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Kittel, Rebecca N.

Maeto, Kaoru

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Revalidation of *Habrobracon brevicornis* (Wesmael) stat. rest. (Hymenoptera: Braconidae) based on the CO1, 16S and 28S gene fragments

Rebecca N. Kittel[1,2], Kaoru Maeto[1]

[1] *Laboratory of Insect Biodiversity and Ecosystems Science, Graduate School of Agricultural Science, Kobe University, Rokkodai 1-1, Nada, Kobe, 657-8501, Japan.*

[2] *Current address: Museum Wiesbaden, Hessisches Landesmuseum für Kunst und Natur, Friedrich-Ebert-Allee 2, 65185 Wiesbaden, Germany.*

Contact: rebecca.n.kittel@gmail.com

Abstract

Habrobracon hebetor (Say, 1836) is an important biological control agent around the world. Many hundreds of papers have been published about its biology. Due to the global distribution of *H. hebetor* many species have been described over the years in various countries as a sister species to *H. hebetor* but were eventually synonymised with *H. hebetor*. One of which is *Habrobracon brevicornis* (Wesmael, 1838). Here, we revalidate the status of

H. brevicornis stat. rest. based on molecular data. It remains difficult to discriminate the two species based on morphology alone, but with molecular data the differentiation is straight forward. An integrative key is provided to distinguish *H. hebetor* from *H. brevicornis.*

Keywords: *Habrobracon*, *hebetor*, *brevicornis*, key, revalidate

Introduction

Habrobracon hebetor Say, 1836 (sometimes also classified as *Bracon* or *Microbracon* (Loni et al., 2016; Tobias, 1986; van Achterberg & Polaszek, 1996)) is a gregarious ectoparasitoid wasp which is used as a biological control agent for various stored grains pests, like the Mediterranean flour moth, *Ephestia kuehniella*, the Indian meal moth, *Plodia interpunctella*, or the greater wax moth, *Galleria mellonella* (Adarkwah et al., 2008; Kares et al., 2010; Khloptseva, 1991; Na et al., 2005; Nam et al., 2011; Schöller, 2000; Schöller & Prozell, 2001; Yoon et al., 2005). The species is also the focus of intensive research of its biology, e.g. the lifespan (40 days), lifetime fecundity (300-500 eggs), or juvenile development (6 days minimum) (Antolin et al., 2003; Aung & Takasu, 2004; Chen et al., 2011; Eliopoulos & Stathas, 2008; Genchev et al., 2008; Howard & Baker, 2003; Magro et al., 2006; Perez Mendoza et al., 2000; Rafiee Dastjerdi et al., 2009; Tian et al., 2006; Weiser et al., 2004; Yu et al., 2003; Zhong et al., 2009). Since the *H. hebetor* occurs all around the globe (naturally or introduced), it has been accidentally described multiple times, but those "new" species were eventually synonymised with *H. hebetor* (Yu et al., 2012). Heimpel et al. (1997) have mentioned, that there is a cryptic species among *H. hebetor*, which is sexually isolated and has different biologic traits. At the same time, *Habrobracon brevicornis* Wesmael 1838 has been suspected to form a valid species and not a synonym of *H. hebetor*, but it has not been verified yet (Chomphukhiao et al., 2018; Quicke, 2015), although some authors have treated it as a separate species (Haider et al., 2004; van Achterberg, 2014; van Achterberg & Polaszek, 1996). *H. hebetor* and *H. brevicornis* have been formally synonymised by Puttarudriaha & Basavannaa (1956), and then this synonymy has been subsequently used by many authors (Papp, 2012; Tobias, 1986). Here, we can show for the first time that *H. hebetor* and *H. brevicornis* are indeed two different, valid species, based on molecular data from specimens around the world. Additionally, we can show that the cryptic species of Heimpel et al. (1997) is in fact *H. brevicornis*.

Material and methods

We aimed for the following strategy: 1) to sample specimens of both *H. hebetor* and *H. brevicornis* from various wild populations within the natural distribution and to sample specimens from labs in which they have been reared, 2) to derive sequences for a few markers from those specimens, 3) to infer monophyletic groups from the sequence data, 4) to recognise the number of valid species based on the sequence data.

Material

We contacted labs in which either of the two species were reared and ask colleagues for samples from wild populations (see table 1, countries stated are the countries the specimens originated from, not necessarily countries where they were reared). Additional the 16S sequence of the cryptic species from Barbardos as well as the *H. hebetor* sequence from Heimpel et al. were included (see table 2 for material used in this study). For outgroups, we used a species within the subfamily Braconinae (*Atanycolus ulmicola* Viereck, 1906) and a species of the family Braconidae (*Cotesia flavipes* Cameron, 1891) (Banks & Whitfield, 2006; Marsh et al., 2009).

The specimens were identified by using an Olympus SZH10 stereo microscope and the key of van Achterberg & Polaszek (1996).

DNA analysis

DNA was extracted from one leg of the adult wasp, of both males and females. Samples were digested in 20 µl 50 mM NaOH at 95 degrees for 15 minutes, afterwards 20 µl of 0.2 TRIS-HCL mix were added. The extracted DNA was then stored at -20 degrees. Selective polymerase chain reaction (PCR) amplifications of target genes were performed (mitochondrial COI, 16S rDNA, and nuclear 28S rDNA gene; see table 3). PCR amplification was carried out in a 25 µl reaction containing 12.5 µl 2x PCR buffer for KOD FX, 5 µl 2mM each DNTP's , 0.5 µl 10uM forward and reverse primer, 1 µl clean water, 0.5 µl KOD fx Neo enzyme (1.0U/ µl), and 5 µl of DNA. Thermocycling conditions were 2 min at 95 degrees for initial denaturation, followed by 35 cycles of a denaturation step (30 sec at 95 degrees), an annealing step (30 sec at 47 degrees), and an extension step (60 sec at 72 degrees), final extension were at 72 degrees for 7 min. PCR products were purified with the illustra GFX PCR DNA and Gel Band Purification Kit, the DNA purity and concentration was checked using a NanoDrop™ spectrophotometer. BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) was added to the sequences and attached in a sequencing step and analysed on an ABI310 capillary sequencer. Resulting sequences were aligned using the Geneious aligment function and subsequently edited using Geneious R.6 (Drummond et al., 2011). The molecular data was then analysed separately by markers and all markers concatenated in bayesian analyses with four independent runs each and four chains and default prior setting using MrBayes 3.2.5 (Ronquist et al., 2012). The scheme of substitution types was not specified (mixed rates) and different parameters of the model were unlinked to allow each partition to have its own set of estimations for parameters. All analyses were performed for 10,000,000 generations sampling every 1000th generation. The results were summarised in a majority rule consensus tree after discarding the first 25% of the trees (burn-in). The sequences were deposited in the GenBank database under the accession numbers MH766507–MH766614 (see table 2).

Results

Descriptive statistics

We obtained 46 sequences of the two species (*H. hebetor* (25 sequences) and *H. brevicornis* (21 sequences)). In total 24 wild and 22 reared individuals were used *H. hebetor* (6 wild and 19 reared individuals) and *H. brevicornis* (18 wild and 3 reared individuals). We obtained 524 bp of CO1 sequences from 45 individuals (*H. hebetor* (24 sequences) and *H. brevicornis* (21 sequences)), 496 bp of 16S rDNA sequences from 30 individuals (*H. hebetor* (18 sequences) and *H. brevicornis* (12 sequences), and 674 bp of 28S rDNA gene sequences from 34 individuals (*H. hebetor* (18 sequences) and *H. brevicornis* (16 sequences)), resulting in 23 individuals (*H. hebetor* (13 sequences) and *H. brevicornis* (10 sequences)) of which we have obtained all three markers.

DNA analysis

The 524 bp long CO1 gene sequence has 26 unique bp, the 496 bp long 16S gene sequence only nine unique bp and the 674 bp long 28S gene sequence has only a single unique bp to discriminate between *H. hebetor* and *H. brevicornis*. There were no differences in the sequences among the wild and the reared individuals. Bayesian inference analysis was performed to obtain the relationship of *H. hebetor* and *H. brevicornis*. The phylogenetic trees based on the CO1, 16S gene sequences, and a concatenated approach showed the same result: two distinct clades (figure 1) with 100% posterior probabilities. Only the phylogenetic tree based on 28S gene sequences does not show a clear separation of the two clades. One clade contains material from both reared and wild populations, from Germany, Spain, USA, Japan, Egypt, Korea, and Barbados. This clade comprises of individuals which were identified as *H. hebetor*. The other clade also consists of material from wild populations from Thailand, reared material from Uzbekistan, and the cryptic species from Barbados of Heimpel et al. This clade comprises of individuals which were hitherto also partially identified as *H. hebetor*, but actually comprise of individuals belonging to *H. brevicornis*.

Taxonomy

Family Braconidae

Subfamily Braconincae

Genus *Habrobracon*

Here, we treat *Habrobracon* as a separate genus to *Bracon* (and not as a subgenus, as done by some authors, e.g. van Achterberg (2014)). For a complete list of classification see Yu et al. (2012).

Habrobracon brevicornis (Wesmael, 1838) stat. rest.

Type: female, lectotype, Royal Belgian Institute of Natural Sciences, Bruxelles, Belgium.

Type material examined.

For diagnosis and redescription, see van Achterberg & Polaszek (1996).

Key to H. hebetor and H. brevicornis (modified after van Achterberg & Polaszek (1996) and Haider et al. (2004))

Molecular data (position 27: T, 39: C, 48: T, 60: G, 81: T, 168: C, 180: T, 191: A, 192: C, 196: G, 201: A, 222: T, 223: T, 225: A, 238: A, 246: T, 271: T, 348: T, 358: T, 369: C, 387: A or G, 411: C, 423: C, 450: G, 475: C, 492: T; antenna shorter than length of head and mesosoma combined or of equal length; seta of vertex erect, reaching above upper level of posterior ocellus; first flagellar segment of male antenna distinctly longer than the second, the segments beyond the first very little longer than the broad; metasoma smooth and shining, rarely punctate ... *H. hebetor*

Molecular data (position 27: C, 39: T, 48: C, 60: A, 81: C, 168: T, 180: A, 191: G, 192: T, 196: A, 201: T, 222: C, 223: C, 225: T, 238: G, 246: A, 271: A, 348: C, 358: C, 369: T, 387: T, 411: T, 423: T, 450: A, 475: T, 492 A or G; antenna slightly longer than length of head and mesosoma combined; setae of vertex more adpressed, mostly not reaching above upper level of posterior ocellus; first flagellar segment of male antenna not distinctly longer than the second, the segments beyond the first one-half times as long as broad; 3-5th metasomal tergites disctinctly punctate ... *H. brevicornis*

Discussion

Taxonomy

In 1956, the two species *Habrobracon hebetor* and *Habrobracon brevicornis* have been formally synonymised by Puttarudriaha & Basavannaa. Since then, the majority of authors have accepted the synonymisation and studied e.g. the biology of *H. hebetor* (Chen et al., 2011; Eliopoulos & Stathas, 2008; Loni et al., 2016; Nam et al., 2011; Schöller & Prozell, 2001). However, it has been discussed that *H. brevicornis* may not be a junior synonym of *H. hebetor*, but rather should be a valid species (Quicke, 2015). Only very few authors have treated *H. brevicornis* as such (Gabra et al., 2016; van Achterberg, 2014).

Morphology

Over the years many species were synonymised with *H. hebetor*, but only one species has been discussed to form a valid species: *H. brevicornis. H. brevicornis* is not dissimilar to *H. hebetor*, but the morphological differences are only so slight. Van Achterberg mentions in his key to the two species (van Achterberg & Polaszek, 1996) the number of antennal segment first and states that the females of *H. hebetor* have 13-14 segments and the males 20-23, whereas the females of *H. brevicornis* have 15-18 segments and the males 22-27. Therefore, one can - according to this particular key - clearly distinguish between the two species by the number of antennal segments of the females. Another study (Haider et al., 2004) states that the females of *H. hebetor* have 13-15 segments and males 18-23, whereas the females of *H. brevicornis* have 17-19 segments and the males 20-27. Based on this key, one can clearly distinguish the two species easily. The numbers of antennal segments do differ amongst the key from van Achterberg & Polaszek and Haider et al., but there is still no overlap and one can easily discriminate the two species based on the number of antennal segments of the females in those two keys. However, our observations have shown a variation of the amount of antennal segments, which makes it difficult to separate the two species based on this character. The females of *H. hebetor* in our samples had 13-15 antennal segments and the males 17-23. The females of *H. brevicornis* had 14-16 antennal segments and the males 19-23. Additionally, our study showed that the variety of antennal segments is greater than anticipated. Not only do they vary amongst the adults but even within one individual. The females and males of *H. brevicornis* had about the same percentage of individuals with the same number of antennal segments (55% and 48%, respectively). Females of *H. hebetor* are much more likely to have the same number of antenna segments per each individual (91%), but males are less likely (39%). In all cases, if an individual had two different numbers of antennal segments (left antenna vs right antenna), then the difference was never greater than 1. The number of antennal segments is therefore only then a useful character to distinguish the two species, if they are on the lower end of the range (13 antennal segments of females of *H. hebetor*).

Some of the other morphological characters stated in the keys of van Achterberg & Polaszek and Haider et al. are only somewhat useful, e.g. there is an overlap of the ratio of the vein 3-SR to the vein r in the fore wing, with 0.9-1.2 (-1.4) in *H. hebetor* and 1.2-1.8 in *H. brevicornis*. Interestingly, this is exactly the distinguishing character to separate *Habrobracon* and *Bracon* according to Quicke (1987), with fore wing vein 3-SR less than 1.5 times longer than vein r, but usually 1.2 times as long as r (*Habrobracon*) and fore wing vein 3-SR more than 1.6 times longer than vein r, usually more than 1.9 times longer (*Bracon*). In this case, *H. hebetor* would belong to the genus *Habrobracon* and all individuals of the sister species *H. brevicornis* with a ratio 3-SR to r larger than 1.6 would belong to *Bracon*.

The best characters to discriminate the two species are therefore the ones given in the key: length of antenna to length of head and mesosoma; orientation and length of seta of vertex; length of first flagellar segment of male antenna to length of second flagellar segment; suface structure of metasoma.

Biology and host ranges

Chomphukhiao et al. (2018) tested for reproductive success and it showed that the two populations (Japan and Thailand) or in other words the two species *H. hebetor* and *H. brevicornis* did not reproduce or only produced males. They confirmed the results of Heimpel et al., that the cryptic species aka *H. brevicornis* is sexually isolated from *H. hebetor*.

Both species have a cosmopolitan distribution and both are polyphagous ectoparasites, attacking Crambidae and Pyralidae in stored products and also other lepidopterous families like Noctuidae, Tortricidae, or Gelechiidae in the wild (see below). Although *H. hebetor* and *H. brevicornis* do perform differently as biological control agents depending on the host. For example, according to Chomphukhiao et al. (2018) both species *H. hebetor* and *H. brevicornis* attack *Corcyra cephalonica* and *P. interpunctella* (Pyralidae), however, *H. brevicornis* seemed to lay more eggs with a higher adult emergence rate. This confirms the observation of Heimpel et al. that that the cryptic species aka *H. brevicornis* produces more eggs.

Given the differences in their biology and the importance as biological control agents, it is crucial to test whether the observations in a (past) study has been conducted with *H. hebetor* or with *H. brevicornis*.

H. hebetor has a large range of hosts (for a complete list, see Yu et al., 2012), but is mainly used against Crambidae: *Glyphodes pyloalis*, *Nacoleia octasema;* Noctuidae: *Adisura atkinsoni, Agrotis segetum, Heliocheilus albipunctella*, *Helicoverpa armigera*; Pyralidae: *Cadra calidella, Cadra cautella, Corcyra cephalonica, Ephestia elutella, Ephestia kuehniella, Homoeosoma nebulella, Plodia interpunctella* (Bartlett et al., 1978; Bhatnagar, 1987; Divakar & Pawar, 1983; Gupta & Sharma, 2004; Horvath, 1993; Khloptseva, 1991; Klapal, 2002; Mirzaeva et al., 2007; Patel et al., 1982; Press et al., 1974; Schöller, 2000; Takahashi, 1973; Usman, 1960). *Habrobracon brevicornis* is also used as a biological control agent and attacks in the lab mostly the same host species. In the wild, it has been found on hosts different to the hosts of *H. hebetor*. Autostichidae: *Nephantis serinopa*; Crambidae: *Diatraea saccharalis*, *Ostrinia nubilalis*; Cryptophasidae: *Opisina arenosella;* Gelechiidae: *Pectinophora gossypiella*; Noctuidae: *Adisura atkinsoni*, *Sesamia cretica* (Criddle, 1923; Dharmarju, 1952; Ingram & Bynum, 1941; Kares et al., 2010; Krishnamurti & Appana, 1944; Noble & Hunt, 1937; Rukhsana & Sebastian, 2015). It appears that *H. hebetor* attacks more pest species, but *H. brevicornis* performs slightly better on their preferred hosts, especially on the Coconut Black Headed Caterpillar, *Opisina arenosella*, which is not attacked by *H. hebetor*.

DNA analysis

Of the three markers, the 28S gene sequences did not show two distinct clades. This is probably due to the fact that both CO1 and 16S evolve at a higher rate than 28S (Klopfstein et al., 2010).

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Table 1. Abbreviations of depositories/labs from which material has been used.

Abbreviation	Institution
BB	Biologische Beratung, Germany
KNU	Kyungpook National University, Korea
RAS	Siberian Branch of Russian Academy of Science, Russia
IRTA	Institute of Agrifood Research and Technology, Spain
UM	University of Minnesota, USA
CSU	Colorado State University, USA
KU	Kasetsart University, Thailand
UB	University of Bremen, Germany

Table 2. Material sequenced and used in this study.

Species	Individual code	Country	wild/ reared	Genbank CO1	Genbank 28S	Genbank 16S
H. brevicornis	RK324	Germany BK15	wild (BU)	MH766507	MH766551	MH766585
H. brevicornis	RK325	Germany BK15	wild (BU)	MH766508	MH766552	
H. brevicornis	RK327	Germany BK15	wild (BU)		MH766553	MH766586
H. brevicornis	RK328	Germany BK15	wild (BU)	MH766509		MH766587
H. brevicornis	RK329	Germany L06	wild (BU)	MH766510	MH766554	
H. brevicornis	RK330	Germany L06	wild (BU)	MH766511	MH766555	MH766588
H. brevicornis	RK331	Germany L06	wild (BU)	MH766512	MH766556	
H. brevicornis	RK332	Germany L06	wild (BU)	MH766513	MH766557	MH766589
H. brevicornis	RK333	Germany L06	wild (BU)	MH766514	MH766558	MH766590
H. brevicornis	RK115	Uzbekistan	reared (RAS)	MH766515	MH766559	MH766591
H. brevicornis	RK117	Uzbekistan	reared (RAS)	MH766516	MH766560	
H. brevicornis	RK59	Uzbekistan	reared (RAS)	MH766517	MH766561	MH766592
H. brevicornis	RK295	Spain	wild (IRTA)	MH766518	MH766562	
H. brevicornis	RK123	Thailand TCC	wild (KU)	MH766519	MH766563	MH766593
H. brevicornis	RK124	Thailand TCC	wild (KU)	MH766520	MH766564	MH766594
H. brevicornis	RK125	Thailand TCC	wild (KU)	MH766521		
H. brevicornis	RK126	Thailand TCC	wild (KU)	MH766522		
H. brevicornis	RK127	Thailand TCC	wild (KU)	MH766523	MH766565	MH766595
H. brevicornis	RK133	Thailand TOA	wild (KU)	MH766524	MH766566	MH766596
H. brevicornis	RK134	Thailand TOA	wild (KU)	MH766525		

Species	Individual code	Country	wild/ reared	Genbank CO1	Genbank 28S	Genbank 16S
H. brevicornis	RK135	Thailand TOA	wild (KU)	MH766526		
H. hebetor	RK100	Egypt	Reared (BB)	MH766527	MH766567	MH766597
H. hebetor	RK102	Egypt	reared (BB)	MH766528	MH766568	MH766598
H. hebetor	RK25	Egypt	Reared (BB)	MH766529	MH766569	MH766599
H. hebetor	RK334	Germany AMW	wild (BU)	MH766530	MH766570	MH766600
H. hebetor	RK335	Germany AMW	wild (BU)	MH766531		MH766601
H. hebetor	RK336	Germany AMW	wild (BU)	MH766532	MH766571	MH766602
H. hebetor	RK337	Germany AMW	wild (BU)	MH766533	MH766572	MH766603
H. hebetor	RK338	Germany AMW	wild (BU)	MH766534	MH766573	MH766604
H. hebetor	RK27	Germany	reared (BB)			MH766605
H. hebetor	RK96	Germany	reared (BB)	MH766535	MH766574	
H. hebetor	RK118	Japan	Reared (KU)	MH766536	MH766575	
H. hebetor	RK119	Japan	Reared (KU)	MH766537	MH766576	MH766606
H. hebetor	RK120	Japan	reared (KU)	MH766538	MH766577	MH766607
H. hebetor	RK121	Japan	reared (KU)	MH766539		MH766608
H. hebetor	RK02	Korea	reared (KNU)	MH766540	MH766578	
H. hebetor	RK103	Spain	wild (IRTA)	MH766541	MH766579	

H. hebetor	RK26	Spain	wild (IRTA)	MH766542	MH766580	MH766609
Species	**Individual code**	**Country**	**wild/ reared**	**Genbank CO1**	**Genbank 28S**	**Genbank 16S**
H. hebetor	RK298	USA	reared (UM)	MH766543	MH766581	MH766610
H. hebetor	RK299	USA	reared (CSU)	MH766544		
H. hebetor	RK300	USA	reared (CSU)	MH766545		MH766611
H. hebetor	RK301	USA	reared (CSU)	MH766546	MH766582	MH766612
H. hebetor	RK128	USA WE	reared (UM)	MH766547		
H. hebetor	RK129	USA WE	reared (UM)	MH766548	MH766583	MH766613
H. hebetor	RK131	USA WE	reared (UM)	MH766549		
H. hebetor	RK132	USA WE	reared (UM)	MH766550	MH766584	MH766614
H. hebetor	Heimpel et al.	USA	reared			
H. hebetor "cryptic"	Heimpel et al.	Barbados	wild			
Outgroups						
Cotesia flavipes				DQ538818	DQ538530	DQ538977
Atanycolus ulmicola				GU173833		GU135650

Sequences from Heimpel, *Cotesia flavipes* and *Atanycolus ulmicola* were obtained from the respective publication or obtained off Genbank (Banks & Whitfield, 2006; Heimpel et al., 1997; Marsh et al., 2009).

Table 3. Table with markers and primers

Region	Name	Sequence
co1 - F	CI-J-1718	GGAGGATTTGGAAATTGATTAGTTCC
28S - F	28S D2F	AGAGAGAGTTCAAGAGTACGTG
16S - F	Wb	CACCTGTTTATCAAAAACAT
co1 - R	1 CI-N-2191	CCCGGTAAAATTAAAATATAAACTTC
28S - R	28S D3R	TAGTTCACCATCTTTCGGGTCCC
16S - R	SH	AGATTTTAAAAGTCGAACAG

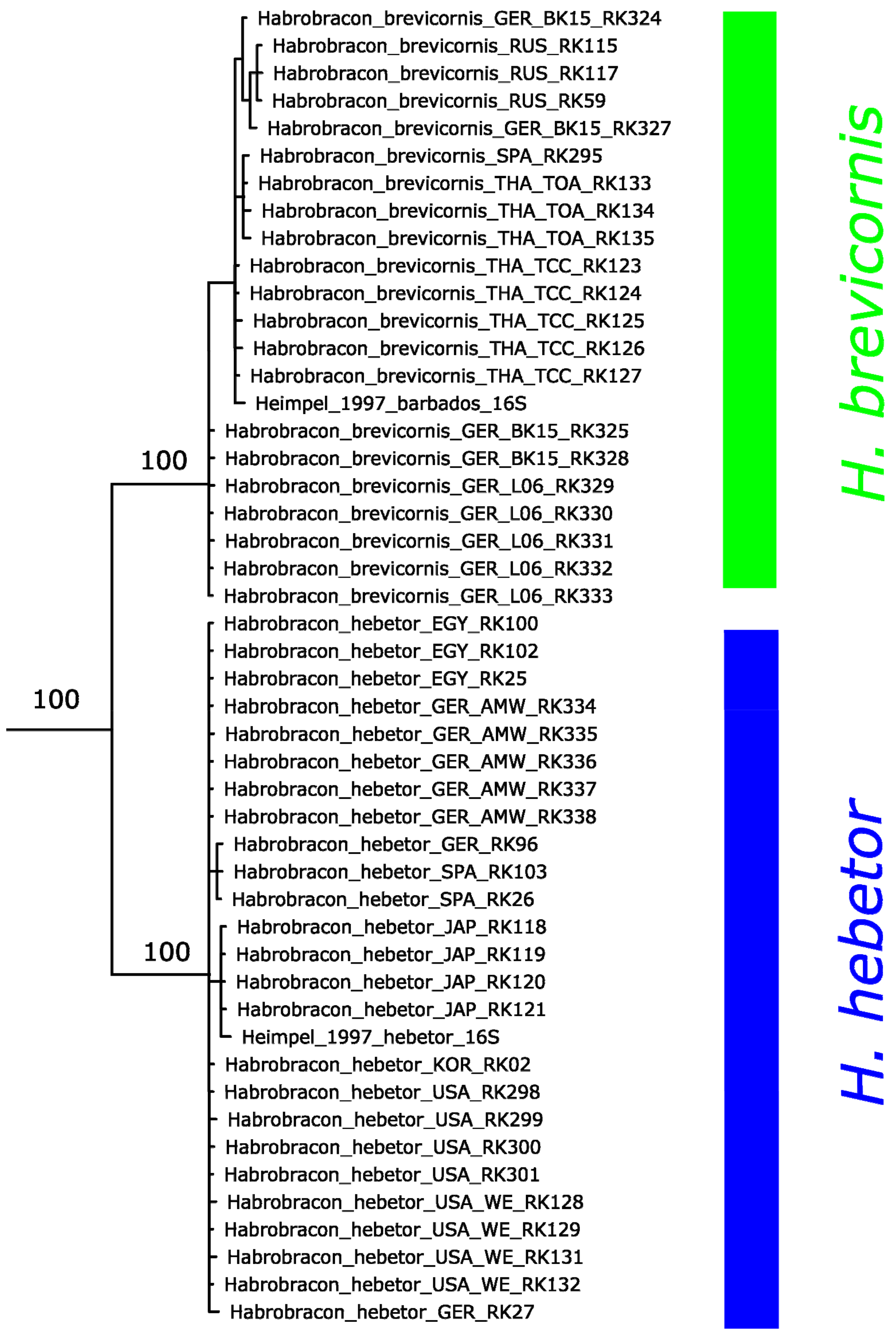

Figure 1 Bayesian consensus tree based on the concatenated molecular data (CO1, 16S, and 28S gene fragments). Terminal Bayesian posterior probabilities not shown. Posterior probabilities are the same for the markers CO1 and 16S, but not 28S.