Can parasites halt the invader? Mermithid nematodes parasitizing the yellow-legged Asian hornet in France

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Since its introduction in France 10 years ago, the Yellow-legged Asian bee-hawking hornet *Vespa velutina* has rapidly spread to neighboring countries (Spain, Portugal, Belgium, Italy, and Germany), becoming a new threat to beekeeping activities. While introduced species often leave behind natural enemies from their original home, which benefits them in their new environment, they can also suffer local recruitment of natural enemies. Three mermithid postparasitic juveniles were obtained from *V. velutina* adults in 2012, from two French localities. However, these are the only parasitic nematodes reported up to now in Europe, in spite of the huge numbers of nests destroyed each year and the recent examination of 33,000 adult hornets. This suggests that the infection of *V. velutina* by these nematodes is exceptional. Morphological criteria assigned the specimens to the genus *Pheromermis* and molecular data (18S sequences) to the Mermithidae, due to the lack of *Pheromermis* spp. sequences in GenBank. The species is probably *Pheromermis vesparum,* a parasite of social wasps in Europe. This nematode is the second native enemy of *Vespa velutina* recorded in France, after a conopid fly whose larvae develop as internal parasitoids of adult wasps and bumblebees. In this paper, we provide arguments for the local origin of the nematode parasite and its limited impact on hornet colony survival. We also clarify why these parasites (mermithids and conopids) most likely could not hamper the hornet invasion nor be used in biological control programs against this invasive species.

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Introduction

The recent introduction of the Yellow-legged Asian hornet *Vespa velutina* in France was the first successful invasion of an exotic Vespidae in Europe (Rasplus et al., 2010; Beggs et al., 2011). This species is of great concern among public authorities and beekeepers because of its rapid multiplication and high impact on beekeeping due to its predatory action on honeybees (Perrard et al., 2009) and its hawking behavior that disrupts bee colony foraging (Rortais et al., 2010; Monceau et al., 2013; Arca et al., 2014). This invasive hornet was first observed in 2004 in Southwest France (Villemant et al., 2011); since then it has spread out across 67 French departments (ca. 340 000 km²) (Rome et al., 2013; INPN, 2015). In addition, it spread to Spain in 2010, to Portugal and Belgium in 2011 (Rome et al., 2013), to Italy in 2012 (Demichelis et al., 2014), and arrived in Germany in 2014 (R. Witt pers. comm., 2014) (Fig. 1). It is expected to eventually spread throughout Europe (Villemant et al., 2011) and with recent climate change scenarios, future movements are even more pessimistic (Barbet-Massin et al., 2013).

Multiple biotic factors, including resources, competition, and natural enemies can affect the demographics of an invader, either independently or interactively and thus play a role in its establishment. Introduced species often leave behind natural enemies from their original home, thus benefiting them in the new environment and resulting in an increase of growth, reproduction and competitive ability (Holway, Suarez & Case, 1998; Colautti et al., 2004; Torchin & Mitchell, 2004; Lee & Klasing, 2004; Roy et al., 2011). Local recruitment of natural enemies in their new home can also affect the development of the invasion and lessen their effect on local hosts (Prenter et al., 2004; Girardoz, Kenis & Quicke, 2006; Dunn, 2009; Kenis et al., 2009; Péré et al., 2011).

The first native enemy of *Vespa velutina* reported in Europe was a thick-headed fly (family Conopidae) whose larvae develop as internal parasitoids of adult wasps and bumblebees (Darrouzet, Gévar & Dupont, 2015). We report here a new parasite, a mermithid nematode of the genus *Pheromermis* that was obtained from *V. velutina* adults in 2012 in two different French localities. As far as we know, no other nematode parasites of *V. velutina* have been reported up to now in Europe.

In this paper, we discuss how these parasites could potentially hamper the hornet invasion and whether they could be used in biological control programs against this invasive species.

Materials and methods

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Origin of specimens

The invasive progress of the alien hornet has been monitored since 2006 through an online biodiversity database maintained by the Muséum National d'Histoire Naturelle (MNHN) and regularly updated by one of us, Q.R. (Rome et al., 2013; INPN, 2015). This monitoring showed that more than 7000 nests were discovered from 2006 to 2014. Nests are mainly observed in autumn after leaf fall, when the colonies reach maturity and contain several hundred to two thousand adult hornets (Rome et al., 2015). This surveillance network provides useful information but the hornets were not regularly surveyed for parasites. However, in order to study seasonal changes in *V. velutina* colony structure, we dissected 77 nests between 2007 and 2011 (Rome et al., 2015); we sorted and weighed some 33,000 adult hornets without discovering any individual infested by nematodes. The only parasitized adult we obtained contained a conopid pupa that has not been identified (Villemant et al., 2008).

Nematode parasites were unexpectedly noticed in hornets by local observers on two occasions. In November 2012, one mermithid was obtained from ten adult hornets dissected from a nest collected at Dompierre-sur-Besbre, Allier (Ph. Noireterre pers. comm., 2012). In January 2013, two mermithids were obtained from dead adults in an advanced state of decomposition, from a nest at Issigeac, Dordogne (P. Doumanjou-Laroque pers. comm., 2013). The mermithids were sent to the MNHN for identification. Specimens are deposited in the MNHN collection as MNHN JL50 (Dompierre-sur-Besbre) and MNHN JL51A and JL51B (Issigeac).

Morphology

The mermithid nematode from Dompierre-sur-Besbre (Allier) was photographed (Fig. 2), then part of its body was sampled for the molecular study, and the remainder was submitted to one of us (G.O.P.) for further identification. Mermithid nematodes, including the present species, are quite large at maturity and often exceed the length of their host. The specimen from the Asian hornet was a postparasitic juvenile and had morphological characters that aligned it with species of the genus *Pheromermis* Poinar, Lane and Thomas, 1976.

Molecular identification

Total genomic DNA was extracted from a 5 mm long medial segment sampled from each specimen, using the Qiagen DNA Mini Kit and following the manufacturer's protocol. Three candidate genes

90   were selected for PCR amplification: the mitochondrial cytochrome oxidase I (COI) and the nuclear

91   large and small subunit rRNA genes (28S-rRNA and 18S-rRNA). The choice was dictated by the large

92   number of nematode sequences already available in GenBank for comparison.

93   The genes were amplified using standard primers and amplification profiles (Table 1). The

94   PCRs were conducted in 20 µl reaction volume, containing 1-5 ng of DNA and to a final concentration

95   of 1X reaction buffer, 2.5 mM MgCl2, 0.26 mM dNTP, 0.3 µM of each primer, 5% DMSO and 1.5

96   units of Qiagen Taq polymerase. For all primer pair combinations, the amplification profile was: 5 min

97   initial denaturation at 94 °C, 40 cycles of 40 s at 94 °C, 40 s at primer annealing temperature (see Table

98   1) and 60 s at 72 °C, followed by a final extension of 5 min at 72 °C. PCR products were visualized on

99   a 1.5% agarose gel stained with ethidium bromide and the positive PCRs were sequenced in both

100 directions using the Sanger method.

101   A preliminary BLAST search suggested that the 18S and 28S sequences obtained from the

102 samples were rather similar to those of Mermithidae present in GenBank. However, only two out of the

103 twelve 28S GenBank sequences overlapped significantly with our sequences, so further comparisons

104 were restricted to the 18S gene. All 18S sequences of Mermithidae available in GenBank were then

105 downloaded and aligned with the sequences we obtained, resulting in a 1322 bp alignment. The 18S

106 GenBank sequences proved to be rather variable in length, some of them with minimal overlap with the

107 region amplified using our primers. The dataset was then reduced, retaining only the sequences longer

108 that 400 bp.

109   All new sequences have been submitted to GenBank under the accession numbers xxx-xxx.

110   The final matrix, including 26 ingroup sequences and five outgroups (Aulolaimidae:

111 *Aulolaimus* and Isolaimiidae: *Isolaimium*) was analyzed under the maximum likelihood criteria, using

112 RAxML v. 7.0.3 (Stamatakis, 2006), selecting a GTR+Γ+I model and random starting tree, with

113 empirical base frequencies and estimated α-shape parameters and GTR-rates. Nodal support was

114 estimated using 100 bootstrap replicates.

115

116 **Results**

117

118 *Morphology*

119 Only the specimen from Dompierre-sur-Besbre revealed morphological features (Fig. 2); the other two

120 specimens from Issigeac were too damaged by putrefaction. The morphological features of the single

121   juvenile examined were consistent with those of juveniles of the European wasp mermithid,

122   *Pheromermis vesparum* Kaiser, 1987. This species specializes on social Hymenoptera and has been

123   reported from *Vespa* spp. and *Polistes* sp.

124   This genus is characterized by the presence of four submedian cephalic papillae; large

125   anteriorly placed cup-shaped amphids; an S-shaped vagina not bent in a transverse plane to the body;

126   six hypodermal cords; paired, short, separate spicules; cuticle with cross fibers; and eggs lacking

127   processes (Poinar, Lane & Thomas, 1976). Because most of these are characters of the adults and the

128   specimens from the Asian hornet were postparasitic juveniles, it is possible that more than one species

129   of *Pheromermis* was involved.

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131   *Molecular identification*

132   Of the three specimens analyzed, one from Issigeac was probably too decomposed and the resulting

133   DNA likely too degraded because all amplification attempts failed. Instead we recovered sequences for

134   the 18S and 28S genes from the other two specimens i.e. from two localities. The 18S sequences were

135   identical, while the 28S differed only by 1.6%, with all variable positions concentrated in the loop

136   regions. Despite several attempts, the amplification of the COI gene also failed, suggesting that the

137   standard primers are ineffective for amplifying the COI gene in this species.

138   The tree obtained from the maximum likelihood analysis recovers the two 18S sequences well

139   nested within the family Mermithidae, and sisters to three specimens identified as *Mermis nigrescens*

140   (Fig. 3), a parasite of grasshoppers (Baker & Capinera, 1997). These results are congruent with the

141   morphological identification, although no molecular data of *Pheromermis* are currently available for

142   comparison and thus, we cannot identify the specimens at the species level. We have to consider them

143   as belonging to *Pheromermis* sp. However, larvae collected from *V. velutina* most probably belong to

144   *Pheromermis vesparum* Kaiser, 1987, a well-known parasite of social wasps. It has been recorded from

145   *Vespa crabro, Vespula vulgaris, V. germanica, Dolichovespula saxonica* and *Polistes* sp. (Kaiser,

146   1987).

147

148   **Discussion**

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150   In a ten year span, only 3 nematodes have been collected from hornets in two distant localities in

151   France. Nevertheless, numerous and very populous colonies of *V. velutina* are destroyed and dropped

152 from tree crowns each year in France. In spite of these huge numbers of destroyed nests, the rarity of

153 records suggests that the infection of the hornet by these nematodes is exceptional.

154   The development of the *Pheromermis* species is unique among the Mermithidae because a

155 second host (paratenic or transport host) is required for life cycle completion (Poinar, Lane & Thomas,

156 1976; Kaiser, 1987; Martin, 2004). The adult nematodes occur in water or saturated soils and the eggs

157 are fully embryonated at oviposition. The eggs hatch in the gut of various aquatic or semi-aquatic

158 insects and infective juvenile stages penetrate the gut wall to enter a quiescent state in the tissues of the

159 paratenic hosts, even during host metamorphosis to adult form. Wasp larvae are probably infected

160 when they are fed with adult paratenic hosts captured by worker wasps. The nematode larvae become

161 active and start feeding on non-vital tissues of the developing wasp. This coincides with the period

162 when social wasps are raising their sexual brood (Kaiser, 1987). As nematodes rarely kill their juvenile

163 wasp hosts, the adult wasp emerges and the nematode matures in the abdomen of the wasp, rendering

164 sexual individuals sterile or inactive. When the infected wasp visits water, the mature worm leaves its

165 host, molts into the adult stage, mates and lay eggs, so completing the life cycle. It is not yet known

166 whether the reproductive wasps normally visit wet areas in the fall before the queens enter hibernation

167 or whether the mermithids cause hosts to seek water (Poinar, 1976).

168   Two hypotheses can be made to explain the presence of the nematode parasites in *V. velutina*

169 adults. The parasite (1) may have been introduced in the new range by the invader itself, or (2) may

170 have been acquired from the local fauna. The first hypothesis is unlikely because hornet queens

171 parasitized at the time of their introduction would have died without descendants. Moreover, genetic

172 data have shown that only very few hornet queens (or even a single one) have been introduced in

173 France (Arca et al., 2015), therefore making the hypothesis of an introduced parasite even less possible.

174 In addition, the exotic parasite would have had to adapt to one (or several) local insect species whose

175 larvae are aquatic. The second hypothesis seems to be more likely. The invasive species was infested

176 by an autochthonous nematode whose paratenic hosts are various aquatic insects and main hosts are

177 autochthonous social wasps. *Pheromermis* species that attack social wasps in Europe have a wide range

178 of hosts (Molloy, Vinikour & Anderson, 1999) and thus are more likely to infect a new host than more

179 specific nematodes.

180   Known paratenic hosts of *Pheromermis* spp. attacking wasps include notably larvae of

181 caddisflies (Trichoptera), stoneflies (Plecoptera), craneflies (Tipulidae) and mayflies (Ephemeroptera),

182 as well as various Coleoptera larvae (Poinar, Lane & Thomas, 1976; Poinar, 1981; Molloy, Vinikour &

183   Anderson, 1999). In the course of an ongoing study, we collected more than 2000 prey flesh pellets at

184   the time they were brought back to the nest by worker wasps. Their identification showed that

185   caddisflies are part of the *V. velutina* prey spectrum but only in tiny proportions (0.2%) compared to

186   other insect preys (unpublished data from the authors).

187   Local recruitment of natural enemies like mermithid nematodes obviously leads to the

188   following question: are they able to control hornet populations? The fact that *Pheromermis* spp. kill

189   their hosts makes these nematodes important as biological control agents of social wasps. However,

190   Martin (2004) noted that contrary to the 50% ratio quoted by Poinar, Lane & Thomas (1976) and

191   Moller et al. (1991), levels of infection vary from 0-7% in workers and males, and 8-35% in social

192   wasp queens (Blackith & Stevenson, 1958; Kaiser, 1987). In 1893, all males in one large *Vespula* wasp

193   nest were found infected (Fox-Wilson, 1946) by *Gordius* worms (*Gordius* belong to the

194   Nematomorpha, a phylum distinct from the Nematodes to which mermithid belong, but with a similar

195   life cycle). However, the dissections of thousands of adults from hundreds of *Vespula* and *Vespa* nests

196   by various wasp researchers indicate that such extreme levels of infection are very rare (Martin, 2004).

197   Our extensive survey (Rome et al., 2015) led to the same conclusion.

198   The degree of infection in any nest also depends on the proximity of the wasp nest to an

199   abundant source of the nematodes' paratenic hosts (Rose, Harris & Glare, 1999; Martin, 2004). Kaiser

200   (1987) found that 1/3 of *Vespula* nests were infected with *Pheromermis* when, and only when, they

201   were within 200 m of water. Also, the rarity of potential paratenic hosts in *V. velutina*'s prey spectrum

202   does not enable the nematode to greatly infest a colony, even if hornet workers generally fed several

203   larvae with every flesh pellet brought back to the nest (Janet, 1903; Spradbery, 1973). Moreover, a

204   high mortality of nestmates is not sufficient to ensure the total destruction of a colony, and even with

205   75% mortality, recovery is possible (Gambino, Pierluisi & Poinar, 1992; Toft & Harris, 2004; Gouge,

206   2005). The founder queen also cannot be infected since female sexual adults cannot over-winter. The

207   maturing nematode, which eventually occupies all the gaster when the sexual stages emerge, severely

208   interferes with the amount of fat deposited (Martin, 2004). Finally, unlike many entomopathogenic

209   nematodes, *Pheromermis* spp. do not seem to serve as vectors for symbiotic insect-pathogenic bacteria

210   (Poinar, 1979), whose presence may increase the virulence of the infection (Lacey et al., 2001).

211   The possible use of *Pheromermis* spp. as a biological control agent against social wasps was

212   tested with a simulation model (Martin, 2004) which predicted that the production of sexual individuals

213   would be reduced in colonies undergoing early and high levels of infection. However, even highly

214 infected (80%) colonies can still produce some reproductive offspring, indicating that they are resilient

215 to infection. Moreover, an increase of the infestation level raises the larva/worker ratio so that less fed

216 larvae produce sexual females of lesser quality, with fewer over-wintering and nest founding successes

217 (Harris & Beggs, 1995). The low quality of some sexual females may inadvertently permit a greater

218 founding success via density-dependent compensation: the healthy females are less numerous but they

219 experience lower mortality due to weaker competition for fat storage (Harris & Beggs, 1995) and

220 reduced nest usurpation in the spring (Martin, 1991, 2004; Archer, 2012).

221 The reduction of usurpation disputes which lead to the death of a high number of founder

222 queens (Spradbery, 1991; Archer, 2012) also explains why conopid flies would not be efficient control

223 agents even if they were able to directly attack adult queens. Thus, the local recruitment of *Conops*

224 *vesicularis* as a parasitoid of *Vespa velutina* in France (Darrouzet, Gévar & Dupont, 2015) would not

225 make it a potential control agent of the invasive hornet. Moreover, conopids mainly fly in summer

226 from June to September (Schmid-Hempel et al., 1990) and are thus more likely to attack foraging

227 workers than mother queens, which do not leave the nest after their first workers emerge in June

228 (Matsuura & Yamane, 1990; Rome et al., 2015).

229 Many parasites of social wasps, such as the ichneumonids *Sphecophaga* spp. (Donovan et al.,

230 2002; Beggs et al., 2008), the stylops *Xenos* spp. (Matsuura & Yamane, 1990), or the conopid flies like

231 *C. vesicularis*, attack only single individuals within the colony. By contrast, other parasites such as

232 *Varroa destructor* mites can kill a bee colony of more than 30,000 individuals by transmitting viral

233 pathogens as they move between bees within a colony (Sumpter & Martin, 2004). Infection levels of

234 parasites which attack only single individuals need to be very high (>50%) to kill or significantly

235 reduce the productivity of social wasp colonies (Matsuura & Yamane, 1990; Barlow, Beggs & Barron,

236 2002), because their populations have high reproductive efficiency and undergo density-dependent

237 compensation in spring (Martin, 1991, 2004).

238 On the other hand, the introduction of an alternative (invading) host, rather than diluting the

239 effects of a parasite, may act as a reservoir for infection, a factor exacerbated by high densities of the

240 invading hosts. The arrival of an alternative host could thus favor the multiplication of the native

241 parasite, resulting in reduced population growth of susceptible hosts (Holt & Lawton, 1993). Such

242 indirect host competition can lead to the extinction of the most parasitized host (Prenter et al., 2004;

243 Dunn, 2009). Nematodes are considered to have limited effects on social wasps (Gouge, 2005) whereas

244 conopid flies which rarely attack social wasps (Spradbery, 1973; Matsuura & Yamane, 1990) can

245 locally be extremely destructive to bumblebee colonies in Europe (Schmid-Hempel, 2001). The
246 negative effects of parasitoids on their hosts are however not always clear-cut and immediately visible.
247 Parasite effects often depend on host condition and may only be expressed when the host population is
248 in poor condition (Schmid-Hempel, 2001), a threat which nowadays may apply more to bumblebees
249 whose populations are more susceptible to decline (Gillespie, 2010) than those of social wasps.
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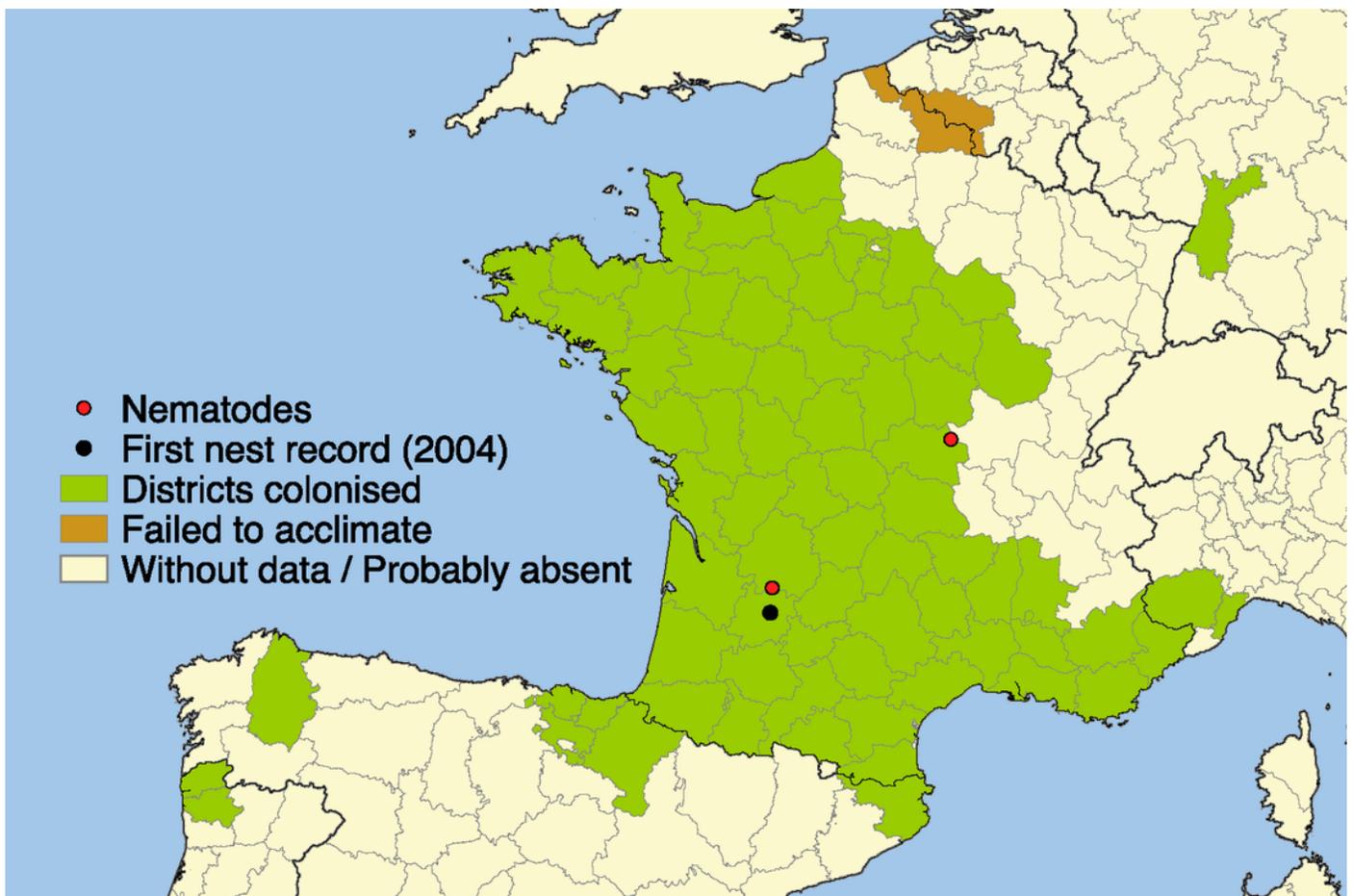
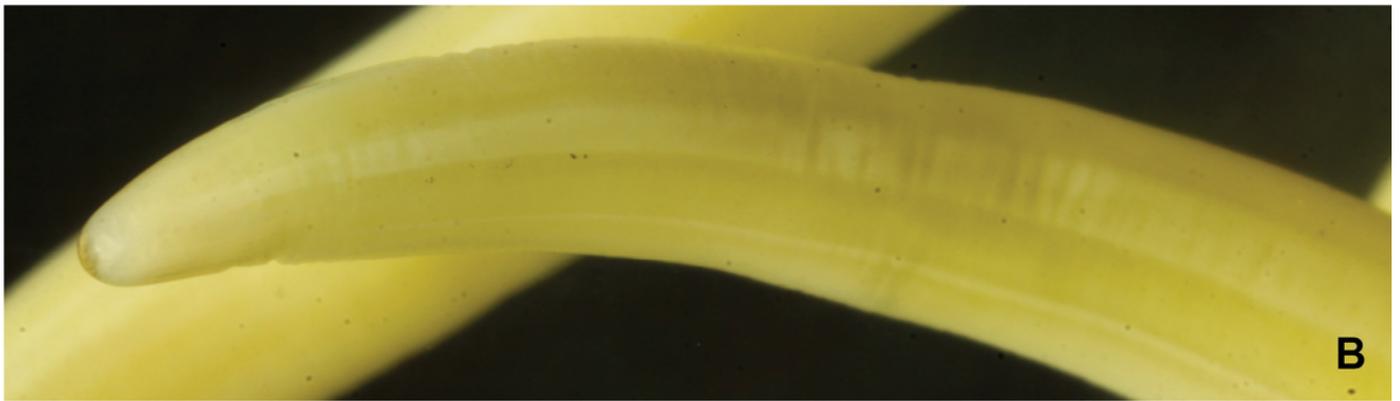
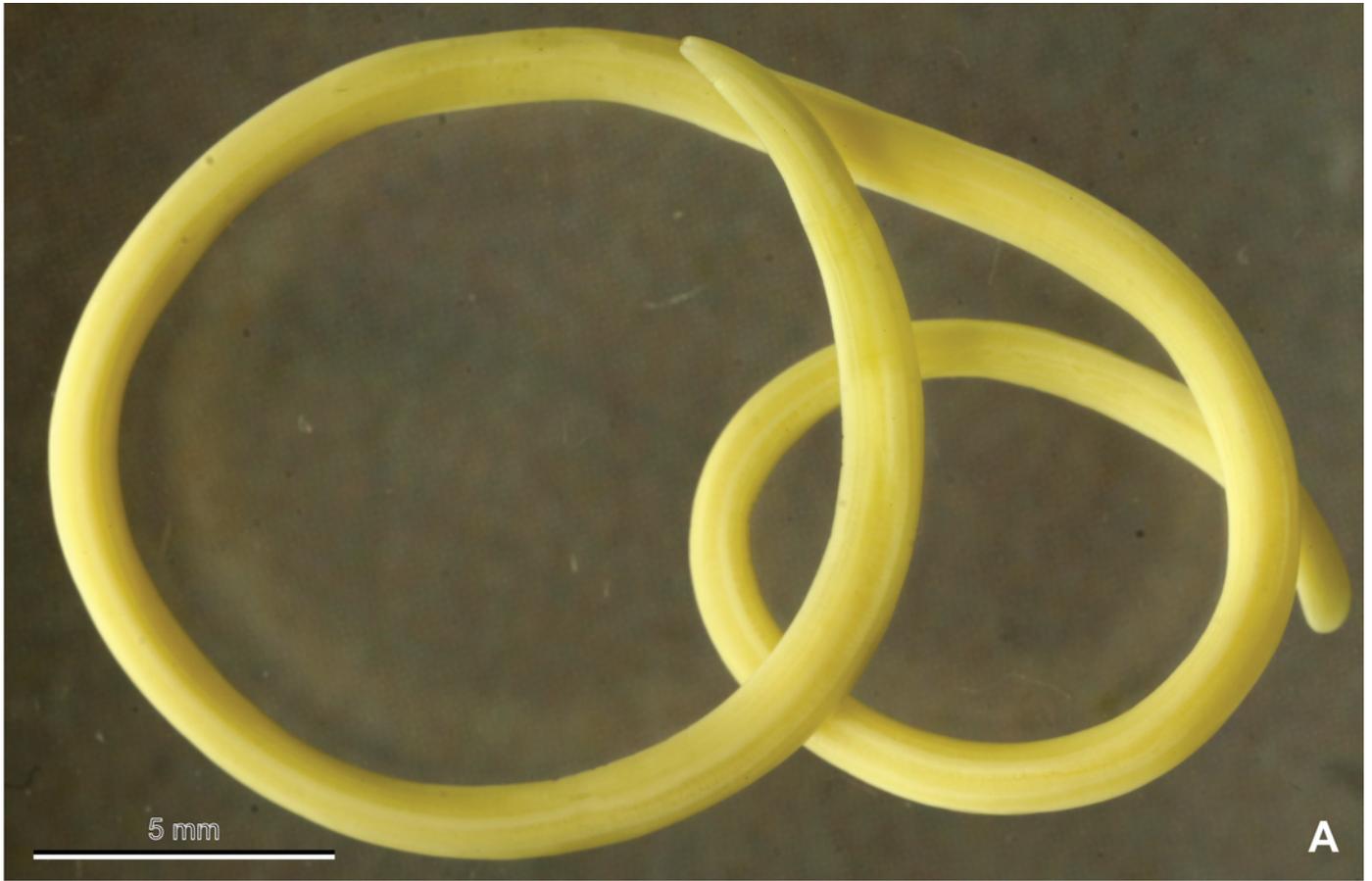
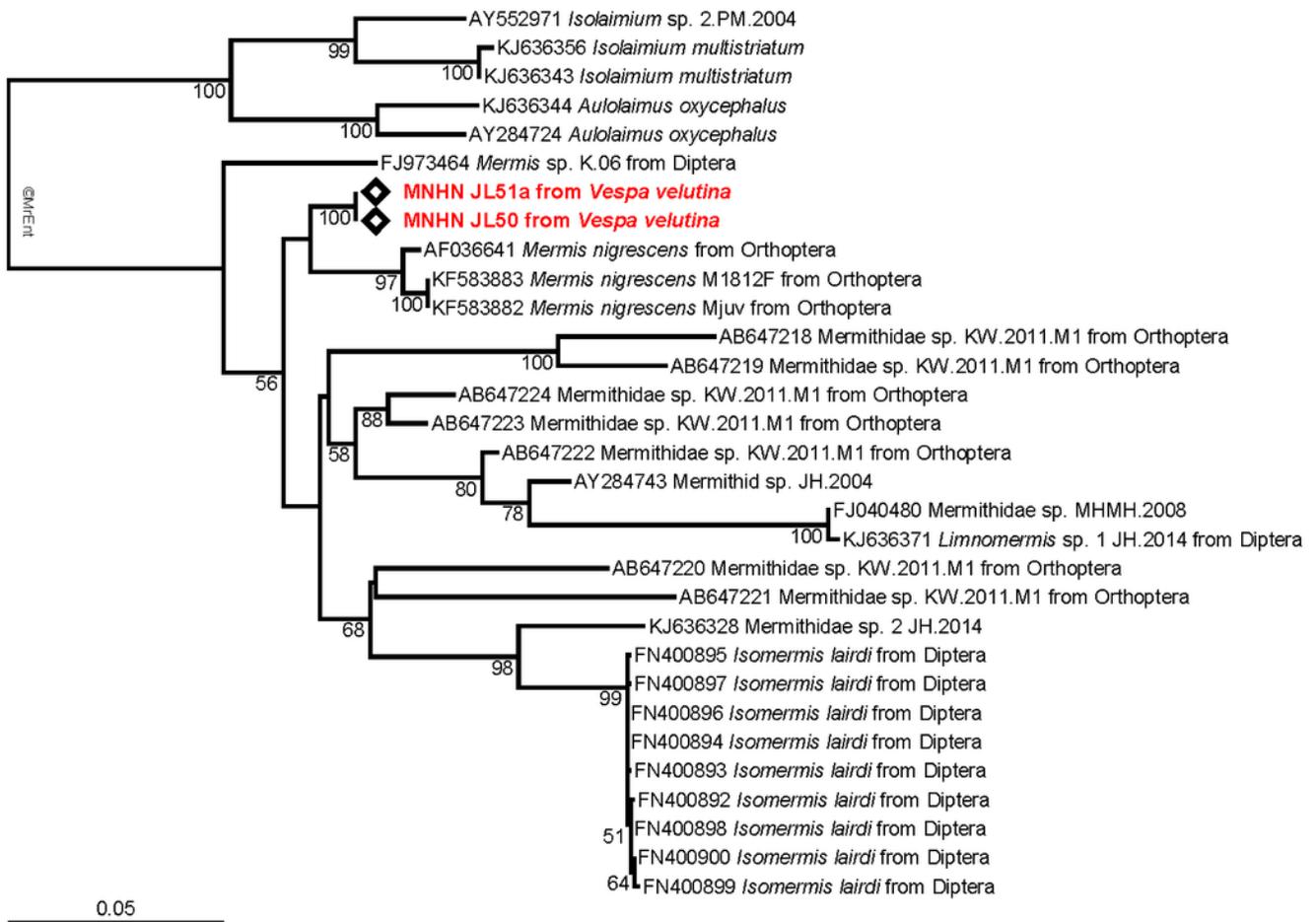
Current distribution of *Vespa velutina.*

Distribution of the invasive yellow-legged Asian hornet *Vespa velutina* in Europe in 2014. Black spot: first occurrence of *V. velutina* in Europe. Red spots: localities where the nematodes have been found.

Mermithid Nematode from *Vespa velutina*.

Photographs of a postparasitic juvenile of the mermithid nematode (MNHN JL50) from *Vespa velutina*, collected in Dompierre-sur-Besbre, France. A, whole worm; B, head; C, tail.

3

Molecular analysis.

Maximum-likelihood 18S tree showing the relationships of the parasite infecting *Vespa velutina* (red) with other mermithids. When available, the main host insects are indicated after the parasite name. The bootstrap support values are indicated at the node.

Primers used.

Primer pairs used in this study with their annealing temperatures. The 18S was amplified in two overlapping fragments.

Gene	Primers		Annealing T	Reference
18S	18S-1F	TACCTGGTTGATCCTGCCAGTAG	51	Giribet et al., 1996
	18S-5R	CTTGCAAAGCTGCTTTCGC		
	18S-3F	GTTCGATTCCGGAGAGGGA	51	
	18S-Bi	GAGTCTCGTTCGTTATCGGA		
28S	28S-C1	ACCCGCTGAATTTAAGCAT	55	Dayrat et al., 2001
	28S-D2	TCCGTGTTTCAAGACGGG		
COI	AnCO1-F	ATTTGGTCTTTGATCTGGTATGG	48	Cross et al., 2006
	AnCO1-R	TGGCAGAAATAACATCCAAACTAG		
	LCO1490	GGTCAACAAATCATAAAGATATTGG	48	Folmer et al., 1994
	HCO2198	TAAACTTCAGGGTGACCAAAAAATCA		