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ORIGINAL PAPER

The microsporidian parasites *Nosema ceranae and Nosema apis* are widespread in honeybee (*Apis mellifera*) colonies across Scotland

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Abstract *Nosema ceranae* is spreading into areas where *Nosema apis* already exists. *N. ceranae* has been reported to cause an asymptomatic infection that may lead, ultimately, to colony collapse. It is thought that there may be a temperature barrier to its infiltration into countries in colder climates. In this study, 71 colonies from Scottish Beekeeper's Association members have been screened for the presence of *N. apis* and *N. ceranae* across Scotland. We find that only 11 of the 71 colonies tested positive for spores by microscopy. However, 70.4 % of colonies screened by PCR revealed the presence of both *N. ceranae* and *N. apis*, with only 4.2 or 7 % having either strain alone and 18.3 % being *Nosema* free. A range of geographically separated colonies testing positive for *N. ceranae* were sequenced to confirm their identity. All nine sequences confirmed the presence of *N. ceranae* and indicated the presence of a single new variant. Furthermore, two of the spore-containing colonies had only *N. ceranae* present, and these exhibited the presence of smaller spores that could be distinguished from *N. apis* by the analysis of average spore size. Differential quantification of the PCR product revealed *N. ceranae* to be the dominant species in all seven samples tested. In conclusion, *N. ceranae* is widespread in Scotland where it exists in combination with the endemic *N. apis*. A single variant, identical to that found in France (DQ374655) except for the addition of a single nucleotide polymorphism, is present in Scotland.

Introduction

Insect pollinators provide essential ecosystem services to both world crops and native flora by increasing their quality and yield of fruit and seeds (Garibaldi et al. 2011). As such, insect pollinators make an estimated contribution of $216 billion to worldwide economies every year (Gallai et al. 2009).

Honeybees are managed commercially and recreationally for their pollination services and honey production. Although the magnitude of the honeybee contribution to both crop and wild flower pollination is not yet known, it is argued that our need for managed bees (both honeybees and bumblebees) may increase along with the rapid expansion of the cultivation of insect-pollinated crops (Aizen and Harder 2009; Potts et al. 2010).

The major threat to the health of the western honeybee (*Apis mellifera*) is the ectoparasite, *Varroa destructor* (Richards et al. 2011) and the viruses that it transmits

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(Moore et al. 2011). However, there is an increasing and unknown risk from poor nutrition (Alaux et al. 2010a), as a result of habitat destruction (Potts et al. 2010) and exposure to dietary toxins (Wright et al. 2010) that accumulate within the hive (Mullin et al. 2010). In addition to these stress factors, *A. mellifera* are endemically infected with a unicellular microsporidian parasite, *Nosema apis*, that multiplies in the epithelial cells of the mid-gut and is spread by faecal contamination. This parasite is controlled by natural mechanisms within the colony itself (Malone et al. 2001). When this fails, treatment with Fumagillin is often used (Katznelson and Jamieson 1952). However, within the last decade, *Nosema* (*ceranae*) that infects the eastern honeybee, *Apis cerana*, has been detected in *A. mellifera* (Higes et al. 2006; Paxton et al. 2007) and the Argentine bumblebees, *Bombus atratus*, *Bombus morio*, and *Bombus bellicosus* (Plischuk et al. 2009).

In contrast to the clinical symptoms of *N. apis*, such as crawling bees and dysentery (Liu 1988), infection with *N. ceranae* is symptomless apart from reports of a massive depopulation of colonies (Higes et al. 2006) and reduced honey production (Higes et al. 2008). The impact of *N. ceranae* infection on colony survival is unclear and has been found in both healthy colonies (Vanengelsdorp et al. 2009; Cox-Fosteret et al. 2007; Gisder et al. 2010) and those undergoing sudden collapses (Higes et al. 2008, 2009; Martin-Hernandez et al. 2007). However, there may be contributory effects of climate (Gisder et al. 2010; Fenoy et al. 2009; Fries 2010) or altered virulence amongst different strains of *N. ceranae* (Williams et al. 2008; Huang et al. 2008). Moreover, virulence may be affected by its coincidence with other factors stressing the colony. In this light, *N. ceranae* imposes an energetic burden on *A. mellifera* (Mayack and Naug 2009), decreasing nutritional benefits from food (Mayack and Naug 2010), leading to a weakened immune system (Alaux et al. 2010a) and an increased vulnerability to pesticides (Alaux et al. 2010b) that has been associated with the rapid decline in honeybee colonies (Higes et al. 2008).

Evidence indicates that *N. ceranae* has been spreading throughout the world populations of *A. mellifera*, unnoticed, since sometime before 1990 (Invernizzi et al. 2009). In 2007, it was detected in the USA, Brazil, China, Taiwan, Vietnam, Spain, Greece, Italy, Serbia, Germany, France, Denmark, Finland, and Sweden (Paxton et al. 2007; Klee et al. 2007) and more recently in Canada, Argentina, Hungary, and the UK (Williams et al. 2010a, b; Medici et al. 2012). In the UK, *N. ceranae* has been reported in England, Wales and Northern Ireland, but not Scotland. In many parts of the world, *N. ceranae* appears to be spreading into areas containing *N. apis* (Fries 2010; Klee et al. 2007; Medici et al. 2012; Stevanovic et al. 2010; Chen et al. 2009; Chen and Huang 2010). However, this is not thought to be occurring in colder climates, with *N. apis* remaining the dominant species (Gisder et al. 2010; Fenoy et al. 2009).

In this study, we screened 71 *A. mellifera* colonies (from 27 apiaries) from Scottish Beekeeper's Association members across Scotland for the presence of *Nosema* spores by microscopy and investigated whether *N. ceranae* had reached Scotland using PCR. We found that, although only 11 of the 71 (15.5 %) samples had spores detectable by light microscopy, 55 of the 71 (77.5 %) samples were positive by PCR using *N. ceranae* selective primers and 52 of the 71 (73.2 %) samples were positive for *N. apis*. There were 50 of the /71 (70.4 %) samples that were coinfected with both *N. ceranae* and *N. apis*. Thus, over 60 % of colonies carried *Nosema* at levels too low to be detected by traditional methods.

Materials and methods

Light microscopy The method is adapted from "OIE Manual of diagnostic tests and vaccines for terrestrial animals 2011, chapter 2.02.04". Honeybee foragers were collected from the hive entrance, frozen on the same day, and then sent to the lab for testing. For each sample, 30 bees were used. Abdomens were removed with forceps, placed in "Bioreba" bags, 15 ml water added, and abdomens crushed with a spatula. The liquid was collected from the other compartment to which the abdomens were added to minimize the debris collected. A 100-μl aliquot was placed on a microscope slide and covered with a coverslip. *Nosema* spores were counted at ×400 magnification. Three randomly selected fields were counted and averaged by two people (six fields counted). Positive samples were recounted for an accurate spore count using a Neubauer hemocytometer. Spore sizes were determined using a calibrated objective and measured manually using a ruler to ensure that it could be reproduced by beekeepers. Size differences were determined by ANOVA (Kruskal–Wallis; Dunnett's post hoc test).

Polymerase chain reaction DNA was extracted using the *Extract-N-Amp Tissue PCR kit* (Sigma). Briefly, to 10 μl of bee sample, 100 μl extraction solution and 25 μl tissue preparation solution were added, vortexed, and incubated at 55 °C (10 min) then 95 °C (3 min). Finally, 100 μl neutralization solution was added and the extract vortexed. PCR amplification was performed as follows: 4 μl tissue extract, 10 μl PCR reaction mix, 1 μl of each primer (200 nM for *N. apis*, 100 nM for *N. ceranae*), and 4 μl H_2O. The PCR program was 94 °C (2 min) 1 cycle; followed by 94 °C (15 s), 55 °C (30 s), 72 °C (45 s) for 30 cycles; and finally 72 °C (7 min) for 1 cycle. For amplification of *N. apis* primers were as follows: APIS-FOR, 5′-GGGGG

CATGTCTTTGACGTACTATGTA-3′ and APIS-REV, 5′-GGGGGGCGTTTAAAATGTGAAACAACTATG-3′; for *N. ceranae*: MITOC-FOR, 5′-CGGCGACGATGTG ATATGAAAATATTAA-3′ and MITOC-REV, 5′-CCCGGTCATTCTCAAACAAAAAACCG-3′ to produce 321 and 219 bp fragments, respectively (Vanenglesdorp et al. 2009). DNA markers (100 bp and 1 kb) were from Promega. In addition to the use of specific primers and fragment size to identify the species present, a selection of fragments (both *N. ceranae* and *N. apis*) were verified by DNA sequencing. This sequence has been submitted to GenBank (accession number JQ406638). To serve as a rigorous negative control, *A. mellifera* heads were used. These should exhibit no (*N. apis*) or at most very low (*N. ceranae*) *Nosema*. In our experiments, no *Nosema* was detected from *A. mellifera* heads. The primary screen of all 71 colonies was performed twice, and identical results were obtained.

DNA quantification of PCR fragments (repeated on three separate occasions and performed in triplicate using independent DNA extractions) was performed on the spore-positive samples using the PicoGreen double-stranded DNA quantitation kit (Invitrogen) as per manufacturer's instructions and the signal determined to be within the linear range for DNA detection.

Results

Detection of Nosema spores with light microscopy Seventy-one samples from 27 apiaries across Scotland were screened for the average number of spores per field of view (3 random fields selected), at a magnification of ×400 (Fig. 1). Of these, 11 of 71 (15.5 %) were positive for spores (samples 15, 17, 18, 27, 31, 34, 42, 66, 67, 70, and 71; Table 1). The positive samples were reanalyzed to obtain accurate spore counts using a Neubauer hemocytometer. Average spore counts per bee were determined to be 6.6×10^5 (sample 15), 2.9×10^5 (sample 17), 2.1×10^5 (sample18), 1.36×10^6 (sample 31), 1×10^5 (sample 34), 3.4×10^5 (sample 42), and 2.7×10^6 (sample 66). In the one sample for which sufficient bees remained (sample 31), we looked for spores in 60 individual bees and discovered only one positive bee with 4.8×10^7 spores.

The spores of *N. ceranae* have been reported to be smaller (~4.4 μm long×2.2 μm wide) than that of *N. apis* (6×3 μm) (Chen and Haung 2010), but a significant overlap was thought to preclude size as a diagnostic tool. In the samples analyzsed in this study, a similar difference in the size of spores was observed. To investigate further the possibility of using spore size diagnostically, by a method that would be accessible to any beekeeping association, we measured 10 spores from each sample to determine whether the average sizes of a few spores could distinguish the two populations (the data from >50 spores per sample were also determined (Supplemental Figure 1). We find that the spore samples appeared to fall into two distinct classes with respect to size (Fig. 1a), suggesting that *N. apis* and *N. ceranae* may be present in Scotland. To explore whether each size group are significantly distinct from each other, we pooled data into groups based on size and the presence of *N. ceranae* (see later) and explored whether the two groups could be distinguished by size alone. The average length (± 1 standard deviation) of group A (samples 31, 34, and 66) spores was 4.81±0.40 μm and that of group B (samples 15, 17, 18, and 42) was 5.82±0.27 μm. The average width of spores was 2.37±0.25 μm (group A) and 2.98±0.21 μm (group B) (Fig. 1b). We did not calculate precise two-dimensional area of spores using imaging software but determined instead to use a simplified method that would be available to ordinary beekeepers to aid in identification. Therefore, we determined the crude area (length×width) of spores within each group and found a value of 11.4± 1.28 μm^2 (group A) and 17.35±1.36 μm^2 (group B). Therefore, spores in group A are significantly shorter ($P<0.001$, Mann–Whitney test), narrower ($P<0.001$, Mann–Whitney test) and, consequently, have a smaller 2D area ($P<0.0001$, Mann–Whitney test) than spores from group B (Fig. 1b). To highlight the spread of values for each sample, scatter plots were generated for length, width, and area (Fig. 1c). Although the same two groups (A: 31, 34, and 66; B: 15, 17, 18, and 42) are evident, significant overlap is observed as expected (Fries 2010; Chen and Huang 2010). The same is true when the two groups are merged for both length and width (Fig. 1d). However, the grouped area scatter plot reveals no overlap between groups. Likewise, when each individual spore is plotted as length against width, two clear groups emerge with no overlap (Fig. 1 e). A more extensive analysis using 51–61 spores for each sample (group A total=171; group B total=221) yielded very similar results (Supplementary Figure 1) supporting the validity of this approach.

Detection and discrimination of N. ceranae and N. apis using PCR analysis To characterize further the identity of which species of *Nosema* was present, we performed PCR using primers specific for either *N. apis* or *N. ceranae* (Martin-Hernandez et al. 2008). Using this more sensitive method, we observed that 57 of 71 (80.2 %) were positive for *Nosema*. We found that *N. ceranae* was present in 55 of 71 (77.5 %), *N. apis* in 52 of 71 (73.2 %), and dual infection in 50 of 71 (70.4 %) of these infected colonies (Table 1). Example PCR fragments are shown in (Fig. 2a). Both *N. apis* and *N. ceranae* were present in every apiary, with the exception of two sites (G83 and G84). However, in both cases, only a single colony was analyzed.

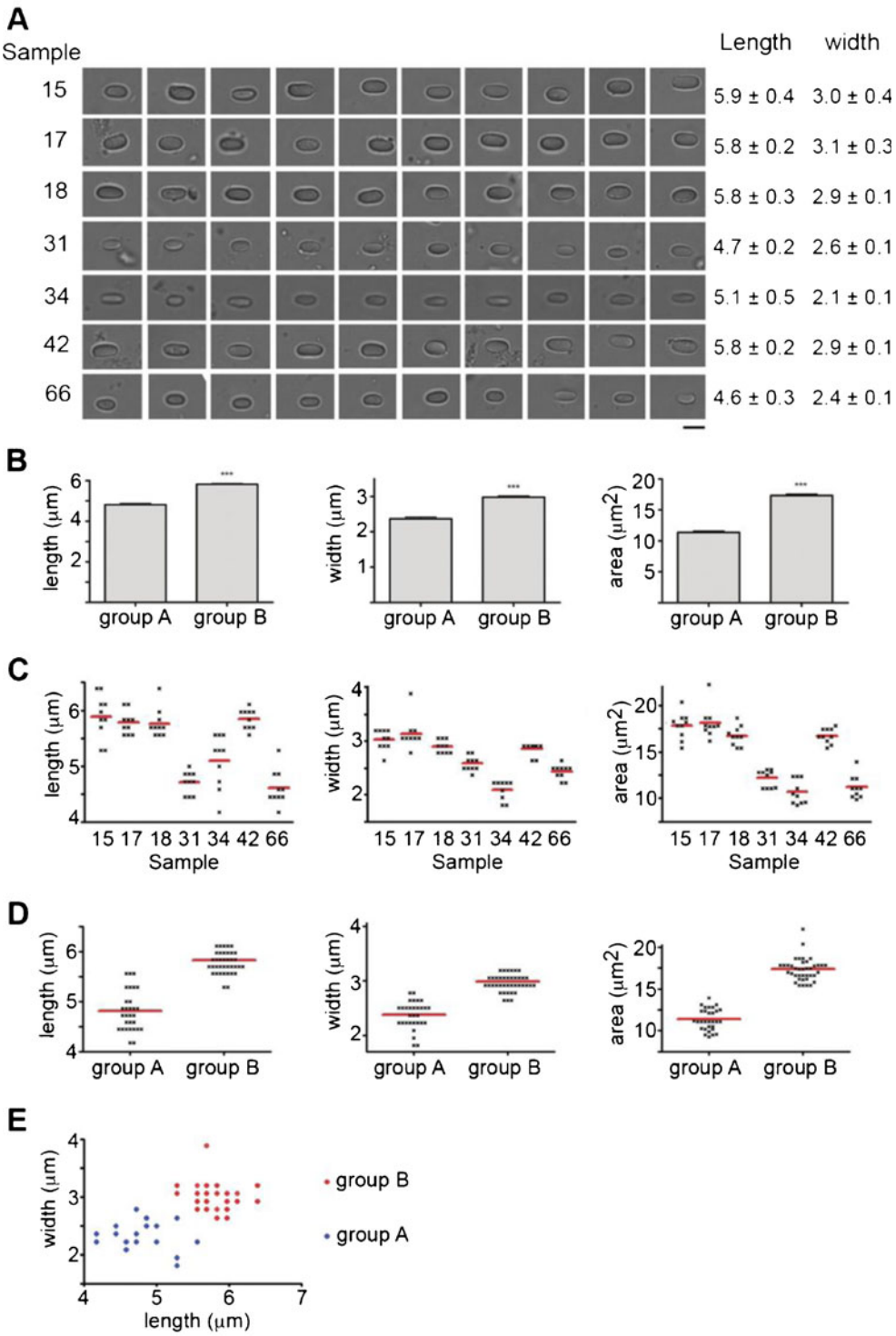

Fig. 1 Size distribution of *Nosema* spores identified in Scotland. **a** Light microscopy (×400 magnification) of seven samples from the postcode regions: G81 (*15*, *17*, and *18*), DD1 (*31*), KY10 (*34*), PA5 (*42*), and KA7 (*66*). The average measurements of length and width were determined (μm) for 10 spores from each sample (values shown are mean ±1 standard deviation). Scale bar=5 μm. **b** Samples were distributed into two groups [(*31*, *34*, and *66*) and (*15*, *17*, *18*, and *42*)] based on size differences determined by ANOVA (Kruskal–Wallis; Dunnett's post hoc test) and recalculated as groups A and B, respectively, for length, width, and square area. Data were analyzed using a Mann–Whitney test (***$P<0.001$). **c**, **d** The scatter of the length, width, and square area of the spores in each sample (**c**), or the samples pooled into groups A and B (**d**) to illustrate their size distribution. *Red lines* represent the average of each dataset. **e** For each individual spore, its length was plotted against its width to illustrate size differences between group A (*blue dots*) and group B (*red dots*)

Given the coexistence of both *N. ceranae* and *N. apis* (Fries 2010; Klee et al. 2007; Medici et al. 2012; Stevanovic et al. 2010), we endeavored to gain some indication of the relative abundance of both species. Therefore, we concentrated on those samples in which we identified spores (Fig. 1). Interestingly, in two of the samples with smaller spore sizes (group A samples 31 and 34), only *N. ceranae* was detected by PCR (Table 1 and Fig. 2a).

To determine the comparative abundance of each species within a colony, we used the dye PicoGreen that exhibits a linear relationship between fluorescence and DNA concentration over four orders of magnitude. To ensure that the PCR amplification did not saturate due to a limited amount of primers, we performed the PCR over 30, 40, and 50 cycles. Quantification (in triplicate) using PicoGreen revealed that linear

Table 1 Summary of *Nosema* screen

			PCR					PCR	
Sample	Postcode	Spores	*N. apis*	*N. ceranae*	Sample	Postcode	Spores	*N. apis*	*N. ceranae*
1	FK21	0	+	+	37	PA34	0	+	+
2	FK21	0	+	+	38	PA34	0	+	+
3	IV12	0	+	+	39	PA34	0	+	+
4	G84	0	−	−	40	PA34	0	+	−
5	G84	0	+	+	41	PA34	0	−	−
6	G84	0	−	−	42	PA34	0	+	+
7	G84	0	+	+	43	PA5	0	+	+
8	G84	0	+	+	44	PA5	2.7±1.2	+	+
9	EH48	0	+	+	45	PA5	0	+	+
10	EH48	0	+	+	46	PA75	0	+	+
11	EH48	0	+	+	47	PA75	0	−	−
12	G81	0	−	±	48	PA75	0	+	+
13	G81	0	+	+	49	PA75	0	+	+
14	G81	0	+	+	50	PA75	0	+	+
15	G81	32.5±11.5	+	+	51	PA75	0	−	−
16	G81	0	+	+	52	PA75	0	+	+
17	G81	6±1.4	+	+	53	KY15	0	+	+
18	G81	4.8±2.6	+	+	54	KY15	0	+	+
19	EH46	0	+	+	55	FK14	0	+	+
20	KY12	0	−	−	56	EH45	0	+	+
21	KY12	0	−	−	57	EH45	0	+	+
22	KY12	0	−	±	58	EH45	0	−	−
23	KY12	0	−	−	59	KY16	0	+	+
24	KY12	0	+	+	60	G84	0	−	−
25	TD4	0	+	+	61	G84	0	+	+
26	TD4	0	+	+	62	G83	0	−	−
27	DD1	1.2±1.2	+	+	63	KY11	0	−	−
28	DD1	0	+	+	64	KY11	0	+	+
29	DD1	0	+	+	65	KY16	0	+	+
30	DD1	0	+	+	66	KA7	7.8±2.3	+	+
31	DD1	17±3.2	−	+	67	KA7	0.3±0.5	±	+
32	DD1	0	−	+	68	KA7	0	±	+
33	KY10	0	+	+	69	KA7	0	±	−
34	KY10	1±1.1	−	+	70	KY5	2±0.9	+	+
35	KA2	0	−	−	71	KY5	0.5±0.5	−	−
36	KA2	0	+	±					

Seventy-one samples from across Scotland were screened for the presence of *Nosema* spores by light microscopy (×400 magnification). Spore counts from six independent fields of view were determined (mean±1 standard deviation) and classified as to the number of spores present: 0 (−), 0–5 (+), 6–15 (++) and >15 (+++). All samples were screened by PCR, using primers specific for *N. apis* or *N. ceranae* and recorded as amplifying the appropriate size PCR fragment (+) or not (−). Those samples with a clear, yet weak, band were designated (±)

amplification was still occurring after 40 cycles for all samples tested (not shown), and these conditions were used to estimate the relative abundance of *N. apis* and *N. ceranae*. In all samples, *N. ceranae* band quantification revealed a higher intensity than those of *N. apis* (Fig. 2b). This relationship persisted when all samples for each species were pooled (Fig. 2c) and found to be statistically significant ($P<0.001$, Mann–Whitney).

Sequencing of N. ceranae strain The presence of *N. ceranae* in Scotland has not been reported in the literature. Therefore, we confirmed our findings by the DNA

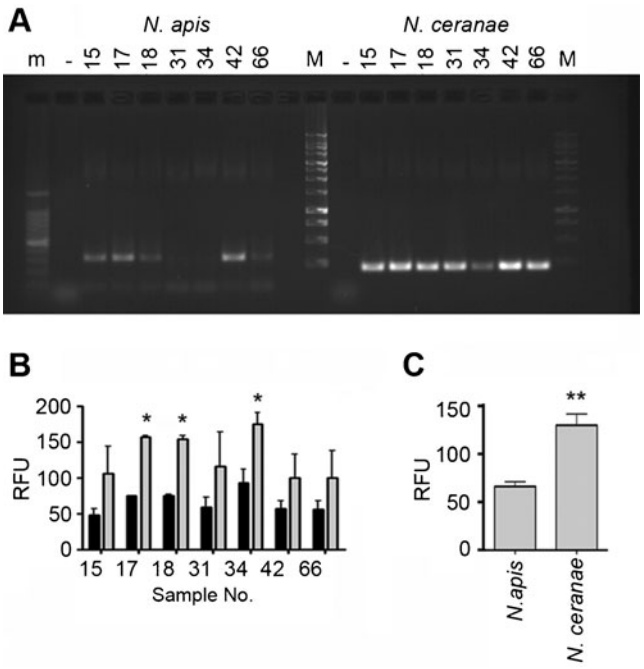

Fig. 2 PCR analysis of *N. apis* and *N. ceranae* distribution is spore-positive samples. PCR analysis was performed on DNA extracts from samples 15, 17, 18, 31, 34, 42, and 66 using DNA primers specific for *N. apis* or *N. ceranae*. **a** PCR fragments of ~320 bp for *N. apis* (*left*) or 220 bp for *N. ceranae* (*right*) were separated by electrophoresis on a 1 % agarose gel. The negative control (−) represents DNA extracted from *A. mellifera* heads (from uninfected bees). DNA size markers were 100 bp (m) and 1 kb (*M*). **b** PCR fragments (triplicate) were quantified using PicoGreen. Data represent relative fluorescence units (RFU) for PCR fragments generated using specific primers for *N apis* (*gray bars*) or *N. ceranae* (*black bars*). **c** Pooled data from all PCR samples for either *N. apis* or *N. ceranae*. **P*<0.05, ***P*<0.001 comparing *N. apis* with *N. ceranae* within each sample or group (Mann–Whitney test)

sequencing of a selection of samples from geographically distinct locations throughout Scotland. Sequence confirmation of the presence of *N. ceranae* was obtained for the following postcodes: KY5, KY10, KY16, DD1, EH48, IV12, FK21, KA7, PA5, PA75, G8, and TD4 from normal, apparently healthy, colonies. A similar number of samples possessing *N. apis* (by PCR) were sequenced and, in all cases, were found to be identical to existing sequences (GenBank U97150.1).

In contrast to that observed in other countries (Medici et al. 2012), we observed no intraspecific variation in the 16S SSU of *N. ceranae* in Scotland. To identify which variant is present, we performed a *Blastn* search of *Nosema* sequences (Zhang et al. 2000). We found 100 % homology of the Scotland strain with a variant reported in France (DQ374655), Germany (DQ374656), Italy (HM859898), Iran (JF431546), Indonesia (FJ789802), FJ227957 (Argentina), and Turkey (Whitaker et al. 2010) but not with other haplotypes (Medici et al. 2012). In keeping with this homology, alignment to the six variants (G1-G6) of *N. ceranae* (Zhang et al. 2000) revealed that the Scottish variant (JQ406638) is G2 (Fig. 3). However, a single nucleotide polymorphism (SNP) A31C (Fig. 3, indicated as M using the International Union of Pure and Applied Chemistry code) exists along with the A31 and is present in all samples. As the 16S SSU is likely to be present in multicopies in *N. ceranae*, as observed in *N. bombi* (O'Mahony et al. 2007), both variants are likely to exist within a single genome. The presence of the C31 variant in all samples may provide an historical indicator of the original source of *N. ceranae* introduction into Scotland. However, a *Balstn* screen for this C31 variant did not identify its reported existence elsewhere.

Discussion

N. ceranae was first identified as a pathogen of *A. mellifera* in Europe in 2006 (Higes et al. 2006) and appeared to be highly pathogenic (Paxton et al. 2007, Higes et al. 2007). However, *N. ceranae* has been reported in historical samples dating back to 1990 (Invernizzi et al. 2009) and may have been present for a significant period before then.

It appears that the spread of *N. ceranae* across the globe (Fries 2010; Klee et al. 2007; Medici et al. 2012; Stevanovic et al. 2010; Williams et al. 2008) is curtailed in colder climates (Fries 2010; Gisder et al. 2010) as *N. ceranae* spores are capable of surviving high temperatures (60 °C) and dessication (Fenoy et al. 2009; Martin-Hernandez et al. 2009) but are intolerant to cold (4 °C) (Fenoy et al. 2009; Fries 2010; Gisder et al. 2010). Although these characteristics may influence the spread of *N. ceranae* across the environment, they are likely to have little impact within an active, thermostatically controlled colony.

In support of a climatic barrier, in Sweden, 83 % of colonies had *N.* apis only and 17 % had both *N. apis* and *N. ceranae* (Fries 2010). In contrast, we find that *N. ceranae* is present in 94.3 % and *N. apis* in 89.7 %, of infected colonies in Scotland (2011). Moreover, *N. ceranae* has only arrived in Finland within the last decade, during which time its prevalence has increased from 0 % pre-1995 (compared to *N. apis* 21.4 %) to 17.8 % in 2006, by which time *N. apis* was only found in coinfections with both strains (Paxton et al. 2007). Similarly, in Canada, 8 of 12 (67 %) colonies screened had *N. Ceranae*, and 1 of 12 (8.3 %) had both (Williams et al. 2010a). Similarly, 66 % of infected colonies in France possess *N. ceranae* (Chauzat et al. 2007). In another study, the distribution of *N. ceranae*/*N. apis*/both was found to be 62 %/25 %/13 % (Spain), 82 %/0 %/18 % (France), 96 %/4 %/0 % (Switzerland), and 89 %/8 %/2 % (Germany) (Martin-Hernandez et al. 2007). Within England and Wales (2007), the prevalence of these *Nosema* species

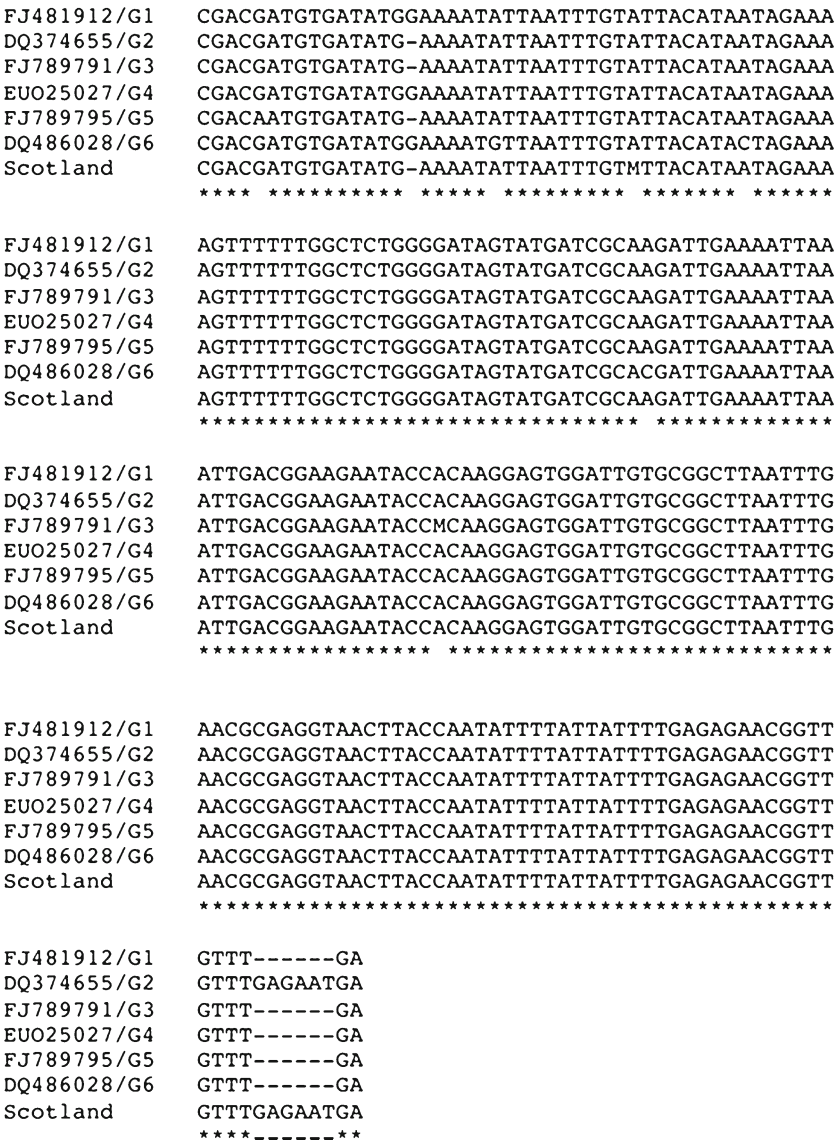

Fig. 3 Sequence alignment of the Scotland variant. All samples of *N. ceranae* found in Scotland and sequenced (GenBank accession number JQ406638), were determined to possess the same variant with 100 % homology to variant G2 of the16S SSU of *N. ceranae* (DQ374655). However, in all Scotland samples, a single nucleotide polymorphism (SNP) exists at position 31 where either adenine or cytosine is found (*M*). A similar SNP is observed in variant G3 at position 116

was determined (https://secure.fera.defra.gov.uk/beebase) to be 4.5 %/10 %/1 %. However, caution must be applied to direct comparisons of the published incidence of each strain as some studies may only perform PCR from samples with positive spore counts and the level of sensitivity may vary between studies.

It is possible that *N. ceranae* is still colonizing and supplanting *N. apis* across the globe, regardless of climatic conditions. The differing prevalence of *N. ceranae* may simply reflect its time of arrival, by natural spread or the importation of infected honeybees, and mobility of bees within a country. Canada, Scandinavia, and Scotland may be ideal locations to study how the spread of this disease correlates to climatic conditions and how it moves to particularly remote areas. However, a simplified technique to distinguish the species is required to enable local beekeeping associations to monitor the spread of *N. ceranae*.

Given the lack of clinical symptoms for *N.* ceranae, distinguishing *N. ceranae* from *N. apis* requires sophisticated equipment to perform PCR. Such equipment is not available to most beekeepers or their local associations, and this will hamper the monitoring of *N. ceranae*. In this study, we quantified the size variation, taking advantage of the knowledge that two samples had only *N. ceranae* infection and found that the *N. ceranae* group had statistically smaller spores than those in the *N. apis* group by analyzing as few as 10 spores from each sample and using basic measurements.

Although screening by microscopy is limited to high levels of infection, the high incidence of vegetation forms of *Nosema* suggests that spore detection may be more relevant. Indeed, four of the spore-positive samples that possess predominantly *N. ceranae* (by PCR) had larger spores indicative of *N. apis* infection. This highlights the fact that PCR identifies vegetative as well as spore forms of *Nosema*.

Thus, the definition of whether a colony is infected needs to consider the severity of infection (individual bee or averaged spore count) and the number of bees infected rather than the presence of the organism by PCR analysis. Importantly, confidence in the accuracy of the result depends on the number of bees sacrificed for the screen. Given our (single) finding that only 1 of 60 bees was positive by microscopy, it may be necessary to screen a large number of bees.

An intriguing hypothesis is that *N. ceranae* infection leads to increased hunger and decreased food sharing and therefore creates isolated "sinks," with respect to infectiousness. Such a mechanism would explain a low frequency of spore-positive bees and may limit colony losses (Naug and Gibb 2009), especially as a single bee with very high *Nosema* counts is unlikely to survive long. Thus, from a practical point of view, unless the frequency of such highly infected bees remains stable (a serious problem requiring intervention), high variability of microscopic detection may result. Perhaps, multiple weekly positive spore counts would be more indicative of a productive infection. More investigation into the development of a robust screening method, which is not heavily biased by individual bees, and alternative therapeutic interventions are necessary. In the meantime, the use of an "integrated management system," including the sterilization and replacement of hive frames, will be required to reduce the build-up of disease.

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