *N. ceranae* and FJ536221, FJ536222, and FJ536223 for *N. apis*).

R-T PCR assay using SYBR® Green I dye

Simplex primer optimization (forward and reverse) was obtained for NAPIS and NCERANE at equimolar concentrations (350 nM); however, optimal conditions for duplex R-T PCR occurred when NAPIS was one half the concentration of NCERANAE (175 and 350 nM, respectively) (data not shown).

Repeatability (SD < 0.5) and specificity (T_m) for NAPIS and NCERANAE are outlined in Table 2 (simplex and duplex R-T PCR) and Figure 2 (duplex R-T PCR) using 10-fold dilutions of composite-bee DNA co-infected with *N. apis* and *N. ceranae*. Overall, simplex assays detected the presence of

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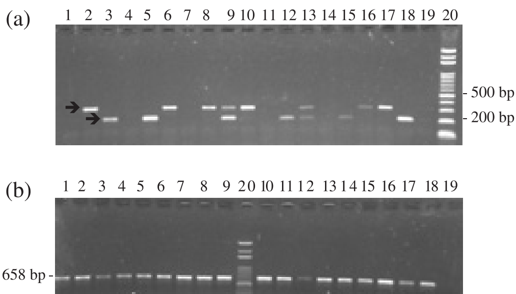
Fig. 1. (*a*) Conventional SYBR® Green I dye PCR for purified honey bee (*Apis mellifera*) DNA from individual workers suspected to be infected with *Nosema apis* and *Nosema ceranae* spores. Duplex PCR with the primer sets NAPIS and NCERANAE to detect *Nosema* spp. infection or co-infection (the arrows indicate PCR products: 321 bp for *N. apis*, 219 bp for *N. ceranae*, and both PCR products for co-infection). (*b*) Simplex PCR with the universal cytochrome *c* oxidase primers LCO1490 and LCO2198 (PCR product 658 bp) to confirm the absence of PCR inhibitors (lanes 1–9, PEI-1 to PEI-9; lanes 10–18, NS-1 to NS- 9; lane 19, no template control; lane 20, 100-bp DNA ladder).



Nosema spp. in <10 pg of composite-bee DNA. The lowest mean C_t values, indicating the highest target DNA (C_t values are inversely related to the amount of initial DNA template), were found in the duplex assay. The ranking of C_t values over all DNA dilutions was as follows: duplex < simplex NCERANAE < simplex NAPIS. Melting-curve analysis yielded consistent T_m values for each primer set over 5 orders of dilution (78.5 °C for NAPIS and 77.5 °C for NCERANAE); however, a 100-fold dilution (110 pg) caused T_m values to decrease from 78.5 to 77.5 °C for the duplex assay despite the two dilutions having similar melting-curve profiles (Fig. 2*b*). A C_t value of 38.2 was produced for the NTC sample using duplex R-T PCR; melting-curve analysis indicated that the corresponding peak (a small, wide peak at a lower temperature, T_m = 72.5 °C) was due to the presence of primer–dimers (a double-strand primer association causing a signal). The presence of primer–dimers in the NTC is acceptable because peaks were not found in experimental samples. Primer–dimers were not observed in the simplex R-T PCR assays (data not shown).

Standard curves for the duplex R-T PCR assay (Fig. 2*c*) had a linear dynamic range between 10 ng and 10 pg of composite-bee DNA with an acceptable fit (R^2 = 0.98). However, the Y- intercept had a theoretical LOD at C_t = 26.36, corresponding to a total genomic DNA concentration <110 pg. Simplex-assay mean C_t values (Table 2) indicated that there was more *N. ceranae* than *N. apis* DNA template in the sample; therefore, the Y-intercept and melting-curve analysis showed that the primer set NAPIS was not able to detect *N. apis* in a duplex R-T PCR reaction when the DNA dilution was ≤110 pg (the minimum recommended range of template DNA for R-T PCR is 100 pg – 1 µg; Invitrogen Corporation 2008). The efficiency calculated from the slope was 102.8%, suggesting that the duplex R-T PCR assay performed within the optimal efficiency range, 90%–110%, and that the results were not influenced by contaminants or poor optimization.

Nosema spp. survey using optimized duplex R-T PCR

All bee DNA samples previously determined to contain *Nosema* spp. by means of conventional PCR (*n* = 32) produced R-T PCR C_t values ranging from 25.2 to 33 and from 22 to 32 for individual- and composite-bee samples, respectively (data not shown). Melting-curve analysis yielded two T_m values, 77.5 and 78.5 °C, among the individual- and composite-bee samples, with melting-curve profiles placed in three groups by observing *T* where $-R'(T)$ began to increase (Figs. 3*a*, 3b). Among individual-bee samples, for 27.8% (*n* = 5), T_m = 77.5 °C and *T* = 72 °C where $-R'(T)$ increased (group *i*); for 11.1% (*n* = 2), T_m = 78.5 °C and *T* = 72 °C where $-R'(T)$ increased (group *ii*); and for 33.3% (*n* = 6), T_m = 78.5 °C and *T* = 74 °C where $-R'(T)$ increased (group *iii*). No T_m value was obtained for 5 samples, indicating the absence of the target PCR product (Fig. 3*c*). Composite-bee samples were divided into two groups: for 72.2% (*n* = 13), T_m = 77.5 °C (group *i*) and for 27.8% (*n* = 5), T_m = 78.5 °C (group

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Table 2. Repeatability and specificity of simplex and duplex real-time PCR (SYBR® Green I dye) for DNA of honey bees (*Apis mellifera*) co-infected with *Nosema apis* and *Nosema ceranae*, using the primer pairs NAPIS and NCERANAE, at 10-fold serial dilutions.

		Simplex NAPIS C_t value					Simplex NCERANAE C_t value					Duplex NAPIS/NCERANAE C_t value				
		Replicate					Replicate					Replicate				
DNA	No. of spores	1	2	3	Mean ± SD	T_m	1	2	3	Mean ± SD	T_m	1	2	3	Mean ± SD	T_m
11.0 ng	22 800 000	23.9	24.0	23.7	23.9±0.1	78.5	22.5	22.5	22.9	22.7±0.2	77.5	19.0	19.1	19.0	19.1±0.1	78.5
1.1 ng	2 280 000	28.6	28.0	28.3	28.3±0.3	78.5	26.0	26.2	26.4	26.2±0.2	77.5	23.3	23.1	23.0	23.2±0.1	78.5
110.0 pg	228 000	32.1	32.4	32.2	32.3±0.2	78.5	30.2	30.3	30.7	30.4±0.3	77.5	27.2	27.0	27.0	27.1±0.1	77.5
11.0 pg	22 800	35.3	35.0	35.7	35.4±0.4	78.5	33.8	34.4	34.0	34.2±0.4	77.5	30.5	30.1	30.6	30.5±0.3	77.5
1.1 pg	2 280	No C_t	39.2	39.5	39.6±0.5	78.5	36.4	37.4	37.0	37.0±0.5	77.5	31.5	32.0	32.6	32.1±0.5	77.5
NTC		No C_t	No C_t	No C_t	No C_t	59.5	No C_t	No C_t	No C_t	No C_t	61.5	39.3	38.2	37.7	38.4±0.8	72.5

*Ct, threshold cycle number; No C_t, no C_t value after 40 cycles; NTC, no template control.

ii); for all composite samples, $T = 72$ °C where $-R'(T)$ increased (Fig. 3*c*). Two composite samples (NSC-6.a and NSC-6.b) that were previously determined with conventional PCR to be infected with *N. ceranae* or *N. apis* were now found in group *ii* with co-infected samples. Agarose-gel analysis of duplex R-T PCR products (Figs. 3*e*, 3*f*) confirms the assignment of melting-curve analysis profile groups. Samples in group *i* contained a *N. ceranae* PCR product band at 218 bp, samples in group *ii* contained both *N. cerane* and *N. apis* PCR bands at 218 and 321 bp, respectively, and samples in group *iii* contained a *N. apis* PCR band at 321 bp. A BLAST search of GenBank revealed that the sequenced R-T PCR products from sample PEI-6 and the 321-bp PCR band from NSC-6.a yielded 100% matches for *N. apis* (accession Nos. DQ235446 and NAU97150), and that sample NS-3 and the 218-bp PCR band from NSC-6.a yielded 100% matches for *N. ceranae* (accession Nos. EU545141, EF584424, EF584422, EF584421, EF584420, and EF584419). Sequence data confirmed the presence of *Nosema* spp. in total genomic bee DNA samples, and identical results were obtained with agarose-gel or melting-curve analysis alone. Overall, R-T PCR determined *Nosema* spp. prevalence to be 86.5% (32/37) and *N. apis, N. ceranae*, and co-infection prevalence to be 21.9% (7/32), 56.2% (18/32), and 21.9% (7/32), respectively.

Discussion

A sensitive, reliable, and relatively fast method for identifying *N. apis* and *N. ceranae* in samples containing complex honey bee DNA is important for monitoring *Nosema*-induced stress in honey bee colonies. Here we show that *Nosema* spp. prevalence determined using duplex R-T PCR with previously published primers for *N. apis* and *N. ceranae* (Martín-Hernández *et al.* 2007) (86.5%) was similar to that determined using conventional duplex PCR. A comparison of these methods is summarized in Table 3 for the individual- and composite-bee DNA samples. With both conventional and R-T duplex PCR, prevalence of *N. ceranae* infection was more than 2.5

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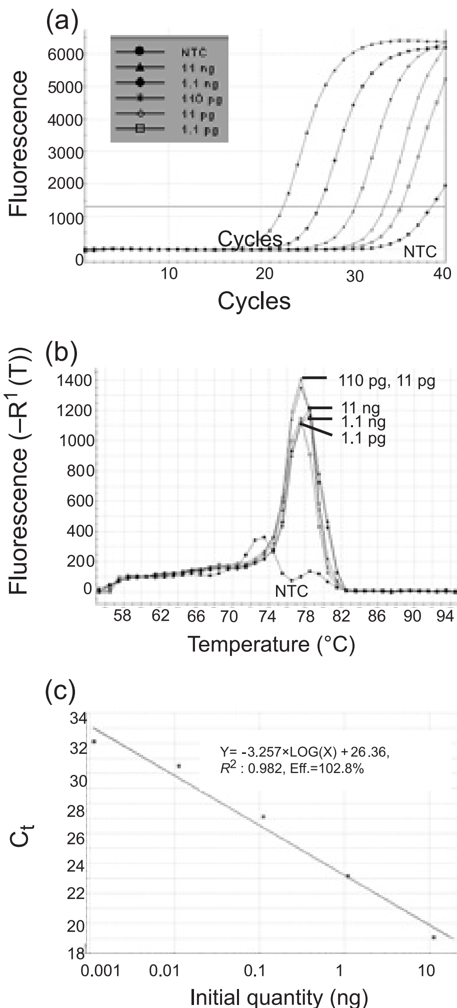
Fig. 2. SYBR® Green I dye duplex real-time PCR assay optimization with the primer sets NAPIS and NCERANAE, showing amplification plots of 10-fold serial dilutions of purified honey bee (*Apis mellifera*) DNA co-infected with *Nosema apis* and *Nosema ceranae* spores (*a*), melting-curve analysis (*b*), and standard curve (*c*) from the same experiment, illustrating the sensitivity, specificity, and linearity of the assay (NTC, no template control; standard-curve parameters: slope, −3.257; correlation coefficient, 0.982; PCR efficiency, 102.8%).



times greater in the composite-bee DNA samples than in the individual-bee DNA samples, whereas with R-T duplex PCR, *N. apis* infection only was found in the individual-bee DNA samples. However, conclusions about the rate of *Nosema* spp.infection should not be derived from this study, as the colonies selected were previously known to be infected with both *N. apis* and *N. ceranae* and were only sampled once. We found that the R-T PCR assay with melting-curve analysis was more sensitive than conventional PCR because it detected two co-infections in composite-bee DNA samples that were not previously observed with conventional agarose-gel analysis.

Melting-curve analysis is important for interpreting the specificity of R-T PCR results. Raw data plots (not shown) demonstrate that fluorescence declined as temperature increased, with a sharp decline and end point at the exact T_m value for each PCR product because SYBR® Green I dye emits a fluorescent signal only when associated with double-stranded DNA. Negative first derivatives of the raw data ($-R'(T)$) are plotted as a curve with a peak T_m value that clearly demonstrates the temperature at which the PCR product starts to melt. Each PCR product has a specific T_m value, based on sequence composition (GC content) and length; ideally, a single T_m value is observed for optimized specific primers. Although primer sets NAPIS and NCERANAE were originally designed for conventional PCR, they are specific and thus suitable for R-T PCR because the simplex R-T PCR assays had one T_m value for each primer pair (T_m = 77.5 and 78.5 °C for *N. ceranae* and *N. apis*, respectively). Duplex R-T PCR distinguished single *Nosema* infections using T_m values and it was observed that there was a delay in T where an increase in $-R'(T)$ occurs in the samples that had a T_m value of 78.5 °C. If, in a mixture of two PCR products, their T_m values differ by >2 °C, two T_m peaks with two distinct values will be observed in the melting-curve analysis (Ririe *et al.* 1997). We observed only one melting curve, because T_m values differ by only 1 °C for primer sets NAPIS and NCERANAE; however, by observing the melting curve we could identify group *ii* (Figs. 3*a*, 3*c*, 3*d*), where $-R'(T)$ increased at T = 72 °C (same value for *N. ceranae* infection only) and T_m = 78.5 °C (same value for *N. apis* infection). Thus, the melting-curve profile indicated the presence of both PCR products

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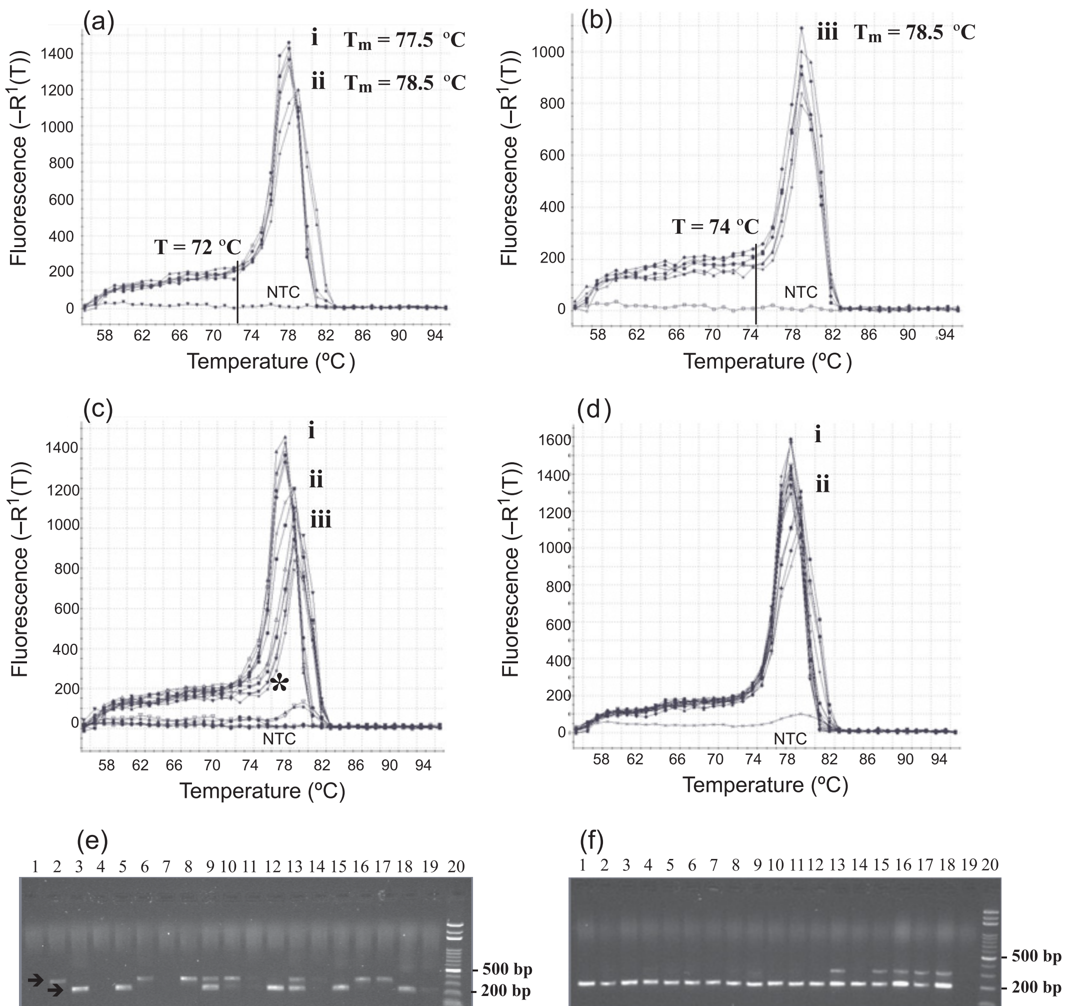
Fig. 3. Qualitative duplex real-time PCR (R-T PCR) assay identifying infection or co-infection of *Nosema apis* and *Nosema ceranae* with the primer sets NAPIS and NCERANAE in individual (n = 18) and composite (n = 18) samples of purified honey bee (*Apis mellifera*) DNA and no template control (NTC) samples. (*a*, *b*) Melting-curve analysis (derivative change in fluorescence divided by change in temperature ($-R'(T)$) *vs.* temperature (T)), with melting-temperature (T_m) peaks and three curve profiles (*i*, T = 72 °C and T_m = 77.5 °C, *N. ceranae*; *ii*, T = 72 °C and T_m = 78.5 °C, co-infection (*a*); and *iii*, T = 74 °C and T_m = 77.5 °C, *N. apis* (*b*)). (*c* , *d*) Melting-curve analysis of individual (*c*) and composite (*d*) purified honey bee DNA (the asterisk indicates delayed T for $-R'(T)$ increase to identify *N. apis* from co-infected samples). (*e*, *f*) Agarose-gel analysis of the same duplex R-T PCR products (arrows): 321 bp for *N. apis* and 219 bp for *N. ceranae* and both PCR products for co-infection) for honey bee DNA from individual samples (lanes 1−9, PEI-1 to PEI-9; lanes 10–18, NS-1 to NS-9; lane 19, NTC; lane 20, 100-bp DNA ladder) (*e*) and composite samples (lanes 1−18, NSC-1 to NSC-6; lane 19, NTC; lane 20, 100-bp DNA ladder) (*f*).



during co-infection. The R-T PCR sequence results for the two bands in co-infected bee DNA sample NSC-6.a (321 and 218 bp for *N. apis* and *N. ceranae*, respectively) showed that the melting-curve profile of group *ii* could reliably identify both *Nosema* species. The simplex R-T PCR data for primer pairs NAPIS and NCERANAE confirmed this finding; the method-comparison summary (Table 3) shows that 5 composite DNA

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Table 3. Comparison of PCR methods (conventional and real-time with SYBR® Green I dye), with the primer pairs NAPIS and NCERANAE, used to detect infection or co-infection with *Nosema apis* and *Nosema ceranae* in purified DNA from individual (n = 19) and composite (n = 18) worker honey bee samples (*Apis mellifera*).

	Conventional duplex PCR		Simplex real-time PCR				Optimized duplex real-time PCR	
			NAPIS		NCERANAE			
	Individual	Composite	Individual	Composite	Individual	Composite	Individual	Composite
N. apis	7	1	9	5	–	–	7	0
N. ceranae	5	14	–	–	7	18	5	13
Co-infection	2	3	–	–	–	–	2	5
Negative samples	5	0	10	13	12	0	5	0

samples yielded positive results for NAPIS and all 18 yielded positive results for NCERANAE; 5 composite samples must therefore be co-infected.

Although qualitative R-T PCR with species-specific primers can only detect the presence of the target DNA template, it is essential that assay performance characteristics are evaluated with special attention paid to the linear dynamic range and the LOD to confirm the low-end sensitivity of the assay (Bustin *et al*. 2009). A 5-fold dilution series of total genomic DNA showed that both simplex R-T PCR and duplex R-T PCR were efficient, repeatable, and sensitive because the linear interval for the duplex assay contained bee DNA concentrations >110 pg, which is near the recommended lower limit for R-T PCR (each PCR reaction in this study contained over 100 times this amount of bee DNA). The calibration curve presented here is not a true standard curve for *Nosema* target DNA correlated with spore count; the exact proportion of *Nosema* spp. DNA in the sample is unknown because all life stages (*i.e*., not just the spores detected by light microscopy) of the parasite will contribute to the DNA yield. Nevertheless, we were able to demonstrate that one or both species can be detected in DNA from individual bees with spore counts as low as 150 000 (increasing the DNA concentration in bee samples may lower the LOD). R-T PCR will improve identification of *Nosema* infection because spore counts alone may not be reliable for estimating *Nosema* infection intensity, especially using individual-bee samples (we found that two samples (PEI-1 and PEI-4) had low spore counts but yielded no positive results for conventional PCR or R-T PCR). This may have been due to sample contamination during spore counting and (or) the assumption that *Nosema* spores contributed most of the *Nosema* DNA found in the total DNA sample. We have observed (unpublished data) a poor correlation of spore count with R-T PCR C_t values when *Nosema* intensity is <10 million spores, suggesting that in early infection, other life stages of the parasite may contribute most of the *Nosema* DNA. It would be useful to compare *Nosema* intensity determined using molecular techniques and light microscopy with colony strength or health. Recently, Martín–Hernández *et al.* (2009) reported that in Spain, *N. ceranae* had a greater biotic potential to infect honey bees than *N. apis*, even when spore counts for the two species were similar; therefore it is recommended that qPCR methods be used to accurately quantify *Nosema* infection.

Currently, this method cannot quantify each *Nosema* species in bee DNA samples because SYBR® Green I dye produces one fluorescent signal for duplex R-T PCR even when both *Nosema* species are present. The benefit of duplex qPCR with TaqMan® probes as described by Chen *et al.* (2009) is that it can simultaneously quantify both species; however, our additional work (unpublished data) using simplex R-T PCR with

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standards of cloned PCR product for standard-curve analysis has shown promise for qPCR. This method with primer pair NCERANAE will be useful, as *N. ceranae* appears to be the more virulent parasite and has been identified as a major agent of colony decline and bee depopulation worldwide (Klee *et al.* 2007; Higes *et al.* 2008).

Here we offer a sensitive and rapid (approximately 4–5 h) protocol for extracting genomic DNA and simultaneously detecting both *N. apis* and *N. ceranae* in individual and composite samples of honey bees with qualitative duplex R-T PCR and melting-curve analysis. The use of this method with the specific primer pairs NCERANAE and NAPIS can be considered a cost-effective molecular tool for researchers needing to identify *Nosema* species for monitoring the pathology and control of infection in honey bees.

Acknowledgements

Research was supported by the Canadian Bee Research Fund, a Natural Sciences and Engineering Research Council of Canada (NSERC) Industrial Postgraduate Scholarship to G.R.W., and a NSERC Discovery Grant to D.S. We thank mathematician Jeff Franklin for assistance with graphical description, and beekeepers who allowed us access to their colonies.

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