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ORIGINAL PAPER

High genetic variability of *Nosema ceranae* populations in *Apis mellifera* from East Asia compared to central Asia and the Americas

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Abstract *Nosema ceranae* is believed to have been originally a parasite of the Asian honey bee, *Apis cerana*, in East Asia that later infected the Western honey bee, *Apis mellifera*, followed by a worldwide spread. To examine if that possibly affected the genetics of the parasite, *A. mellifera* samples infected with *N. ceranae* were collected from seven locations across the world to compare the genetic variation of the parasite within its putative invasive range (Iran, Ontario-Canada, Alberta-Canada, New York-United States of America, Mexico and Argentina) to its native range (Vietnam) using SNPs in the translation elongation factor-1 alpha sequence and three SSRs. Both type of markers detected the highest genetic variation in Vietnam. The SNPs revealed that the most common variant in Vietnam was the type found in all other locations with no variation detected in the invasive range. The SSRs, however, showed variation in populations in the invasive range with three groups: Iran with Alberta-Canada, Mexico with Argentina, and Ontario-Canada with New York-United States of America. These groupings may be related to the international movement of bees and beekeeping products. The genetic variation of *N. ceranae* supports the hypothesis that the most likely origin of *N. ceranae* was East Asia, and that the parasite subsequently spread throughout the world.

Keywords *Apis mellifera* · Founder effect · Genetic variability · Microsporidia · *Nosema ceranae*

Introduction

Nosema disease of honey bees is caused by two species of microsporidia, *Nosema apis* and *Nosema ceranae* (Fries et al. 1996). Both parasites enter the digestive system when honey bees clean fecal spots or exchange food by trophallaxis, and then infect ventriculus epithelial cells, reproducing asexually, resulting in spores that are released in the feces (Fries et al. 1996). Both parasites may reduce the life span of bees (Fries 1997; Emsen et al. 2020; Valizadeh et al. 2020). The morphological characteristics of the two species are almost identical, but *N. apis* and *N. ceranae* can be differentiated by their SSU rRNA, ITS and LSU rRNA sequences (Huang et al. 2007).

N. apis was first identified in the Western honey bee, *Apis mellifera*, in Germany (Zander 1909),

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whereas *N. ceranae* was first identified almost a century later in the Asian honey bee, *Apis cerana*, in China (Fries et al. 1996). It was first believed that *N. ceranae* was restricted to *A. cerana*, but there have been numerous reports of *N. ceranae* in *A. mellifera* wherever *A. mellifera* is managed (e.g., Klee et al. 2007; Huang et al. 2007; Paxton et al. 2007; Chen et al. 2008; Currie et al. 2010; Guzman-Novoa et al. 2011; Nabian et al. 2011; Teixeira et al. 2013). It has been proposed that *N. ceranae* moved from *A. cerana* to *A. mellifera* and subsequently spread worldwide (Botias et al. 2012). If correct, then those events could have impacted the population genetics of the parasite. Invasive populations of a species often have decreased genetic diversity compared to native populations (Besnard et al. 2007). Thus, *N. ceranae* could show more genetic variability in its native range in eastern Asia, compared to its invasive range in the rest of the world.

Several *N. ceranae* sequences have previously been tested as molecular markers to examine its genetic variability. However, *N. ceranae* sequences for the IGS of the rDNA (Huang et al. 2008) and polar tube protein 1 (PTP1) (Chaimanee et al. 2011) have shown limited polymorphisms, while sequences for PTP3 (Hatjina et al. 2011), SSU-rDNA (Sagastume et al. 2011; Roudel et al. 2013), spore wall protein 25 (SWP25) and SWP30 (Roudel et al. 2013), have shown high variability, even within a single honey bee.

An alternative marker would be the sequence of the translation elongation factor-1 alpha. Pombert et al. (2013) compared the sequences of 22 genes of the microsporidian parasite, *Encephalitozoon cuniculi*, and the translation elongation factor-1 alpha was chosen as one of the genes to study its population genetics because it had a minimum length of 1,000 bp, a minimum of seven single nucleotide polymorphisms (SNPs), no paralogs and did not encode a protein with a virulence function or was involved in antigenic selection by its mammalian hosts. The translation elongation factor-1 alpha has been used as a marker with other microsporidians, including the insect parasite, *Nosema locustae* (Tanabe et al. 2002), but it has not yet been used with *N. ceranae*. Another type of marker is length polymorphisms in simple sequence repeats (SSRs), which are repeats of 1–6 DNA motifs found throughout the genome in both encoding and non-coding regions, and because the number of repeats can vary, SSR lengths can be used for detecting variation (Li et al. 2004). While SSRs have also been used to detect variation of another microsporidian parasite of insects, such as *Nosema bombycis* infecting silkworms (Rao et al. 2005), there are no reports of them being used with *N. ceranae*.

The goal of this study was to compare the genetic variability of *N. ceranae* populations from *A. mellifera* samples collected from several locations worldwide using SNP and SSR markers based on sequences of the draft genome of *N. ceranae* strain BRL01. Vietnam was chosen as part of the possible native range of *N. ceranae* where Asian honey bees are found, and Iran, Alberta-Canada, Ontario-Canada, New York-United States, Mexico and Argentina were chosen as parts of the presumed invasive range of the parasite in the Middle East, North America and South America, respectively. If *N. ceranae* spread from its native range, then the greatest variation would be found in its native range, whereas the least genetic variation would be found in its invasive range. If spread of this parasite was associated with the shipment of honey bee queens and/or contaminated beekeeping equipment or pollen, then *N. ceranae* populations would be more closely related between those locations with a history of long distance movement of honey bees, beekeeping equipment or pollen.

Materials and methods

Samples

Forager honey bees from *N. ceranae* infected colonies were collected from hives in Vietnam, Iran, Ontario-Canada, Alberta-Canada, New York-United States, Mexico and Argentina (Table 1). From each hive, 5–100 bees were collected, and a sample was defined as a pooled collection of five bees from a single hive. Samples were placed in 70% ethanol and stored at − 20 °C.

DNA extraction

DNA was extracted from each sample using five excised abdomens as per Hamiduzzaman et al. (2010). After washing the abdomens in DNA extraction buffer (0.03 M hexadecyltrimethyl ammonium bromide, 0.05 M tris hydroxymethyl aminomethane,

Table 1 Source of *Nosema ceranae*-infected honey bee samples used in this study

Designation	City, Province/State	Country	Apiary	Collected (Year)
AB 1	Peace, Alberta	Canada	Peace	2010
AB 2	Fairview, Alberta	Canada	Fairview	2010
AB 3	Grand Prairie, Alberta	Canada	Grand Prairie	2010
AB 4	Smoky River, Alberta	Canada	Smoky River	2010
AB 5	Birch Hills, Alberta	Canada	Birch Hills	2010
AG 1	Resistencia, Chaco	Argentina	Chaco 1	2014
AG 2	Resistencia, Chaco	Argentina	Chaco 2	2014
AG 3	Corrientes, Corrientes	Argentina	Salades	2014
AG 4	Posadas, Misiones	Argentina	Misiones 1	2014
AG 5	Posadas, Misiones	Argentina	Misiones 2	2014
IR 1	Karaj, Alborz	Iran	Mashayekhi	2009
IR 2	Isfahan, Isfahan	Iran	Arabi	2009
IR 3	Takab, West Azarbaijan	Iran	Ebadian	2009
IR 4	Sarein, Ardabil	Iran	Ghesmati	2009
IR 5	Astara, Gilan	Iran	Gohari	2009
MX 1	Delicias, Chihuahua	Mexico	Criadora 008	2009
MX 2	Villa de Tezontepec, Hidalgo	Mexico	Choy Montero	2009
MX 3	Mojarras, Nuevo León	Mexico	Mojarras	2009
MX 4	Delicias, Chihuahua	Mexico	Criadora 008	2009
MX 5	Villa de Tezontepec, Hidalgo	Mexico	Choy Montero	2009
NY 1	Trumansburg, New York	USA	House	2009
NY 2	Ithaca, New York	USA	Burgette	2009
NY 3	Ithaca, New York	USA	Ferris	2009
NY 4	Trumansburg, New York	USA	House	2009
NY 5	Spencer, New York	USA	Chestnut	2009
ON 1	Guelph, Ontario	Canada	Townsend House	2013
ON 2	Guelph, Ontario	Canada	Townsend House	2013
ON 3	Watford, Ontario	Canada	Davisdon	2013
ON 4	Owen sound, Ontario	Canada	Berger	2013
ON 5	Niagara, Ontario	Canada	Perker	2013
VI 1	Chieng Ve, Moc Chau	Vietnam	Hao	2007
VI 2	Van Ho, Van Ho	Vietnam	Thanh	2007
VI 3	Chieng Ve, Moc Chau	Vietnam	Hao	2007
VI 4	Chieng Ve, Moc Chau	Vietnam	Hieu	2007
VI 5	Chieng Ve, Moc Chau	Vietnam	Hao	2007
VI 6	Chieng Ve, Moc Chau	Vietnam	Hai	2007
VI 7	Van Ho, Van Ho	Vietnam	Thanh	2007
VI 8	Van Ho, Van Ho	Vietnam	Thanh	2007
VI 9	Chieng Ve, Moc Chau	Vietnam	Hao	2007
VI 10	Chieng Ve, Moc Chau	Vietnam	Hieu	2007
VI 11	Chieng Ve, Moc Chau	Vietnam	Hieu	2007
VI 12	Chieng Ve, Moc Chau	Vietnam	Hieu	2007
VI 13	Chieng Ve, Moc Chau	Vietnam	Hao	2007
VI 14	Chieng Ve, Moc Chau	Vietnam	Hai	2007
VI 15	Chieng Ve, Moc Chau	Vietnam	Hai	2007
VI 16	Chieng Ve, Moc Chau	Vietnam	Hai	2007

Each sample was collected from one colony and determined to contain *N. ceranae* based on PCR of the 16S RNA (Hamiduzzaman et al. 2010)

0.01 M ethylenediamine tetra–acetic acid and 1.1 M NaCl in d H_2O, pH 8.0), they were homogenized in 900 µl DNA extraction buffer with a mortar and pestle, and then 20 µl proteinase K solution (20 mg/ml) was added to the macerate. After incubation at 60 °C for 3 h, an equal volume of phenol–chloroform mix (1:1) was added, mixed gently, and centrifuged at 20,200 g for 15 min (Eppendorf, Model 2417R, West Chester, PA, USA). The supernatant was then extracted a second time with phenol–chloroform followed by chloroform extraction. The supernatant was mixed with 1/10 volume of 3 M NaOAc and 2 volumes of 95% ethanol. This was incubated overnight at −20 °C, and the supernatant was removed after centrifugation at 12,000 g for 15 min. The air-dried pellet was dissolved in 25 µl sterile dH_2O and stored at −20 °C.

Identification of *Nosema* species

Species identification was done using PCR and sequencing of the 16S rRNA of *N. ceranae* and *N. apis* as per Hamiduzzaman et al. (2010). DNA obtained from newly emerged honey bees was used as negative control.

SNP analysis

The predicted AA sequence from the *E. cuniculi* GB-M1 translation elongation factor 1-alpha (ECU06_1440) partial mRNA sequence (NM_001041522) (Pombert et al. 2013) was used as a query in a tblastn search against the genome of *N. ceranae* BRL01(Ref Seq NZ_ACOL00000000.1) to obtain the *N. ceranae* sequence (Cornman et al. 2009) (http://www.ncbi.nlm.nih.gov/). Primers NcEcU2-forward (5′ CACGTAGATGCAGGAAAATCG 3′) and NcEcU2-reverse (5′ ATGAAGGGGTTCCTG GTGAT 3′) were designed from the sequence of the closest match in the tblastn search. Primers were obtained from Laboratory Services of the University of Guelph (Guelph, ON, CA).

PCR was performed with a Mastercycler (Eppendorf, Mississauga, ON, CA) using 1 µl of DNA in a reaction containing l.5 µl 10×PCR buffer (New England Biolabs, Pickering, ON, CA), 1 µl 10 µM of each primer, 0.5 µl 10 mM dNTPs (Bio Basics, Markham, ON, CA), 0.2 µl 5U/µl Taq polymerase (New England Biolabs, Pickering, ON, CA) and 9.8 µl dH_2O. Amplification conditions were 94 °C for 3 min, then 35 cycles of 30 s at 94 °C, followed by 60 s at 58 °C and 60 s at 72 °C and then 10 min at 72 °C. The PCR products were separated in a 1.5% TAE agarose gel and stained with 0.4 µg/ml ethidium bromide. A 100 bp to 1 kb DNA ladder (Bio Basics) was included. DNA fragments were excised from the gel and purified using an EZ-10 Spin Column DNA Gel Extraction Kit (Bio Basics, Markham, ON, CA) according to the manufacturer′s protocol. Sequencing was performed by Laboratory Services Division at the University of Guelph, Guelph, ON, CA.

Alignment of the translation elongation factor 1-alpha sequences was performed by ClustalW version 2.0 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Trees based on the alignments were created using MEGA version 6 (Tamura et al. 2013) using the UPGMA method (http://www.megasoftware.net/). One thousand bootstrap replicates were assessed to test the robustness of the trees.

SSR analysis

To identify SSRs, the whole genome sequences of *N. ceranae* strain BRL01 (PRJNA4832) was downloaded from GenBank. A FASTA version was parsed using Sputnik (http://archive.is/http://espressosoftware.com/pages/sputnik.jsp) (Abajian 1994; La Rota et al. 2005). After all SSR-containing sequences were obtained from Sputnik, the full sequences were obtained using a Perl script to retrieve the original FASTA sequences of the SSR-containing sequences. Any duplicated SSR-containing sequences were removed prior to generating the primer sequences.

Primer sequences flanking the SSRs were obtained by submitting the selected FASTA entries to BatchPrimer3 (http://probes.pw.usda.gov/batchprimer3) (You et al. 2008) with the primer type set to “SSR screening and primers”. The results were sorted using Microsoft Excel based on the predicted PCR product length between 200 and 700 bp for those sequences with a nucleotide repeat between 3 and 5 bp. Selected primers were examined for quality using GeneRunner (Version 3.04) (http://www.generunner.net/) (Spruyt and Buquicchio 1994). To examine whether there was more than one sequence in the genome of *N. ceranae* strain BRL01 where the primer sequence appeared, BLASTn of the selected primers was done using a BLAST database created from

the downloaded FASTA file of GenBank accession ADDG00000000.1 using BLAST2.2.29 + package (ftp://ftp.ncbi.nlm.nih.gov/blast/executables/blast+/2.2.29/). The lengths of the potential PCR products were predicted using the same BLAST database with BLASTn using the selected SSR primer sequences.

Three SSR primer pairs (Table 2) were used to obtain PCR products from the samples (Table 1). PCR reactions were performed with a Mastercycler (Eppendorf) using the same conditions previously described for SNP analysis. The PCR products were separated using 3.5% Super Fine Resolution (SFR) TAE agarose (Amresco, Solon, OH, USA), and then stained with 0.4 µg/ml ethidium bromide. A 100 bp to 1 kb DNA ladder (Bio Basics) was included. Variation in length of the PCR products was analyzed by visually determining the approximate sizes of the bands. A distance matrix was calculated by Microsat program version 2.0 (http://genetics.stanford.edu/hpgl/projects/microsat/), and phylogenetic trees were drawn using PHYLIP multiple alignment format (version 3.695) (http://evolution.genetics.washington.edu/phylip/getme.html) with the UPGMA method. One thousand bootstrap replicates were assessed to test the robustness of the trees.

Results

SNP analysis

A tblastn search of the genome of *N. ceranae* BRL01 with the protein sequence of the translation elongation factor 1-alpha of *E. cuniculi* (ECU06_1440) (NM_001041522.1) showed that the most similar sequence was NCER_101081 (XP_002995903.1) with an e-value of 2e-131 and 58% predicted aa identity. Primers were designed to amplify only within the

Table 2 Simple sequence repeats (SSR) markers used to assess genetic variability in *N. ceranae* in this study

SSR Marker	Forward (F) and Reverse (R) primer sequences	Sequence ID*	Location of SSR within the genbank sequence**	SSR motif***	Length of SSR within the PCR product****	Productlength (bp)
T4	T4F 5′ ACGAAG GAAGCAACA GTCT 3′ T4R 5′ CCGGAT CGAAGTAAACAC T 3′	NW_003313386.1	478–489 909–920	TTTA ATTA	12 12	658
T5	T5F 5′ AGTATCGCT GGCCATATTTA 3′ T5R 5′ TTCAAC CAAAGACGC AAT 3′	NW_003313699.1	1534–1545 1735–1746	TTC GACT	12 12	394
M8	M8F 5′ ACGGGA CTACGAATACGA T 3′ M8R 5′ TTTAAA CAATGCGCACAA G 3′	NW_003310946.1	97–108 337–348	TAAA ATAA	12 12	402

The SSR marker number, primer sequence, sequence ID from Genbank, location within the sequence, repeating SSR motifs, length of the SSR and predicted PCR product length are listed

*Sequence ID was obtained by using the program Sputnik in the whole genome shotgun sequence sequences of *N. ceranae* strain BRL01 (BioProject accession PRJNA48321)

** Single marker is defined as indicating that the primer sequences were unique in the list. Whereas multiple marker is defined as indicating that there was more than one sequence in the genome shotgun sequences of *N. ceranae* strain BRL01 where both the forward and reverse primer appeared

***The SSR motif is the repeating unit of nt that form the SSR

****The total SSR length was determined by calculating the number between beginning and ending of the location of the SSR within the GenBank

predicted coding region of this *N. ceranae* homolog of the translation elongation factor 1-alpha, and a single band of the predicted size, 888 bp, was amplified from each of the honey bee samples.

Among the 32 samples (Table 1), all were identified as containing *N. ceranae* and not *N. apis* based on a species-specific PCR assay (data not shown). Based on the SNP analysis, six types with identical translation elongation factor 1-alpha nt sequences were observed (Supplementary Fig. 1). Identical sequences were found in 27 samples, and these were designated as the IR_ON_AB_NY_AG_MX_VI1-6_VI12-16 type, which contained all the samples from Iran, Ontario-Canada, Alberta-Canada, New York-United States, Argentina and Mexico but only 11 of the 16 samples from Vietnam. The remaining 5 sequences were all from Vietnam samples, and each had a unique sequence. These were designated the VI7 type and VI8 type, each with one sample from the Thanh apiary, the VI9 type with one sample from the Hao apiary, and the VI10 type and VI11 type, each with one sample from the Hieu apiary. A total of 19 nucleotide differences were detected at 5 locations in the sequences (Supplementary Fig. 1).

Compared to the IR_ON_AB_NY_AG_MX_VI1-6_VI12-16 type, the most similar sequence was the VI7 type with 1 SNP, followed by the VI10 type with 2 SNPs, the VI8 type with 5 SNPs, the VI11 type with 5 SNPs, and the VI 9 type with 6 SNPs (Fig. 1). However, these differences were relatively small compared to the *E. cuniculi* sequence, which had over 400 SNPs when compared to the sequence of the IR_ON_AB_NY_AG_MX_VI1-6_VI12-16 type (data not shown).

Among the four apiaries in Vietnam, four of the five samples from the Hao apiary were in the IR_ON_AB_NY_AG_MX_VI1-6_VI12-16 type and one sample was in the VI9 type, whereas all 4 samples from the Hai apiary were in the IR_ON_AB_NY_AG_MX_VI1-6_VI12-16 type. For the Hieu apiary, 2 of the 4 samples were in the IR_ON_AB_NY_AG_MX_VI1-6_VI12-16 type, one was in VI 10 type and one was in the VI 11 type, and for the Thanh apiary, 1 sample belonged to the IR_ON_AB_NY_AG_MX_VI1-6_VI12-16 type, 1 to the VI7 type and 1 to the VI8 type. Thus, samples in the IR_ON_AB_NY_AG_MX_VI1-6_VI12-16 type were found in all the Vietnam apiaries with samples from the Hieu apiary being the most diverse with 7 SNPs, followed by the Thanh and Hao apiaries with 6 SNPs each and then the Hai apiary with no SNPs compared to the IR_ON_AB_NY_AG_MX_VI1-6_VI12-16 type.

SSR analysis

As only one location in the genome would have been examined using SNPs in the translation elongation factor 1-alpha gene, several SSRs were chosen in order to presumably examine variation in multiple locations in the *N. ceranae* genome. An examination of the whole genome shotgun sequences of *N. ceranae* strain BRL01 identified 731 different SSRs with nucleotide repeat motifs between 2 and 6 bp. Among those, 5 SSRs had 6 bp motifs, 67 SSRs had 5 bp

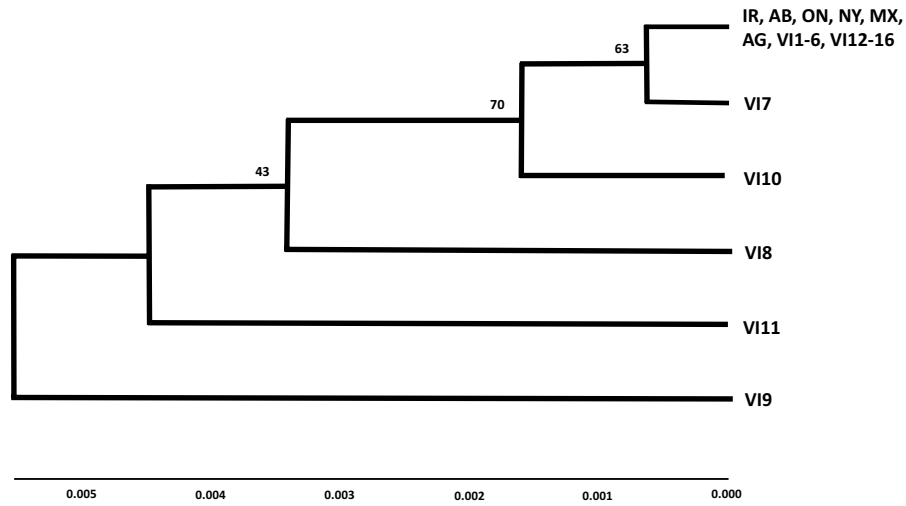

Fig. 1 Tree of the *N. ceranae* translation elongation factor 1-alpha sequences from Vietnam (VI), Iran (IR), Ontario-Canada (ON), Alberta-Canada (AB), New York-United States (NY), Mexico (MX) and Argentina (AG). Values at branch points indicate support based on 1000 bootstrap replicates

motifs, 427 SSRs had 4 bp motifs, 200 SSRs had 3 bp motifs and 32 SSRs had 2 bp motifs (Supplementary Table 1). A comparison of the motifs showed that there was a strong preference for motifs only containing A and T, motifs that are 4–5 bp long, and motifs repeated 3–5 times resulting in SSR lengths of 12–18 bp (Supplementary Table 2).

Screening primer pairs for 11 SSRs showed that primer pairs T4, T5 and M8 gave the most consistent PCR bands and length variation between samples. Primers T4F and T4R, and primers T5F and T5R were predicted to span multiple tandem SSRs in one location in the *N. ceranae* strain BRL01 genome, while primers M8F and M8R were predicted to span SSRs in two locations in the *N. ceranae* strain BRL01 genome (Table 2). The T4 SSR marker yielded a single band of 595 bp for the 30 samples from Iran, Alberta-Canada, Ontario-Canada, New York-United States, Mexico and Argentina, but no band for any of the 10 samples from Vietnam (Table 3). The T5 SSR marker yielded bands of 210, 280, 394 and 658 bp. Vietnam samples had the 210, 280 and 394 bp bands, whereas the 210 and 658 bp bands were found only in samples from other locations. The M8 SSR marker yielded bands of 74, 200, 402 and 690 bp. Vietnam samples had bands of 74, 200 and 402 bp, while only the 74 and 402 bp band was detected in samples from other locations. Several samples had identical banding patterns and were placed into types designated the VI1-2 type (samples VI1 and VI2), VI5-6 type (samples VI5 and VI6), VI7-10 type (samples VI7, VI8, VI9 and VI10), IR_AB type (all samples from Iran with Alberta-Canada), ON_NY type (all samples from Ontario-Canada and New York-United States) and AG_MX type (all samples from Argentina and Mexico).

A tree generated from the SSR polymorphisms showed that the samples were divided into two main clusters (Fig. 2). One cluster only contained Vietnam samples, while the other cluster contained samples from all the other locations. Samples within the Vietnam cluster were divided into 2 subclusters composed of a cluster with the VI1-2 and VI3 types, a cluster with the VI4 and VI5-6 types, and a cluster with the VI7-10 type. Among the apiaries in Vietnam, the Thanh apiary had one sample in the VI2 type and two in the VI7-10 type, the Hao apiary had one sample each in the VI1, VI3, VI5 and VI9 types, the Hieu apiary had one sample each in the VI4 and VI10 types, and the Hai apiary had one sample in the VI6 type. There was no correlation between apiary location and the clustering based on the SSR markers. The cluster for samples outside of Vietnam was divided into two major subclusters, one of which could be further divided. The first sub-cluster contained all samples from Iran and Alberta-Canada, and the second cluster contained all samples from Ontario-Canada, New York-United States of America, Argentina and Mexico, with the Argentina-Mexico samples being distinct from those from Ontario-Canada and New York-United States of America.

Table 3 Percentage of the occurrence of PCR product with T4, T5 and M8 primers in samples from Vietnam (VI), Iran (IR), Ontario-Canada (ON), Alberta-Canada (AB), New York-United States (NY), Mexico (MX) and Argentina (AG)

SSR marker	Size (bp)	VI (%)	IR (%)	ON (%)	AB (%)	NY (%)	MX (%)	AG (%)
T4	595	0	100	100	100	100	100	100
T5	210	60	100	0	100	0	0	0
T5	280	60	0	0	0	0	0	0
T5	394	80	0	0	0	0	0	0
T5	658	0	100	100	100	100	100	100
M8	74	30	100	0	100	0	0	0
M8	200	70	0	0	0	0	0	0
M8	402	20	100	100	100	100	100	100
M8	690	0	100	100	100	100	0	0

Sample sizes were 10 for Vietnam and 5 for all other locations. M4 primers produced one single band of 595 bp, M5 primers produced up to 4 bands of 210 bp, 280 bp, 380 bp and 658 bp, and M8 primers produced up to four bands of 80 bp, 200 bp, 402 bp and 690 bp

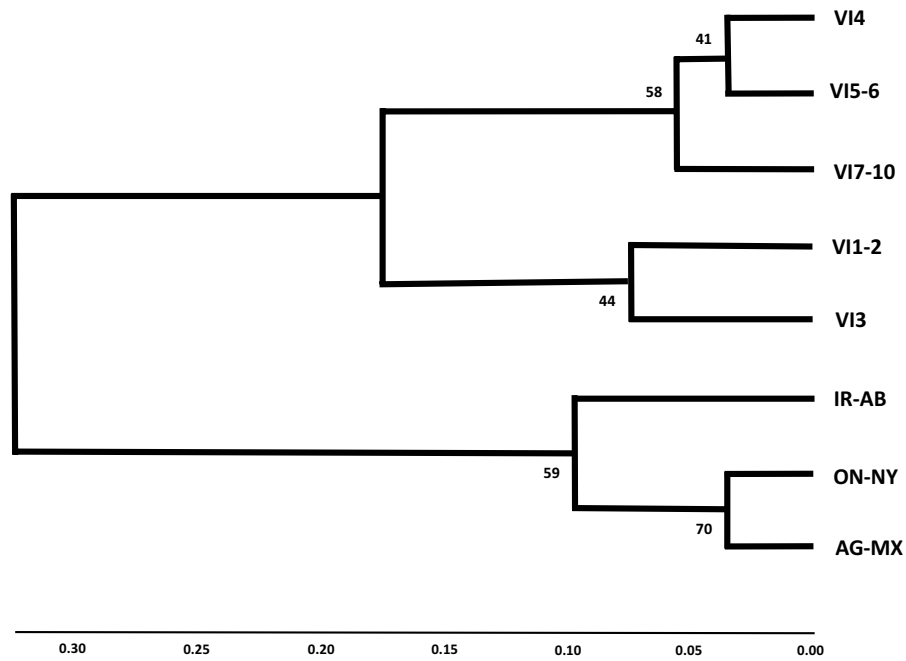

Fig. 2 Tree of *N. ceranae* SSR markers from Vietnam (VI), Iran (IR), Ontario-Canada (ON), Alberta-Canada (AB), New York-United States (NY), Mexico (MX) and Argentina (AG). The results are from the combined banding patterns of SSR markers T4, T5 and M8. Sample size for all the geographical locations was five, except for Vietnam which was 10. IR, ON, AB, NY, MX and AG each contained samples 1–5. Values at branch points indicate support based on 1000 bootstrap replicates

Discussion

SNPs in the translation elongation factor-1 alpha gene were used as a measurement of the genetic variation of *N. ceranae* in this study from locations in the possible native and invasive ranges of the parasite. The translation elongation factor-1 alpha gene was one of the best markers to detect variation of the microsporidian, *E. cuniculi* when complete genomes of three strains of the mammalian pathogen were compared (Pombert et al. 2013). It encodes for a protein involved in the delivery of aminoacyl tRNAs to ribosomes (Mingot et al. 2013) and thus is highly conserved making it useful in phylogenetic studies. In this study, it contained sufficient SNPs to show genetic variation in *N. ceranae* samples within apiaries from a region hypothesized to be the origin of the parasite (Vietnam), but the sequence was not too variable so that it was also able to show similarities in populations from different locations.

A total of 19 SNPs in 18 locations in the 822 bp sequence of the translation elongation factor 1-alpha of *N. ceranae* was observed in this study. The 16 samples from Vietnam were clearly the most variable with all 19 SNPs detected that were divided into 6 types. For samples from other locations, all had the same sequence matching the most common type in Vietnam (11 out of 16 Vietnam samples). All except one of the apiaries in Vietnam had more than one type.

The other genetic marker used in this study was length polymorphisms using SSRs identified in the *N. ceranae* genome. SSRs can vary in length between individuals, repeating approximately from 5–80 times (Li et al. 2002). If inside a coding region, the expansion and contraction of the number of repeats can directly affect the corresponding gene products possibly causing phenotypic changes (Li et al. 2004). They are multi-allelic, abundant, and co-dominant, while being highly informative and relatively simple to interpret (Rafalski et al. 1996; Belaj et al. 2003; Zhan et al. 2015). For microsporidians, *N. bombycis* isolates infecting silkworm were distinguished using 28 SSRs, whereas the SSU-rRNA sequence was able to detect only one genotype demonstrating that SSRs were more useful to detect variation within the species (Rao et al. 2005). SSRs have been used to compare organisms in their invasive versus native ranges, such as Okada et al. (2007), who found that invasive pampas grass (*Cortaderia selloana*) had less variation compared to the cultivated pampas grass in California. The only report of using SSRs with *N. ceranae* was Al-Hameed and Hadi (2020), who examined samples from Iraq, but it appears that they in fact

sequenced the SSU rDNA to differentiate between *N. ceranae* and *N. apis* rather than used SSRs to examine its variability. Thus, SSRs have not yet been used to look at the genetic variability of *N. ceranae*. In this study, an examination of SSRs in the genome of *N. ceranae* resulted in three SSRs, which were able to detect variability in *N. ceranae* samples within apiaries in the proposed native range of the parasite as well as variation within its invasive range.

Like the SNPs, the SSRs detected the most diversity in the Vietnam samples, but the SSR markers were also able to detect variation in other locations. The samples from Iran had the same SSR pattern as those from Alberta-Canada, samples from Ontario-Canada had the same SSR pattern as those from New York-United States of America, and samples from Mexico had the same SSR pattern as those from Argentina. None of those samples showed variation within a location, but the Vietnam samples did show variation within a location with five different types spread across different apiaries. The Hieu, Thanh and Hao apiaries in Vietnam showed variation in each apiary with both the SSR and SNP markers. However, unlike the SNP markers in this study, the SSR markers did not show any of the samples from outside of Vietnam having a direct match with any of the Vietnam samples. Thus, the SNP markers appeared to be better than the SSR markers for indicating the possible originating strains that spread worldwide form East Asia. However, SSR markers appeared to be better than SNP markers for showing variation for *N. ceranae* outside of Vietnam.

There are many reports that *N. ceranae* has been spreading and replacing *N. apis* worldwide as a parasite of *A. mellifera*. For example, PCR detection of *N. ceranae* in *A. mellifera* was negative in samples collected before the 1960's from eastern Australia and New Zealand (Malone et al. 1994; Gatehouse et al. 1999; Rice 1999, 2001), but were positive for *N. ceranae* in samples collected in 2007 (Giersch et al. 2009). Similar shifts from *N. apis* to *N. ceranae* over time have been reported in other countries, including Finland (Paxton et al. 2007), Chile (Martinez et al. 2012) and Canada (Emsen et al. 2016).

The apparently recent spread of *N. ceranae* has been proposed to be due to a jump to *A. mellifera* from *A. cerana*, which is native to southern and southeastern Asia where it likely long co-existed with *N. ceranae* (Fries et al. 1996; Fries 2010). In East Asia, *A. cerana* has been domesticated for several thousand years (Dinh and Pham 2001; Xu et al. 2009), whereas *A. mellifera* was probably first introduced there from Hong Kong in 1947 but was mostly introduced during the 1960–1980s when *A. mellifera* was imported in relatively large numbers from Russia and Cuba (Dinh and Pham 2001; P. Duc, personal communication). Thus, the spread of *N. ceranae* from *A. mellifera* and *A. cerana* may have occurred during that period, indicating a relatively rapid spread globally afterwards. Supporting a spread between the two bee species was the discovery that polymorphisms in the PTP1 sequence of *N. ceranae* from *A. mellifera* and *A. cerana* clustered together, while those from *A. florea* and *A. dorsata* clustered separately, suggesting that *N. ceranae* strains in *A. mellifera* and *A. cerana* are similar and distinct from those in other bee species. A recent population expansion was proposed to be responsible for the majority of *N. ceranae* strains in *A. mellifera* from Hungary and Hawaii having a low number of SNPs in actin, 70 kDa heat shock protein and small subunit of the RNA polymerase II, although no comparison was made with strains from the native range of *N. ceranae* (Gomez-Moracho et al. 2014).

One way to assess if a parasite has recently spread is to compare its genetic diversity between its native and invaded locations as there is a general trend toward reduced genetic diversity of organisms in their invaded versus native ranges (e.g., Amsellem et al. 2000; Kliber and Eckert 2005; Puillandre et al. 2008). Both SSRs (Okada et al. 2007) and DNA sequences (Besnard et al. 2007) have been used to detect higher genetic diversity of organisms in their native versus invasive ranges. Successful invasion often results in low genetic diversity due to factors, such as the founder effect, where genetic variation is reduced due to a low number of ancestors in the introduced population (O'Brien et al. 1994), the highly adapted genotype effect, where genetic diversity is reduced as the newly introduced population spreads due to a greater flexibility and broader acclimatization than native populations, and the aggressive genotype effect, where genetic diversity is reduced as a newly introduced invasive population has greater competitiveness and replaces native populations (Burrell et al. 2015). A founder effect was mentioned to explain fewer *N. ceranae* haplotypes in the Americas compared to its native range in Asia, and the low

frequency of *N. ceranae* variants from *A. mellifera* compared to *A. cerana* and *A. florea* based on PTP1, PTP2 and PTP3 sequences (Gomez-Moracho et al. 2015). The aggressive genotype effect is supported by finding higher populations of *N. ceranae* than *N. apis* in infected bees, resulting in higher populations of *N. ceranae* in bee feces allowing greater spread (Higes et al. 2007; Martin-Hernandez et al. 2009, 2011; Emsen et al. 2016). *N. ceranae* being more aggressive could also explain why mixed infections resulted in approximately 100-fold higher amounts of *N. ceranae* than *N. apis* (Chen et al. 2009).

The SNP results of this study support a founder effect in that a subset of the genotypes from Vietnam was also found in all the other locations examined in the world, but there were no differences in the sequences outside Vietnam. This indicates that only a limited number of *N. ceranae* strains spread from East Asia one or a few times, likely via infected *A. mellifera*. Afterwards, variability would remain low as the parasite reproduces asexually, thus limiting gene flow, with mutations only slowly accumulating over time.

The pattern of genetic variation of *N. ceranae* observed in this study in the invasive range detected by the SSR markers may reflect relatively recent movements of honey bees. One reason why samples from Iran and Alberta-Canada shared the same unique SSR pattern could be the importation of *Nosema*-infected *A. mellifera* queens when they were introduced to Iran and Alberta from New Zealand in the 1980s (Nazeri 2015; M. Nasr, personal communication; British Columbia Ministry of Agriculture 2014). Considering that contaminated pollen can transmit *N. ceranae* (Higes et al. 2008), another source of common introduction of *N. ceranae* to Iran and Alberta-Canada might be the trade of pollen from China (Van Eaton and Law 2000). The samples from Mexico and Argentina may have had the same unique SSR pattern due to the trade of honey bee queens from Mexico to Argentina, whereas no bees were exported from the United States or Canada to Argentina because of regulatory measures (E. Guzman-Novoa, personal communication; M. Nasr, personal communication). Finally, the samples from Ontario-Canada and New York-United States of America may have had the same unique SSR pattern due to the movement of *Nosema*-infected queens between Ontario and New York, as queens are often exported between these nearby locations (D. McRory, personal communication). In contrast, the SSR patterns for *N. ceranae* from Ontario and Alberta were distinct, even though both are provinces in Canada. This is not only because of large areas of boreal forests between them resulting in few apiaries, but also because Alberta beekeepers do not usually move their bees or send beekeeping equipment out of the province and Ontario beekeepers only move their bees and beekeeping equipment out of the province to Atlantic Canada for blueberry pollination (D. McRory, personal communication). Thus, the chances for *N. ceranae* to move in infected colonies between Alberta and Ontario are very low. However, the hypothesis about the patterns of SSRs in this study relative to the movement of honey bees and bee products is only based on a very limited number of samples and thus, remains to be confirmed.

This study showed that novel genetic markers for *N. ceranae* variability based on analysis of several SSRs and SNPs in the translation elongation factor 1-alpha sequence are promising markers of genetic variation of *N. ceranae* related to location. The results for both types of markers are consistent with a possible East Asian origin of *N. ceranae*, and SNPs in the translation elongation factor 1-alpha sequence are consistent with only a small subset of *N. ceranae* genotypes spreading from East Asia. Nevertheless, SSR markers revealed some variation throughout the world consistent with trade associated with beekeeping indicating long distance spread of *N. ceranae*. Further development using these as well as additional SSRs and SNPs is important for tracking *N. ceranae* for regulatory measures and determining the sources of infection for legal liability when outbreaks occur.

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Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare no conflicts of interest.

Consent for publication The authors give consent to publish the manuscript.

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