PLOS ONE

RESEARCH ARTICLE

Prevalence of infection by the microsporidian *Nosema* spp. in native bumblebees (*Bombus* spp.) in northern Thailand

Chainarong Sinpoo[1,2], Terd Disayathanoowat[1], Paul H. Williams[3], Panuwan Chantawannakul[1,4]*

1 Bee Protection Laboratory, Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand, **2** Graduate School, Chiang Mai University, Chiang Mai, Thailand, **3** Department of Life Sciences, Natural History Museum, London, United Kingdom, **4** Environmental Science Research Center (ESRC), Faculty of Science, Chiang Mai University, Thailand

* panuwan@gmail.com

Citation: Sinpoo C, Disayathanoowat T, Williams PH, Chantawannakul P (2019) Prevalence of infection by the microsporidian *Nosema* spp. in native bumblebees (*Bombus* spp.) in northern Thailand. PLoS ONE 14(3): e0213171. https://doi.org/10.1371/journal.pone.0213171

Editor: Bi-Song Yue, Sichuan University, CHINA

Received: July 20, 2018

Accepted: February 16, 2019

Published: March 7, 2019

Data Availability Statement: All relevant data are within the paper.

Funding: This research project is supported by Chiang Mai University.

Competing interests: The authors have declared that no competing interests exist.

Abstract

Bumblebees (tribe Bombini, genus *Bombus* Latreille) play a pivotal role as pollinators in mountain regions for both native plants and for agricultural systems. In our survey of northern Thailand, four species of bumblebees (*Bombus (Megabombus) montivagus* Smith, *B. (Alpigenobombus) breviceps* Smith, *B. (Orientalibombus) haemorrhoidalis* Smith and *B. (Melanobombus) eximius* Smith), were present in 11 localities in 4 provinces (Chiang Mai, Mae Hong Son, Chiang Rai and Nan). We collected and screened 280 foraging worker bumblebees for microsporidia (*Nosema* spp.) and trypanosomes (*Crithidia* spp.). Our study is the first to demonstrate the parasite infection in bumblebees in northern Thailand. We found *N. ceranae* in *B. montivagus* (5.35%), *B. haemorrhoidalis* (4.76%), and *B. breviceps* (14.28%) and *N. bombi* in *B. montivagus* (14.28%), *B. haemorrhoidalis* (11.64%), *and B. breviceps* (28.257%).

Introduction

Bumblebees (tribe Bombini, genus *Bombus* Latreille) play a vitally important role as native pollinators in temperate agricultural ecosystems [1–5]. They are especially important in mountain ecosystems [6] and may be better pollinators than honey bees for many plant species in these areas [7]. Because of this, some species of bumblebees have been employed commercially, especially in greenhouses [3]. From the 1980s onwards, they have been used commercially in greenhouses to pollinate tomatoes, eggplants, and strawberries and also for fruit trees [3, 8]. Several species have been used commercially around the world, including *Bombus terrestris*, *B. lucorum*, *B. occidentalis*, *B. ignitus* and *B. impatiens* [3, 9, 10]. Some bumblebees species (*B. terrestris*, *B. ruderatus*, *B. hortorum*, and *B. subterraneus*) had been released in New Zealand for targeted pollination in the 19th century [11]. Among species used commercially, the most frequent are *B. terrestris* in Europe and *B. impatiens* in North America [3]. The identification of bumblebee species has been difficult because the colour patterns can be highly variable within species and convergent among species [12].

In recent years, molecular approaches have been applied for bumblebee identification using particularly a mitochondrial gene (cytochrome oxidase I (COI)) [7]. COI barcodes provide an easily obtained, dependable and cost-effective solution, especially for morphologically cryptic species [13]. Consequently, the COI gene has been used to re-evaluate species, to estimate phylogenetic relationships and to clarify species complexes in Asian bumblebees [14–18].

Similar to *Apis* bees, bumblebee populations are affected by a number of pathogens and parasites [19]. *Crithidia bombi* (Trypanosomatidae) and *Nosema bombi* are the most common. They are transmitted both horizontally between and vertically within colonies of their hosts [20]. *Nosema bombi* (Microsporidia: Nosematidae) is an obligate intracellular microsporidian parasite infecting a wide range of bumblebee species [20–24]. It is the most widespread bumblebee pathogen worldwide. Thorp (2005) suggested that *N. bombi*, known to infect European *Bombus* species [25], may have invaded North American species [25]. Imhoof et al. (1999) showed that prevalence of *N. bombi* was significantly higher in two declining species, *B. pensylvanicus* and *B. occidentalis*, than in other species [26]. In addition, *Nosema cerana* and *C. bombi* are associated with declining populations of bumble bees in China [27].

In this paper, we aim to study the diversity of native bumblebees in northern Thailand and to report the prevalence of microsporidians and trypanosomes parasitizing bumblebee populations in Thailand.

Materials and methods

The sample locations for which specific permission was not required and bumblebee did not involve endangered or protected species.

Collection and sample preparation

Foraging bumblebees were collected with sweep nets and as random samples from seven sites in four provinces in northern Thailand (Chiang Mai, Mae Hong Son, Chiang Rai and Nan province) in 2015 & 2016 (Table 1). After capture, they were transferred directly into RNA *later* Solution and stored at -20˚C prior to DNA extraction. The following information was recorded for each specimen: GPS coordinates, elevation, collection-site name, and date. The samples were later analyzed in the laboratory. The exact locations are listed in Table 1 and shown in Fig 1. Bumblebee taxa were identified using an updated version of the morphological characters of Williams (2010) [28].

DNA extraction, mitochondrial cytochrome oxidase 1 (COI) gene sequence amplification

DNA extraction was achieved using a single crushed mid leg from each of the bumblebees. For most specimens, legs were ground in a 0.5-mL oxygen tube in liquid nitrogen using a stainless steel pestle, a Proteinase K Digestion kit was used, and the DNA was extracted following a standard phenol-chloroform protocol [29]. DNA extracts were kept at -20˚C until needed as a DNA template for the PCR (polymerase chain reaction). The PCR products of the mitochondrial COI (~685 base pairs) sequence were conducted using the universal primers LCO1490 and HC02198 [30]. The PCR amplification was performed in a total volume of 25 μL containing 2 μL of DNA extract, 12.5 pM of each primer, 0.2 mM of each dNTP, 0.2 mM $MgCl_2$, 1X reaction buffer and 2.5 units of *Taq* DNA polymerase (Invitrogen) under the following thermal conditions: 94˚C for 1 min, 5 cycles of 94˚C for 1 min, 50˚C for 1.5 min, 72˚C for 1 min; 35 cycles of 94˚C for 1 min, 50˚C for 1.5 min, 72˚C for 1 min and final step 72˚C for 5 min. Amplicons were checked on 1% agarose gels stained with ethidium bromide under UV light. PCR products were purified using PureLink Quick PCR Purification Kit (Invitrogen, Lithuania, USA) following the manufacturer's

Table 1. Prevalence of four parasites recovered from *Bombus* species in northern Thailand.

Province population	Code Name	Elevation	Latitude N	Longitude E	N Bees collected	Prevalence of parasites (%)			
						N. apis	*N. ceranae*	*N. bombi*	*C. bombi*
CHIANG MAI									
Doi Suthep 1	DS1	1,378	18˚48′55"	98˚55′13"	60	0.00	3.33	10.00	0
Doi Suthep 2	DS2	1,378	18˚48′55"	98˚55′13"	20	0	5.00	15.00	0
Doi Inthanon 1	DI1	2,118	18˚33′11"	98˚28′55"	25	0	0	12.00	0
Doi Inthanon 2	DI2	1,297	18˚32′41"	98˚30′58"	40	0	7.50	20.00	0
Doi Inthanon 3	DI3	1,070	18˚32′38"	98˚32′53"	40	0	12.50	15.00	0
Doi Mae Tha Man	DMTM	1,610	19˚31′35"	98˚83′26"	5	0	0	20.00	0
Doi Ang Khang	DAK	1,410	19˚54′8"	99˚2′24"	25	0	4.00	8.00	0
Doi Mon Ngao	DMNg	930	19˚10′60"	99˚48′35"	20	0	0	15.00	0
MAE HONG SON									
Doi Mae U Kho	DUK	1,509	18˚53′41"	98˚05′21"	20	0	10.00	20.00	0
CHIANG RAI									
Doi Thong	DT	960	20˚17′18"	99˚48′35"	20	0	5.00	20.00	0
Nan									
Doi Phu Kha	DPK	1,980	19˚12′20″	101˚40′50"	5	0	20.00	0	0
				Total	280	0	5.71	13.57	0

https://doi.org/10.1371/journal.pone.0213171.t001

protocol. The purified PCR products were sequenced. Sequencing reactions were performed, and the sequences were automatically determined in a genetic analyzer (1st Base, Selangor, Malaysia) using PCR primers mentioned above.

DNA Isolation and PCR Detection for pathogen/parasite

The abdomens of 280 individual bumblebees were removed with scissors and individually homogenized in 100 μL of Krebs-Ringer solution with a sterile Eppendorf tube. Total genomic DNA was extracted from 50 μL of the homogenate of each abdomen using a DNA purification kit (PureLink Genomic DNA Mini Kit (Invitrogen)). DNA samples were stored at -20˚C prior to molecular screening for parasites. Primers used for detection of *N. ceranae*, *N. apis*, *N. bombi* and *C. bombi* are listed in Table 2. The PCR amplification was performed in a total volume of 25 μL containing 2 μL DNA extract, 12.5 pM of each primer, 0.2 mM of each dNTP, 0.2 mM $MgCl_2$, 1X reaction buffer and 2.5 unit of Taq DNA polymerase (Invitrogen). Amplification used thermal cycling profiles: initial DNA denaturation step of 4 min at 94˚C followed by 40 cycles of 30s at 94˚C, 30s at 56˚C, and 1 min at 72˚C, and terminated with a final extension step of 72˚C for 10 min. For each run of the PCR reaction, negative (water) and positive (previously identified positive sample) controls were run along with DNA extracts of the samples. PCR products were electrophoresed on 1.2% agarose gels with ethidium bromide and visualized under UV light. Some of the PCR-amplified bands were purified with PureLink Quick PCR Purification Kit (Invitrogen, Lithuania, USA) following the manufacturer's protocol. After the sequencing reactions the sequences were determined automatically in a genetic analyzer (1st Base, Selangor, Malaysia) using the PCR primers mentioned above. The DNA sequences were used for estimating phylogenetic trees.

Data analysis

Sequences were checked manually and aligned using the BioEdit (version v7.2.6; http://www.mbio.ncsu.edu/BioEdit/BioEdit.html, accessed 2017), and the primers removed from both

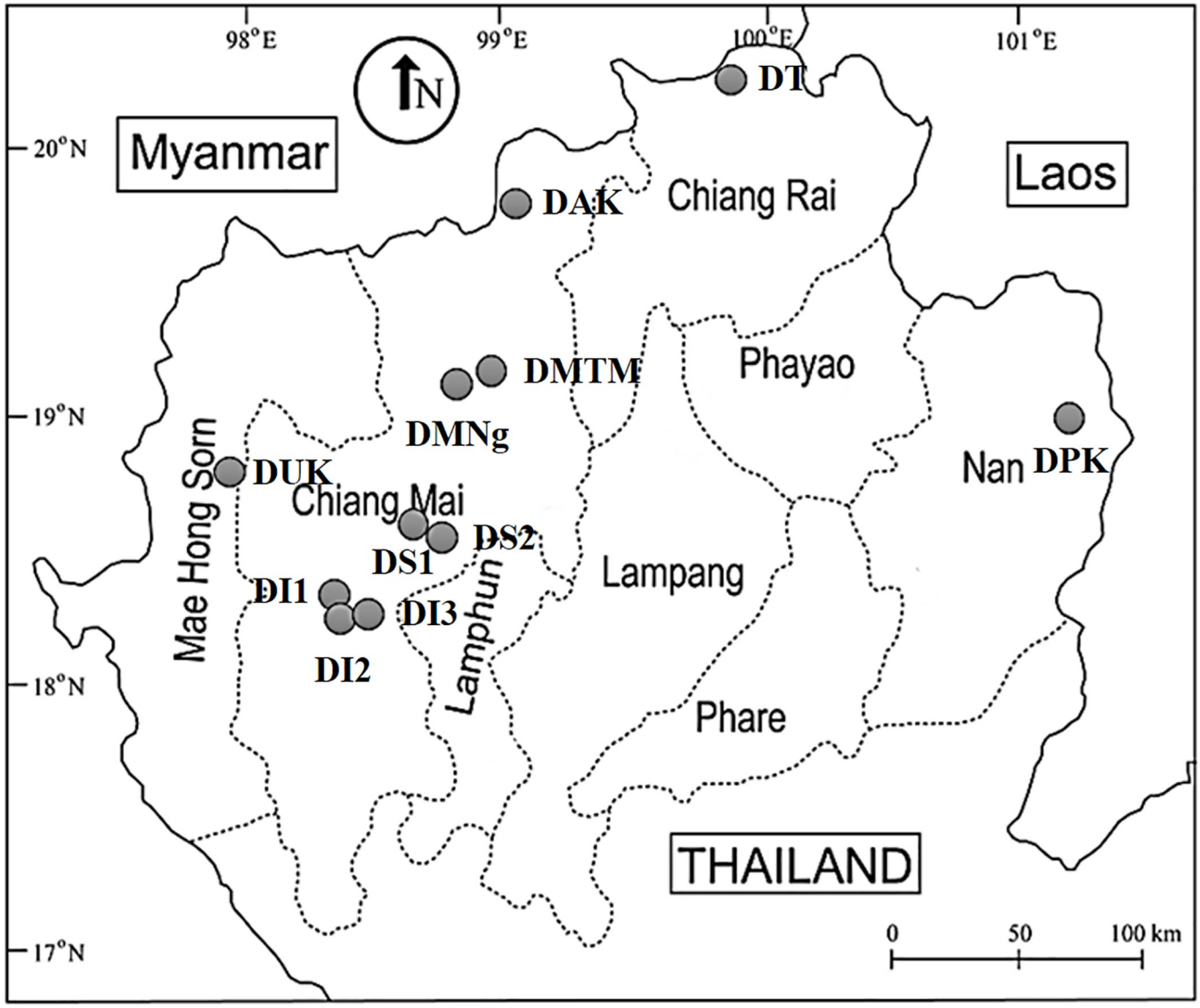

Fig 1. Map of the collection sites (grey dots) of native bumblebees in northern Thailand. Code name are abbreviated as following: DS = Doi Suthep, DI = Doi Inthanon, DMTM = Doi Mae Thaman, DAK = Doi Ang Khang, DMNg = Doi Mon Ngao, DUK = Doi Mae U Kho, DT = Doi Thong, DPK = Doi Phu Kha.

https://doi.org/10.1371/journal.pone.0213171.g001

ends (Table 2). The sequences were aligned using ClustalW and the alignments were refined by visual inspection. Sequences were used to query GenBank via the BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi). All covering DNA cytochrome oxidase I (COI) region and *Nosema* parasites sequences obtained in this study can be accessed as NCBI GenBank entries (http://www.ncbi.nlm.nih.gov; bumblebee species accession number MF582589—MF582628; *Nosema* parasites accession number MF776532-MF776567).

For phylogenetic analysis, multiple alignments of sequences determined in this study and reference sequences obtained from databases were taken together in the calculations of levels of sequence similarity using ClustalX2 program [35], with arithmetic averages tree-making algorithms taken from the MEGA package version 7 [36]. The topologies of the maximum likelihood phylogenetic trees were evaluated based on bootstrap analyses of 1,000 replicates.

Table 2. Primers used for pathogen/parasite and mtDNA detection.

Primer	Sequence 5′-3′	Amplification target	Size (bp)	Reference
RPS5-F	AATTATTTGGTCGCTGGAATTG	Ribosomal protein S5 (reference gene)		Evans (2006)[31]
RPS5-R	TAACGTCCAGCAGAATGTGGTA			
LCO1490	GGTCAACAAATCATAAAGATATTGG	mtDNA	685	Folmer et al. (1994)[30]
HCO2198	TAAACTTCAGGGTGACCAAAAAATCA			
Crith-F	GGAAACCACGGAATCACATAGACC	*Crithidia* (Trypanosome)	500	Li et al. (2012)[32]
Crith-R	AGGAAGCCAAGTCATCCATCGC			
Napis-SSU-Jf1	CCATGCATGTCTTTGACGTACTATG	*N.apis* (Microsporidium)	325	Klee et al. (2007)[33]
Napis-SSU-Jr1	GCTCACATACGTTTAAAATG			
NOS-FOR	TGCCGACGATGTGATATGAG	*N.ceranae* (Microsporidium)	252	Higes et al. (2006)[34]
NOS-REV	CACAGCATCCATTGAAAACG			
Nbombi-SSU-Jf1	CCATGCATGTTTTTGAAGATTATTAT	*N. bombi* (Microsporidium)	323	Klee et al. (2007)[33]
Nbombi-SSU-Jr1	CATATATTTTTAAAATATGAAACAATAA			

https://doi.org/10.1371/journal.pone.0213171.t002

Results

Geographical distribution

Samples were collected from Chiang Mai, Mae Hong Son, Chiang Rai and Nan province, at an elevation range of 700–2,200 m. (sample site; Fig 1, Table 1 and Table 3).

Our study of bumblebees in northern Thailand included 280 female bumblebees. Many of the bumblebees' colour patterns were similar among species within northern Thailand. The dominant colour of the 6th abdominal segment was red in all of the specimens. Of *B. montivagus*, three distinct colour patterns were collected (Fig 2). In this study, similar colour patterns to those of *B. montivagus* were observed in co-occurring species, *B. haemorrhoidalis* and *B. breviceps*. The colour pattern of the thoracic pubescence of the workers was primarily orange. In *B. breviceps*, *B. haemorrhoidalis*, *and B. montivagus*, the described orange colour pattern runs anterior to posterior on the notum of the thorax. However, some species have extensive black hair on the thorax, ranging from a small patch in the center of the thorax to a transverse band between the tegulae (above the wing bases), or (in the case of *B. eximius*) the entire thorax. The sides of the thorax are orange or yellow in all species except *B. eximius*.

COI-sequence-based analyses

DNA was extracted and the COI gene sequence was amplified successfully from 40 individual bumblebee specimens from 11 localities. All of the sequences were 658 base pairs long after removing the primer from both ends. We found a strong A+T bias in the COI gene barcoding from mtDNA. All new sequences have been deposited in GenBank and are accessible via the sequence numbers MF582589–MF582628 (Table 4).

The phylogenetic analysis by maximum likelihood method (Fig 3) with COI barcode data showed strong support for all of the following four conventional *Bombus* subgenera: *B.*

Table 3. A list of *Bombus* subgenera with information on distribution and species number.

Subgenus	Distribution	Species	No. sampled
Alpigenobombus	DS1. DS2 DI1, DI2, DI3	*B. breviceps*	28
Megabombus	DS1, DI2, DI3, DAK, DUK	*B. montivagus*	56
Melanobombus	DI1	*B. eximius*	7
Orientalibombus	DS1,DS2, DI2, DT, DAK, DMNg, DPK	*B. haemorrhoidalis*	189

https://doi.org/10.1371/journal.pone.0213171.t003

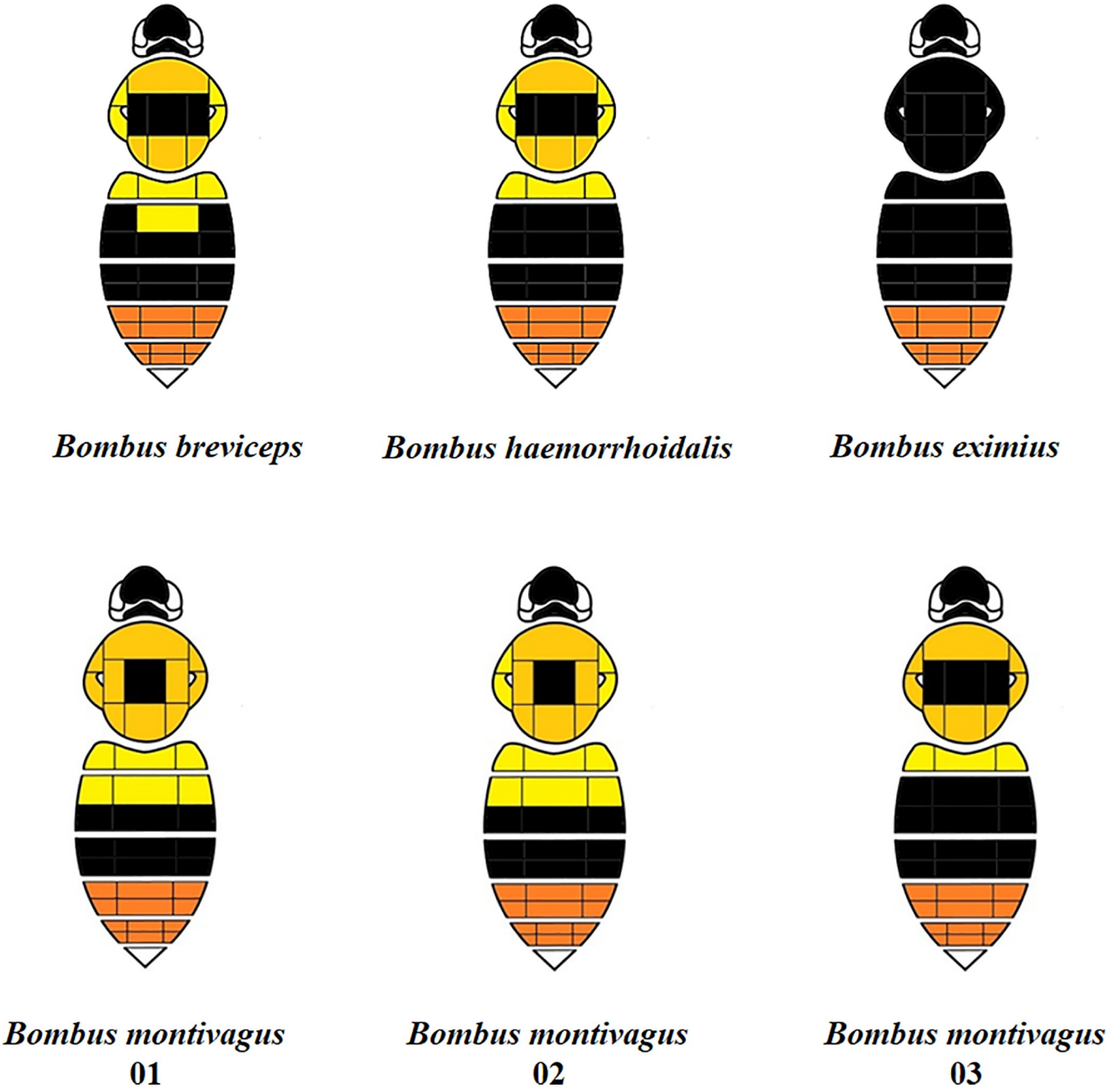

Fig 2. Species identification guide with simplified colour patterns of female workers. The dorsum of the body is artificially divided into an arbitrary set of regions.

https://doi.org/10.1371/journal.pone.0213171.g002

(Megabombus) montivagus Smith (formerly regarded as part of *B. trifasciatus s. l.*), *B. (Alpigenobombus) breviceps* Smith, *B. (Orientalibombus) haemorrhoidalis* Smith *and B. (Melanobombus) eximius* Smith (Fig 3).

Microsporidian and trypanosome parasite frequencies in bumblebees

A total of 280 individual bumblebees representing four species (*B. montivagus*, *B. haemorrhoidalis*, *B. breviceps*, and *B. eximius*) were examined from samples from northern Thailand

Table 4. Material used in the phylogenetic analysis with the sample localities, collector, COI sequence length, depository and GenBank accession number.

Species	Sample name	Sample locality	Collector	Latitude	Longitude	Sequence length (bp)	GenBank acc. no.
Montivagus	DS1-B01	TH, Doi Su Thep CMP	C. Sinpoo	18˚48′55"	98˚55′13"	658	MF582589
haemorrhoidalis	DS1-B16	TH, Doi Su Thep CMP	C. Sinpoo	18˚48′55"	98˚55′13"	658	MF582590
haemorrhoidalis	DS1-B21	TH, Doi Su Thep CMP	C. Sinpoo	18˚48′55"	98˚55′13"	658	MF582591
haemorrhoidalis	DS1-B41	TH, Doi Su Thep CMP	C. Sinpoo	18˚48′55"	98˚55′13"	658	MF582592
montivagus	DI2-B06	TH, Doi Inthanon CMP	C. Sinpoo	18˚32′41"	98˚30′58"	658	MF582593
haemorrhoidalis	DI2-B16	TH, Doi Inthanon CMP	C. Sinpoo	18˚32′41"	98˚30′58"	658	MF582594
haemorrhoidalis	DI2-B31	TH, Doi Inthanon CMP	C. Sinpoo	18˚32′41"	98˚30′58"	658	MF582595
montivagus	DI3-B11	TH, Doi Inthanon CMP	C. Sinpoo	18˚32′38"	98˚32′53"	658	MF582596
montivagus	DI3-B21	TH, Doi Inthanon CMP	C. Sinpoo	18˚32′38"	98˚32′53"	658	MF582597
breviceps	DI3-B27	TH, Doi Inthanon CMP	C. Sinpoo	18˚32′38"	98˚32′53"	658	MF582598
haemorrhoidalis	DMNg-B01	TH, Doi Mon Ngao CMP	C. Sinpoo	19˚10′60"	99˚48′35"	658	MF582599
haemorrhoidalis	DMNg-B11	TH, Doi Mon Ngao CMP	C. Sinpoo	19˚10′60"	99˚48′35"	658	MF582600
haemorrhoidalis	DAK-B01	TH, Doi Ang Khang CMP	C. Sinpoo	19˚54′8"	99˚2′24"	658	MF582601
haemorrhoidalis	DAK-B14	TH, Doi Ang Khang CMP	C. Sinpoo	19˚54′8"	99˚2′24"	658	MF582602
montivagus	DAK-B05	TH, Doi Ang Khang CMP	C. Sinpoo	19˚54′8"	99˚2′24"	658	MF582603
haemorrhoidalis	DAK-B12	TH, Doi Ang Khang CMP	C. Sinpoo	19˚54′8"	99˚2′24"	658	MF582604
montivagus	DAK-B22	TH, Doi Ang Khang CMP	C. Sinpoo	19˚54′8"	99˚2′24"	658	MF582605
haemorrhoidalis	DAK-B06	TH, Doi Ang Khang CMP	C. Sinpoo	19˚54′8"	99˚2′24"	658	MF582606
haemorrhoidalis	DAK-B10	TH, Doi Ang Khang CMP	C. Sinpoo	19˚54′8"	99˚2′24"	658	MF582607
montivagus	DUK-B01	TH, Doi Mae U Kho MHP	C. Sinpoo	18˚53′41"	98˚05′21"	658	MF582608
montivagus	DUK-B08	TH, Doi Mae U Kho MHP	C. Sinpoo	18˚53′41"	98˚05′21"	658	MF582609
haemorrhoidalis	DT-B01	TH, Doi Thong CRP	C. Sinpoo	20˚17′18"	99˚48′35"	658	MF582610
haemorrhoidalis	DT-B04	TH, Doi Thong CRP	C. Sinpoo	20˚17′18"	99˚48′35"	658	MF582611
breviceps	DI2-B20	TH, Doi Inthanon CMP	C. Sinpoo	18˚32′41"	98˚30′58"	658	MF582612
breviceps	DI3-B30	TH, Doi Inthanon CMP	C. Sinpoo	18˚32′38"	98˚32′53"	658	MF582613
haemorrhoidalis	DS2-B01	TH, Doi Su Thep CMP	C. Sinpoo	18˚48′55"	98˚55′13"	658	MF582614
haemorrhoidalis	DS2-B02	TH, Doi Su Thep CMP	C. Sinpoo	18˚48′55"	98˚55′13"	658	MF582615
haemorrhoidalis	DS2-B03	TH, Doi Su Thep CMP	C. Sinpoo	18˚48′55"	98˚55′13"	658	MF582616
breviceps	DS2-B04	TH, Doi Su Thep CMP	C. Sinpoo	18˚48′55"	98˚55′13"	658	MF582617
breviceps	DI3-B01	TH, Doi Inthanon CMP	C. Sinpoo	18˚32′38"	98˚32′53"	658	MF582618
Breviceps	DI3-B03	TH, Doi Inthanon CMP	C. Sinpoo	18˚32′38"	98˚32′53"	658	MF582619
montivagus	DI2-B01	TH, Doi Inthanon CMP	C. Sinpoo	18˚32′41"	98˚30′58"	658	MF582620
haemorrhoidalis	DI2-B03	TH, Doi Inthanon CMP	C. Sinpoo	18˚32′41"	98˚30′58"	658	MF582621
Breviceps	DI2-B04	TH, Doi Inthanon CMP	C. Sinpoo	18˚32′41"	98˚30′58"	658	MF582622
haemorrhoidalis	DI2-B05	TH, Doi Inthanon CMP	C. Sinpoo	18˚32′41"	98˚30′58"	658	MF582623
haemorrhoidalis	DPK-B01	TH, Doi Phu Kha NP	C. Sinpoo	19˚12′20″	101˚40′50"	658	MF582624
haemorrhoidalis	DPK-B02	TH, Doi Phu Kha NP	C. Sinpoo	19˚12′20″	101˚40′50"	658	MF582625
eximius	DI1-B02	TH, Doi Inthanon CMP	C. Sinpoo	18˚33′11"	98˚28′55"	658	MF582626
eximius	DI1-B03	TH, Doi Inthanon CMP	C. Sinpoo	18˚33′11"	98˚28′55"	658	MF582627
Breviceps	DI1-B04	TH, Doi Inthanon CMP	C. Sinpoo	18˚33′11"	98˚28′55"	658	MF582628

https://doi.org/10.1371/journal.pone.0213171.t004

(Chiang Mai, Mae Hong Son, Chiang Rai and Nan province, sampling sites shown in Table 1). We collected and screened for the most common pathogens of foraging worker bumblebees, *Nosema* spp. and *Crithidia* spp..

The results showed that 16 out of 280 individual bumblebees (5.71%) were infected with *N. ceranae*. This parasite was found in specimens of *B. montivagus* (5.35%), *B. breviceps* (14.28%), *and*

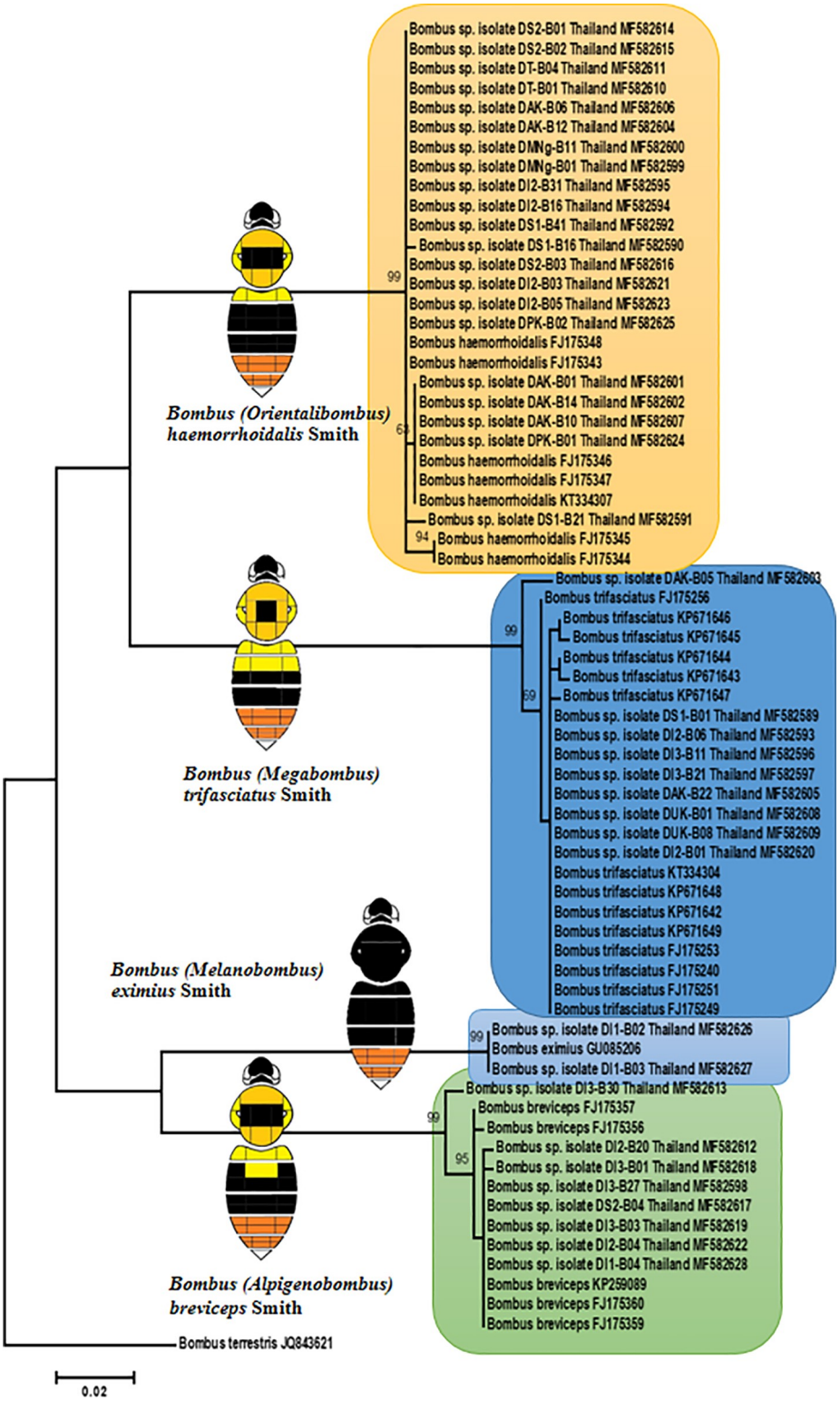

Fig 3. Estimate of phylogenetic relationship of cytochrome oxidase subunit I (COI) from bumblebees (*Bombus* sp.) collected in northern Thailand using maximum likelihood. The sequences of *B. terrestris*–JQ843621 was used as an out group. Numbers at each node represent bootstrap values as percentages and only bootstrap values greater than 70% are shown.

https://doi.org/10.1371/journal.pone.0213171.g003

Table 5. Overall occurrence of four parasites in host species (*Bombus* spp.) (Identities confirmed from barcodes).

Species	N Bees collected	*N. apis* [a]	*N. ceranae*[a]	*N. bombi*[a]	*C. bombi*[a]
B. montivagus	56	0.00	5.35	14.28	0.00
B. haemorrhoidalis	189	0.00	4.76	11.64	0.00
B. breviceps	28	0.00	14.28	28.57	0.00
B. eximius	7	0.00	0.00	0.00	0.00
Total	280	0.00	5.71	13.57	0.00

N = Total number of individual each *Bombus* species collected.

[a] = Prevalence (%)

https://doi.org/10.1371/journal.pone.0213171.t005

B. haemorrhoidalis (4.76%). *Nosema bombi* was found in 38 individuals (13.57%) from the three species of *Bombus* as shown in Table 5. Infection rates of *N. ceranae* and *N. bombi* were higher in *B. breviceps* than in other bumblebee species. *Nosema bombi* was also more prevalent than *N. ceranae* in the three species of bumblebees. When considering the geographical areas, the highest prevalence values of *N. ceranae* (20% and 12.5% respectively) were found at the locations Doi Phu Kha (Nan) and Doi Inthanon 3 (Chiang Mai). Prevalence of *N. bombi* of 20% was found at Doi Inthanon 2, Doi Mae Tha Man (Chiang Mai) and Doi Mae U Kho (Mae Hong Son).

Phylogenetic trees were estimated to assess relationships between the samples of *Nosema* as shown in Fig 4A and 4B. This included a total of 36 sequences from infected *Bombus* with a length of 269 bp for 20 sequences of *N. bombi* and 212 bp for 16 sequences of *N. ceranae*, after removing the primers from both ends. New sequences of *Nosema* have been deposited in GenBank and are accessible with the numbers MF776532–MF776567 (Table 6).

Discussion

In this study we aimed to identify native bumblebees from multiple sites in northern Thailand (Chiang Mai, Mae Hong Son, Chiang Rai and Nan province). Three bumblebee species (*B. montivagus* Smith, *B. haemorrhoidalis* Smith, and *B. breviceps* Smith) show similar colour patterns. These colour patterns are similar to others in Southeast Asia and may have evolved though mutually protective Mullerian mimicry [37]. We have identified similar colour patterns for bumblebee workers (Fig 2) (three of them for *B montivagus* in northern Thailand). Hines and Williams (2012) examined colour-pattern evolution in bumblebees in this Southeast Asian mimicry group, which includes *B.* (*Megabombus*) *montivagus* Smith, *B.* (*Alpigenobombus*) *breviceps* Smith, and *B.* (*Orientalibombus*) *haemorrhoidalis* Smith [37]. Moreover, they reported that because these bumblebees also have high variability of colour patterns within species it is sometimes difficult to make reliable species identifications. Considerable colour variation within bumblebee species has been known for more than a century [38]. Our work reaffirms that only some morphological data can be used to accurately distinguish species.

When possible, additional molecular data should therefore be used to confirm species identification [15, 37, 39, 40]. According to our results, the bumblebee species are supported by groups identified from the (COI) gene. This confirms the value of evidence from barcodes for examining the more closely related bumblebee species despite the variation within species [15, 40, 41].

This study is the first survey of the prevalence of major bumblebee pathogens in native bumblebees in northern Thailand, showing the detection and infection rates of *N. cerana* and *N. bombi* among 280 female bumblebee specimens. In this sample, *N. bombi* was present in three species of *Bombus* (i.e. *B. montivagus*, *B. haemorrhoidalis*, and *B. breviceps*). The complete gene encoding ssrRNA sequences of *Nosema* isolates were identical to those reported

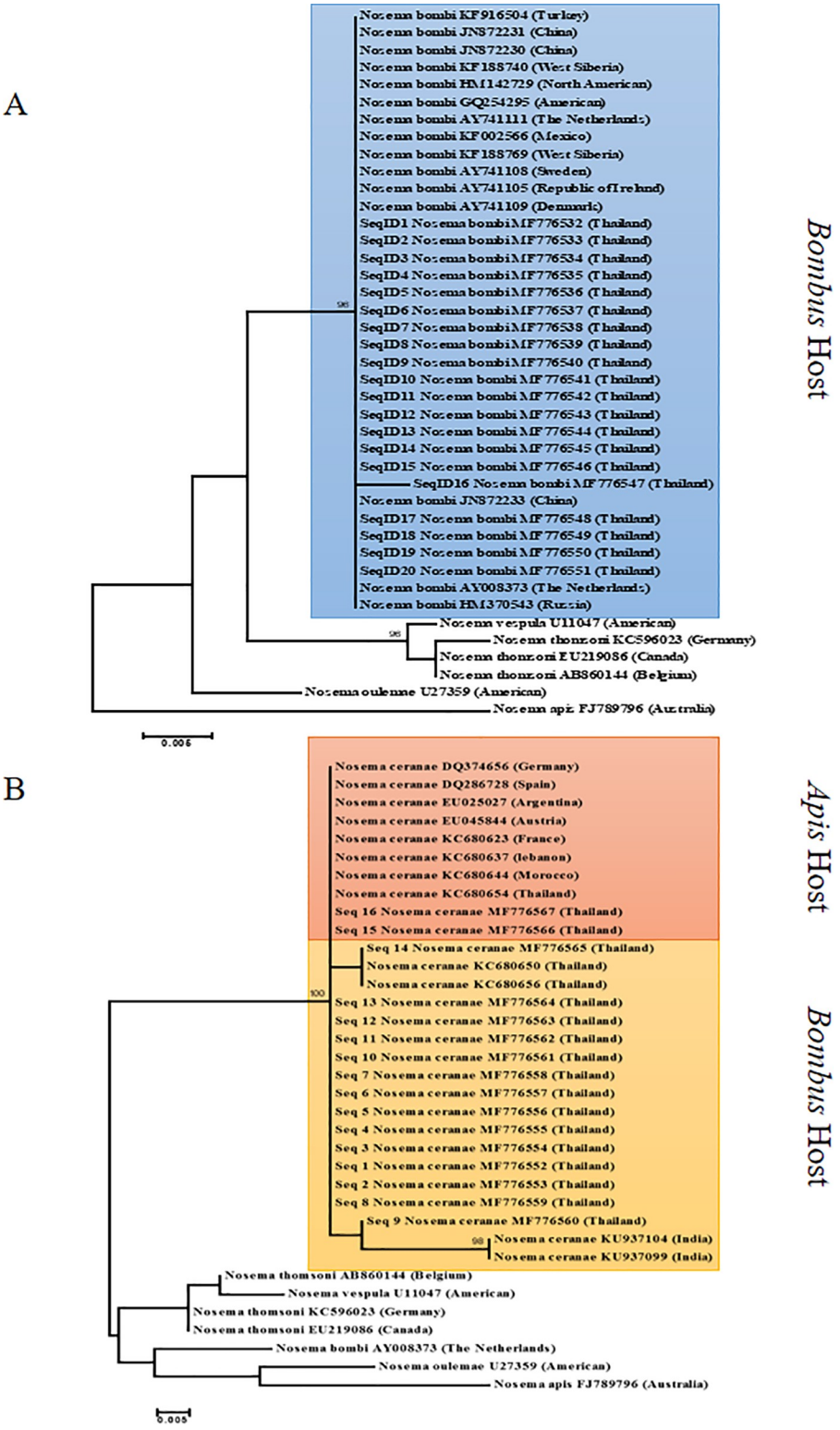

Fig 4. The phylogenetic tree showing the relationship of *Nosema*. Unrooted consensus of phylogenetic tree showing the relationship of *Nosema* isolate the partial sequences of 16S ribosomal RNA of *Nosema* (4-A; *N bombi*, 4-B; *N. ceranae*) from *Bombus* spp. collected in northern Thailand. The tree was estimated using Maximum Likelihood. Numbers at each node represent bootstrap values as percentages and only bootstrap values greater than 70% are shown.

https://doi.org/10.1371/journal.pone.0213171.g004

Table 6. Material used in the phylogenetic analysis with the sample locality, collector, sequence length, depository and GenBank accession number.

Species	Sample name	Sample locality	Collector	Sequence length (bp)	GenBank
1 *N. bombi*	BomDS2-B06	TH, Doi Su Thep CMP	C. Sinpoo	269	MF776532
2 *N. bombi*	BomDS2-B12	TH, Doi Su Thep CMP	C. Sinpoo	269	MF776533
3 *N. bombi*	BomDS2-B20	TH, Doi Su Thep CMP	C. Sinpoo	269	MF776534
4 *N. bombi*	BomDS1-B04	TH, Doi Su Thep CMP	C Sinpoo	269	MF776535
5 *N. bombi*	BomDS1-B10	TH, Doi Su Thep CMP	C. Sinpoo	269	MF776536
6 *N. bombi*	BomDS1-B37	TH, Doi Su Thep CMP	C Sinpoo	269	MF776537
7 *N. bombi*	BomDS1-B45	TH, Doi Su Thep CMP	C. Sinpoo	269	MF776538
8 *N. bombi*	BomDS1-B55	TH, Doi Su Thep CMP	C. Sinpoo	269	MF776539
9 *N. bombi*	BomDI1-B04	TH, Doi Inthanon CMP	C. Sinpoo	269	MF776540
10 *N. bombi*	BomDI1-B07	TH, Doi Inthanon CMP	C. Sinpoo	269	MF776541
11 *N. bombi*	BomDI1-B11	TH, Doi Inthanon CMP	C. Sinpoo	269	MF776542
12 *N. bombi*	BomDI2-B17	TH, Doi Inthanon CMP	C. Sinpoo	269	MF776543
13 *N. bombi*	BomDI2-B24	TH, Doi Inthanon CMP	C. Sinpoo	269	MF776544
14 *N. bombi*	BomDI3-B07	TH, Doi Inthanon CMP	C. Sinpoo	269	MF776545
15 *N. bombi*	BomDMNg-B05	TH, Doi Mon Ngao CMP	C. Sinpoo	269	MF776546
16 *N. bombi*	BomDMNg-B11	TH, Doi Mon Ngao CMP	C. Sinpoo	269	MF776547
17 *N. bombi*	BomDMNg-B15	TH, Doi Mon Ngao CMP	C. Sinpoo	269	MF776548
18 *N. bombi*	BomDMTM-B03	TH, Doi Mae Tha Man CMP	C. Sinpoo	269	MF776549
19 *N. bombi*	BomDAK-B10	TH, Doi Ang Khang CMP	C. Sinpoo	269	MF776550
20 N. *bombi*	BomDAK-B12	TH, Doi Ang Khang CMP	C. Sinpoo	269	MF776551
1 *N. ceranae*	BomDS2-B16	TH, Doi Su Thep CMP	C. Sinpoo	212	MF776552
2 N. *ceranae*	BomDS1-B10	TH, Doi Su Thep CMP	C. Sinpoo	212	MF776553
3 N. *ceranae*	BomDS1-B37	TH, Doi Su Thep CMP	C. Sinpoo	212	MF776554
4 N. *ceranae*	BomDI2-B02	TH, Doi Inthanon CMP	C. Sinpoo	212	MF776555
5 N. *ceranae*	BomDI3-B02	TH, Doi Inthanon CMP	C. Sinpoo	212	MF776556
6 N. *ceranae*	BomDAK-B10	TH, Doi Ang Khang CMP	C. Sinpoo	212	MF776557
7 N. *ceranae*	BomDUK-B10	TH, Doi Mae U Kho CMP	C. Sinpoo	212	MF776558
8 *N. ceranae*	BomDT-B16	TH, Doi Thong CRP	C. Sinpoo	212	MF776559
9 *N. ceranae*	BomDT-B16	TH, Doi Thong CRP	C. Sinpoo	212	MF776560
10 *N. ceranae*	BomDI2-B04	TH, Doi Inthanon CMP	C. Sinpoo	212	MF776561
11 *N. ceranae*	BomDI2-B38	TH, Doi Inthanon CMP	C. Sinpoo	212	MF776562
12 *N. ceranae*	BomDI3-B05	TH, Doi Inthanon CMP	C. Sinpoo	212	MF776563
13 *N. ceranae*	BomDI3-B23	TH, Doi Inthanon CMP	C. Sinpoo	212	MF776564
14 *N. ceranae*	BomDI3-B27	TH, Doi Inthanon CMP	C. Sinpoo	212	MF776565
15 *N. ceranae*	BomDI3-B39	TH, Doi Inthanon CMP	C. Sinpoo	212	MF776566
16 *N. ceranae*	BomDUK-B19	TH, Doi Inthanon MHS	C. Sinpoo	212	MF776567

https://doi.org/10.1371/journal.pone.0213171.t006

previously from the bumblebee species *B. terrestris*, *B. hortorum*, and *B. lucorum* [21]. Cameron et al. (2011) and Kissinger et al. (2011) could only analyze *N. bombi* in samples of various *Bombus* spp. from the southern states of the USA, which were genetically similar to the European isolates screened by these authors [5, 42]. In our results, the gene sequences showed small variations. In the past it was believed that among all *Nosema* taxa identified to date, only *N. bombi* was an established parasite of *Bombus* spp. [21] in which it may be present at varying levels [19, 43]. Thorp (2005) and Tay et al. (2005) suggested that *N. bombi* was the only microsporidian known to infect European *Bombus* species [20, 25].

Our study found that *N. ceranae* was also present in three *Bombus* spp. (*B. montivagus*, *B. haemorrhoidalis*, and *B. breviceps)*. Normally, *N. ceranae* infects honey bees (originally isolated

from *A*. *cerana* [44] now infecting *A*. *mellifera* as well [33, 45]), but Plischuk et al (2009) found *N*. *ceranae* in bumblebees in South America [46]. Our work also is similar to the findings of researchers who have reported the presence of *N*. *ceranae* in native bumblebees of Argentina (*B*. *atratus*, *B*. *bellicosus*, and *B*. *morio*) [46]. Mean prevalence values of *N*. *ceranae* found in *B*. *breviceps* (14.28%) are lower than those reported in *B*. *atratus* (72%) and *B*. *bellicosus* (63%) from Argentina [47] as well as from these same species in other countries [32, 48]. On the other hand, the lower infection intensity found in native bumblebees of northern Thailand may prevent infection from increasing further as natural reservoirs with high prevalence of the pathogen have not yet been found.

We collected and screened the most common pathogens for total of 280 native foraging worker bumblebees. The trypanosome *C*. *bombi* was not observed in this study. Kissinger et al. (2011) also reported few *C*. *bombi* in his extensive survey [42]. Similarly, prevalence of *Crithidia* was less than 10% of all *Bombus* species examined in United States [49].

Previous studies have proposed that *N*. *ceranae* is closer phylogenetically to *N*. *bombi* than to *N*. *apis* [21, 50, 51], although there is a report to the contrary [52]. Shafer et al. (2009) suggest that *N*. *apis* is a basal member of the clade and, therefore, *N*. *bombi* is closer to *N*. *ceranae* [53]. In our study, *N*. *ceranae* strains present in three species of *Bombus* (*B*. *montivagus*, *B*. *haemorrhoidalis*, and *B*. *breviceps*) from northern Thailand were closely related to the *N*. *ceranae* strains reported from *A*. *mellifera*. This reaffirms that *N*. *ceranae* has a broad host range and may cross between host genera. *Nosema ceranae* was first discovered in *A*. *cerana*, however although it is now spreading to *A*. *mellifera*. This pathogen has potential as an emerging threat to bumblebees among the indigenous pollinators [54].

Acknowledgments

This research project is supported by Chiang Mai university.

Author Contributions

Conceptualization: Panuwan Chantawannakul.

Data curation: Chainarong Sinpoo, Terd Disayathanoowat.

Formal analysis: Chainarong Sinpoo, Panuwan Chantawannakul.

Funding acquisition: Panuwan Chantawannakul.

Investigation: Paul H. Williams, Panuwan Chantawannakul.

Methodology: Chainarong Sinpoo, Panuwan Chantawannakul.

Project administration: Panuwan Chantawannakul.

Resources: Panuwan Chantawannakul.

Software: Chainarong Sinpoo.

Supervision: Panuwan Chantawannakul.

Visualization: Panuwan Chantawannakul.

Writing – original draft: Chainarong Sinpoo.

Writing – review & editing: Chainarong Sinpoo, Paul H. Williams, Panuwan Chantawannakul.

References

1. Bingham RA, Orthner AR. Efficient pollination of alpine plants. Nature. 1998; 391(6664):238.
2. Kremen C, Williams NM, Thorp RW. Crop pollination from native bees at risk from agricultural intensification. Proceedings of the National Academy of Sciences. 2002; 99(26):16812–6.
3. Velthuis HH, Van Doorn A. A century of advances in bumblebee domestication and the economic and environmental aspects of its commercialization for pollination. Apidologie. 2006; 37(4):421–51.
4. Williams PH, Osborne JL. Bumblebee vulnerability and conservation world-wide. Apidologie. 2009; 40 (3):367–87.
5. Cameron AC, Gelbach JB, Miller DL. Robust inference with multiway clustering. Journal of Business & Economic Statistics. 2011; 29(2):238–49.
6. Macior LW, Tang Y. A preliminary study of the pollination ecology of Pedicularis in the Chinese Himalaya. Plant Species Biology. 1997; 12(1):1–7.
7. Winter K, Adams L, Thorp R, Inouye D, Day L, Ascher J, et al. Importation of non-native bumble bees into North America: potential consequences of using *Bombus terrestris* and other non-native bumble bees for greenhouse crop pollination in Canada, Mexico, and the United States. San Francisco. 2006;33.
8. Dias B, Raw A, Imperatriz-Fonseca V, editors. International pollinators initiative: The São Paulo declaration on pollinators. Report on the recommendations of the workshop on the conservation and sustainable use of pollinators in agriculture with emphasis on bees; 1999.
9. Ruz L. Bee pollinators introduced to Chile: a review. Pollinating bees. 2002:155–67.
10. Li J, Wu J, Cai W, Peng W, An J, Huang J. Comparison of the colony development of two native bumblebee species *Bombus ignitus* and *Bombus lucorum* as candidates for commercial pollination in China. Journal of apicultural research. 2008; 47(1):22–6.
11. Macfarlane R, Gurr L. Distribution of bumble bees in New Zealand. New Zealand Entomologist. 1995; 18(1):29–36.
12. Williams P. The distribution of bumblebee colour patterns worldwide: possible significance for thermoregulation, crypsis, and warning mimicry. Biological Journal of the Linnean Society. 2007; 92(1):97–118.
13. Hebert PD, Ratnasingham S, de Waard JR. Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. Proceedings of the Royal Society of London B: Biological Sciences. 2003; 270(Suppl 1):S96–S9.
14. Williams PH, An J, Huang J. The bumblebees of the subgenus *Subterraneobombus*: integrating evidence from morphology and DNA barcodes (Hymenoptera, Apidae, *Bombus*). Zoological Journal of the Linnean Society. 2011; 163(3):813–62.
15. Williams PH, An J, Brown MJ, Carolan JC, Goulson D, Huang J, et al. Cryptic bumblebee species: consequences for conservation and the trade in greenhouse pollinators. PloS one. 2012; 7(3):e32992. https://doi.org/10.1371/journal.pone.0032992 PMID: 22427924
16. Williams PH, Byvaltsev A, Sheffield C, Rasmont P. *Bombus cullumanus*—an extinct European bumblebee species? Apidologie. 2013; 44(2):121–32.
17. Huang J, Jie W, Jiandong A, Williams PH. Newly discovered colour-pattern polymorphism of *Bombus koreanus* females (Hymenoptera: Apidae) demonstrated by DNA barcoding. Apidologie. 2015; 46 (2):250–61.
18. Williams PH, Byvaltsev AM, Cederberg B, Berezin MV, Ødegaard F, Rasmussen C, et al. Genes suggest ancestral colour polymorphisms are shared across morphologically cryptic species in arctic bumblebees. PLoS One. 2015; 10(12):e0144544. https://doi.org/10.1371/journal.pone.0144544 PMID: 26657658
19. Schmid-Hempel P. On the evolutionary ecology of host-parasite interactions: addressing the question with regard to bumblebees and their parasites. Naturwissenschaften. 2001; 88(4):147–58. PMID: 11480702
20. Tay WT, O'MAHONY EM, Paxton RJ. Complete rRNA gene sequences reveal that the microsporidium *Nosema bombi* infects diverse bumblebee (*Bombus* spp.) hosts and contains multiple polymorphic sites. Journal of Eukaryotic Microbiology. 2005; 52(6):505–13. https://doi.org/10.1111/j.1550-7408.2005.00057.x PMID: 16313443
21. Fries I, De Ruijter A, Paxton RJ, da Silva AJ, Slemenda SB, Pieniazek NJ. Molecular characterization of *Nosema bombi* (Microsporidia: Nosematidae) and a note on its sites of infection in *Bombus terrestris* (Hymenoptera: Apoidea). Journal of Apicultural research. 2001; 40(3–4):91–6.

22. Larsson JR. Cytological variation and pathogenicity of the bumble bee parasite *Nosema bombi* (Microspora, Nosematidae). Journal of invertebrate pathology. 2007; 94(1):1–11. https://doi.org/10.1016/j.jip.2006.07.006 PMID: 17005191

23. Otti O, Schmid-Hempel P. A field experiment on the effect of *Nosema bombi* in colonies of the bumblebee *Bombus terrestris*. Ecological Entomology. 2008; 33(5):577–82.

24. Rutrecht ST, Brown MJ. Differential virulence in a multiple-host parasite of bumble bees: resolving the paradox of parasite survival? Oikos. 2009; 118(6):941–9.

25. Thorp R, Shepherd M, Vaughan D. Red list of pollinator insects of North America. The Xerces Society for Invertebrate Conservation. 2005.

26. Imhoof B, Schmid-Hempel P. Colony success of the bumble bee, *Bombus terrestris*, in relation to infections by two protozoan parasites, *Crithidia bombi* and *Nosema bombi*. Insectes Sociaux. 1999; 46 (3):233–8.

27. Li J, Chen J, Wang S. Introduction. Risk Management of Supply and Cash Flows in Supply Chains: Springer; 2011. p. 1–48.

28. Williams P.H., Ito M., Matsumura T. & Kudo I. The bumblebees of the Nepal Himalaya (Hymenoptera: Apidae). Insecta Matsumurana. 2010; 66:115–151.

29. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual: Cold spring harbor laboratory press; 1989.

30. Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Molecular marine biology and biotechnology. 1994; 3(5):294–9. PMID: 7881515

31. Evans JD. Beepath: An ordered quantitative-PCR array for exploring honey bee immunity and disease. Journal of Invertebrate Pathology. 2006; 93(2):135–9. https://doi.org/10.1016/j.jip.2006.04.004 PMID: 16737710

32. Li J, Chen W, Wu J, Peng W, An J, Schmid-Hempel P, et al. Diversity of Nosema associated with bumblebees (*Bombus* spp.) from China. International journal for parasitology. 2012; 42(1):49–61. https://doi.org/10.1016/j.ijpara.2011.10.005 PMID: 22138016

33. Klee J, Besana AM, Genersch E, Gisder S, Nanetti A, Tam DQ, et al. Widespread dispersal of the microsporidian *Nosema ceranae*, an emergent pathogen of the western honey bee, *Apis mellifera*. Journal of Invertebrate Pathology. 2007; 96(1):1–10. https://doi.org/10.1016/j.jip.2007.02.014 PMID: 17428493

34. Higes M, Martín R, Meana A. *Nosema ceranae*, a new microsporidian parasite in honeybees in Europe. Journal of Invertebrate Pathology. 2006; 92(2):93–5. https://doi.org/10.1016/j.jip.2006.02.005 PMID: 16574143

35. Larkin MA, Blackshields G Fau—Brown NP, Brown Np Fau—Chenna R, Chenna R Fau—McGettigan PA, McGettigan Pa Fau—McWilliam H, McWilliam H Fau—Valentin F, et al. Clustal W and Clustal X version 2.0. (1367–4811 (Electronic)).

36. Kumar S, Stecher G, Tamura K. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. Molecular biology and evolution. 2016; 33(7):1870–4. https://doi.org/10.1093/molbev/msw054 PMID: 27004904

37. Hines HM, Williams PH. Mimetic colour pattern evolution in the highly polymorphic *Bombus trifasciatus* (Hymenoptera: Apidae) species complex and its comimics. Zoological Journal of the Linnean Society. 2012; 166(4):805–26.

38. Vogt O. Studien über das Artproblem. Über das Variieren der Hummeln. Mitt. 1 u. 2. Sitzgsber Ges naturforsch Freunde Berl. 1909;1911.

39. Duennes MA, Lozier JD, Hines HM, Cameron SA. Geographical patterns of genetic divergence in the widespread Mesoamerican bumble bee *Bombus ephippiatus* (Hymenoptera: Apidae). Molecular Phylogenetics and Evolution. 2012; 64(1):219–31. https://doi.org/10.1016/j.ympev.2012.03.018 PMID: 22521295

40. Williams PH, Brown MJ, Carolan JC, An J, Goulson D, Aytekin AM, et al. Unveiling cryptic species of the bumblebee subgenus *Bombus* s. str. worldwide with COI barcodes (Hymenoptera: Apidae). Systematics and Biodiversity. 2012; 10(1):21–56.

41. Carolan JC, Murray TE, Fitzpatrick Ú, Crossley J, Schmidt H, Cederberg B, et al. Colour patterns do not diagnose species: quantitative evaluation of a DNA barcoded cryptic bumblebee complex. PloS one. 2012; 7(1):e29251. https://doi.org/10.1371/journal.pone.0029251 PMID: 22238595

42. Kissinger CN, Cameron SA, Thorp RW, White B, Solter LF. Survey of bumble bee (Bombus) pathogens and parasites in Illinois and selected areas of northern California and southern Oregon. Journal of invertebrate pathology. 2011; 107(3):220–4. https://doi.org/10.1016/j.jip.2011.04.008 PMID: 21545804

43. Shykoff J, Schmid-Hempel P. Incidence and effects of four parasites in natural populations of bumble bees in Switzerland. Apidologie. 1991; 22(2):117–25.

44. Fries I, Feng F, Silva A, Slemenda SB, Pieniazek NJ. Nosema ceranae (Microspora, Nosematidae), morphological and molecular characterization of a microsporidian parasite of the Asian honeybee Apis cerana (Hymenoptera, Apidae). Eur J Protistol. 1996;32.

45. Chen Y, Evans JD, Smith IB, Pettis JS. *Nosema ceranae* is a long-present and wide-spread microsporidian infection of the European honey bee (*Apis mellifera*) in the United States. Journal of Invertebrate Pathology. 2008; 97(2):186–8. https://doi.org/10.1016/j.jip.2007.07.010 PMID: 17880997

46. Plischuk S, Martín-Hernández R, Prieto L, Lucía M, Botías C, Meana A, et al. South American native bumblebees (Hymenoptera: Apidae) infected by *Nosema ceranae* (Microsporidia), an emerging pathogen of honeybees (*Apis mellifera*). Environmental Microbiology Reports. 2009; 1(2):131–5. https://doi.org/10.1111/j.1758-2229.2009.00018.x PMID: 23765744

47. Arbulo N, Antúnez K, Salvarrey S, Santos E, Branchiccela B, Martín-Hernández R, et al. High prevalence and infection levels of *Nosema ceranae* in bumblebees *Bombus atratus* and *Bombus bellicosus* from Uruguay. Journal of invertebrate pathology. 2015; 130:165–8. https://doi.org/10.1016/j.jip.2015.07.018 PMID: 26248064

48. Graystock P, Yates K, Darvill B, Goulson D, Hughes WO. Emerging dangers: deadly effects of an emergent parasite in a new pollinator host. Journal of invertebrate pathology. 2013; 114(2):114–9. https://doi.org/10.1016/j.jip.2013.06.005 PMID: 23816821

49. Cordes N, Huang W-F, Strange JP, Cameron SA, Griswold TL, Lozier JD, et al. Interspecific geographic distribution and variation of the pathogens *Nosema bombi* and *Crithidia* species in United States bumble bee populations. Journal of invertebrate pathology. 2012; 109(2):209–16. https://doi.org/10.1016/j.jip.2011.11.005 PMID: 22119631

50. Wang LL, Chen KP, Zhang Z, Yao Q, Gao GT, Zhao Y. Phylogenetic analysis of *Nosema antheraeae* (Microsporidia) isolated from Chinese oak silkworm, *Antheraea pernyi*. Journal of Eukaryotic Microbiology. 2006; 53(4):310–3. https://doi.org/10.1111/j.1550-7408.2006.00106.x PMID: 16872300

51. Chen Y, Evans JD, Zhou L, Boncristiani H, Kimura K, Xiao T, et al. Asymmetrical coexistence of *Nosema ceranae* and *Nosema apis* in honey bees. Journal of invertebrate pathology. 2009; 101(3):204–9. https://doi.org/10.1016/j.jip.2009.05.012 PMID: 19467238

52. Slamovits CH, Fast NM, Law JS, Keeling PJ. Genome compaction and stability in microsporidian intracellular parasites. Current Biology. 2004; 14(10):891–6. https://doi.org/10.1016/j.cub.2004.04.041 PMID: 15186746

53. Shafer AB, Williams GR, Shutler D, Rogers RE, Stewart DT. Cophylogeny of Nosema (Microsporidia: Nosematidae) and bees (Hymenoptera: Apidae) suggests both cospeciation and a host-switch. Journal of Parasitology. 2009; 95(1):198–203. https://doi.org/10.1645/GE-1724.1 PMID: 18684016

54. Fürst MA, McMahon DP, Osborne JL, Paxton RJ, Brown MJ. Disease associations between honeybees and bumblebees as a threat to wild pollinators. Nature. 2014 Feb; 506(7488):364. https://doi.org/10.1038/nature12977 PMID: 24553241