# Transfer of four isolates of *Bursaphelenchus doui* (Nematoda: Aphelenchoididae) into *Monochamus alternatus* (Coleoptera: Cerambycidae) and potential vector switching of the nematode

Noritoshi MAEHARA [1,*], Takuya AIKAWA [2], Natsumi KANZAKI [3], and Katsunori NAKAMURA [2]

[1] *Department of Forest Entomology, Forestry and Forest Products Research Institute, 1 Matsunosato, Tsukuba, Ibaraki 305-8687, Japan*

[2] *Tohoku Research Center, Forestry and Forest Products Research Institute, 92-25 Nabeyashiki, Shimo-Kuriyagawa, Morioka, Iwate 020-0123, Japan*

[3] *Kansai Research Center, Forestry and Forest Products Research Institute, 68 Nagaikyutaroh, Momoyama, Fushimi, Kyoto, Kyoto, 612-0855, Japan*

*Corresponding author, e-mail: maehara@ffpri.affrc.go.jp

**Summary –** To reinforce vector-switching potential of *Bursaphelenchus doui* in its ecological and evolutionary contexts, we tested our previous hypothesis that "*B. doui* (or its ancestor) was transferred by *Acalolepta fraudatrix*, *Acalolepta sejuncta*, and/or *Monochamus subfasciatus* (or their ancestral species) from broad-leaved trees to conifers, switched vectors from these cerambycid beetles to *Monochamus* beetles in conifers, and then evolved into the common ancestor of *Bursaphelenchus mucronatus* and *Bursaphelenchus xylophilus*". The affinity of four *B. doui* isolates and one *B. xylophilus* isolate for *Monochamus alternatus* was tested using our simple nematode-loading method to the beetle, and the affinity was assessed based on the nematode loads on the beetles. Phoretic stages of two *B. doui* isolates obtained from *Monochamus saltuarius* and *Pinus densiflora* showed loading levels similar to that of *B. xylophilus*, which were significantly higher than those of the other two *B. doui* isolates obtained from *A. fraudatrix* and *M. subfasciatus*. This result indicates that the first two isolates of *B. doui* derived from a conifer-using beetle and a coniferous tree adapt to *M. alternatus* better than the last two isolates associated with beetles using broad-leaved trees. We reinforced that vector switching of *B. doui* could have occurred during the evolutionary history of the *B. xylophilus* group.

**Keywords** – broad-leaved tree, conifer, fourth-stage dispersal juvenile, phoretic adult, vector beetle

Many *Bursaphelenchus* nematodes belonging to the *Bursaphelenchus xylophilus* group *sensu* Ryss & Subbotin (2017) and Kanzaki & Giblin-Davis (2018) are associated with cerambycid beetles in the tribe Lamiini. The pinewood nematode, *B. xylophilus* (Steiner & Buhrer) Nickle, the causative agent of pine wilt disease (Kiyohara & Tokushige, 1971), and its closest relative, *Bursaphelenchus mucronatus* Mamiya & Enda are primarily vectored by *Monochamus* cerambycid beetles (Mamiya & Enda, 1972; Morimoto & Iwasaki, 1972; Mamiya & Enda, 1979; Linit, 1988; Tomminen, 1990; Sousa *et al*., 2001, 2002; Penas *et al*., 2006). Kanzaki and Futai (2002) proposed that the ancestral species of *B. xylophilus* group, which had originated in the Eurasian Continent, acquired the ability to use tree species of family Pinaceae instead of broad-leaved ones and expanded their distribution throughout the coniferous forests ranging widely in the ancient continent of Eurasia-North America. Molecular phylogenetic analyses inferred from D2-D3 expansion segments of the large subunit of ribosomal RNA of *B. xylophilus* group showed that nematodes in conifers evolved from nematodes in broad-leaved trees (Figure 3 in Kanzaki *et al*., 2012). The higher genetic diversity of *B. mucronatus* may result from its earlier origin in Eurasia, and *B. xylophilus* may have recently evolved from a population of *B. mucronatus* in North America through geographical or reproductive isolation (Pereira *et al*., 2013). For this evolutionary process, cerambycid beetles must have transferred nematodes from broad-leaved trees to conifers.

Nematode-vector combinations of *B. xylophilus* group species in broad-leaved trees include *Bursaphelenchus conicaudatus* Kanzaki, Tsuda & Futai-*Psacothea hilaris* (Pascoe) (Kanzaki *et al*., 2000) and *Bursaphelenchus luxuriosae* Kanzaki & Futai and *Bursaphelenchus acaloleptae* Kanzaki, Ekino, Maehara, Aikawa, & Giblin-Davis-*Acalolepta luxuriosa* (Bates) (Kanzaki & Futai, 2003; Kanzaki *et al*., 2020). In contrast, *Bursaphelenchus doui* Braasch, Gu, Burgermeister, & Zhang is found in association with several species of cerambycid beetles, *i.e.*, *Acalolepta fraudatrix* (Bates) (Kanzaki *et al*., 2013), *Acalolepta sejuncta* (Bates) (Aikawa *et al*.,

2020), *Monochamus subfasciatus* (Bates) (Kanzaki *et al*., 2008), and *Monochamus saltuarius* (Gebler) (Aikawa *et al*., 2020). Because the first three species of the beetles use both broad-leaved trees and conifers (Iwata, 1992; Makihara, 1992), *B. doui* is present in both (Han *et al*., 2009; Kanzaki *et al*., 2008). In contrast, *M. saltuarius* inhabits only coniferous species (Iwata, 1992). Maehara *et al*. (2020) hypothesized that "*B. doui*, or its ancestor, was transferred by *A. fraudatrix*, *A. sejuncta*, and/or *M. subfasciatus* (or ancestral species of these beetles) from broad-leaved trees to conifers, switched the vectors from these beetles to *Monochamus* beetles, *e.g*., *M. saltuarius*, in conifers, and later evolved into the common ancestor of *B. mucronatus* and *B. xylophilus*".

The life cycle of *B. xylophilus* is divided into propagative and dispersal phases (Mamiya, 1975). The fourth-stage dispersal juvenile (dauer juvenile; $J_{IV}$) of *B. xylophilus* is vital in the nematode life cycle as the phoretic stage carried by beetles (Mamiya & Enda, 1972; Morimoto & Iwasaki, 1972). *Bursaphelenchus xylophilus* $J_{IV}$ develops when late pupae and callow adults of *Monochamus* beetles are present (Morimoto & Iwasaki, 1973; Maehara & Futai, 1996; Necibi & Linit, 1998; Maehara & Futai, 2001; Ogura & Nakashima, 2002) and enters the tracheae of the beetles. The third-stage dispersal juvenile ($J_{III}$) of *B. xylophilus* moults into $J_{IV}$ in response to long-chain C16 and C18 fatty acid ethyl esters secreted from the body surface of *Monochamus alternatus* Hope, specifically during adult eclosion (Zhao *et al*., 2013, 2014). The phoretic stages of *B. doui* are both $J_{IV}$ and the phoretic adult (PA) (Kanzaki *et al*., 2013; Ekino *et al*., 2017), and are also induced by the vector beetle *A. fraudatrix* (Maehara *et al*., 2020). Furthermore, the phoretic stages of *B. xylophilus* and *B. doui* are induced not only by their primary vectors *M. alternatus* Hope and *A. fraudatrix*, respectively, but also by their nonvectors, although the numbers and the percentages of the stages varied widely (Maehara & Futai, 2001; Maehara *et al*., 2020).

Nematodes need to develop into the phoretic stages to be carried by vector beetles because

propagative juveniles and adults cannot transfer to beetles even if they are found around the pupal chambers of the beetles. Therefore, the affinity between nematodes and beetles can be examined by the induction of the phoretic stages in the presence of the beetles and the transfer of the stages into the beetles. Our simple nematode-loading method to beetles (Maehara & Kanzaki, 2016) can be used to examine not only the nematodes' affinity for the vector beetles but also the potential affinity for the nonvectors which do not meet the nematodes in the field. Based on the potential affinity between 20 binary combinations of five species of the *B. xylophilus* group and four cerambycid beetle species in the tribe Lamiini, we tested the above hypothesis and showed potential vector switching of nematodes, especially *B. doui* in the evolution of the *B. xylophilus* group (Maehara *et al*., 2020). Because Maehara *et al*. (2020) used only one isolate of *B. doui*, our objective in the present study was to strengthen the potentiality of vector switching of *B. doui* using four conspecific isolates of the species together with *B. xylophilus* and *M. alternatus*, and to elucidate the evolutionary process of the *B. xylophilus* group.

## Materials and Methods

### Beetle cultures

To obtain *M. alternatus* adults, dead logs of Japanese red pine, *Pinus densiflora* Sieb. & Zucc., were collected at the Chiyoda Experimental Station of the Forestry and Forest Products Research Institute (FFPRI), Kasumigaura, Ibaraki, Japan in spring 2015. The logs were placed in a screen cage at the FFPRI, Tsukuba, Ibaraki, Japan. Adults of *M. alternatus*, which emerged from *P. densiflora* logs on June 3-4, 2015, were allowed to feed on *P. densiflora* twigs for two months, mate and oviposit on the same pine species logs that were cut about 2 weeks prior. Eggs of *M. alternatus* were collected from the logs with a chisel, and were placed on wet filter paper with

distilled water at 25°C in the dark until hatching. Artificial diets were modified from the diet for *M. alternatus* proposed by Kosaka & Ogura (1990) and Kosaka & Enda (1991), and contained the following: 8 g of the current and 1-year-old needles of *P. densiflora* dried at 70°C for 1 d and milled into powder, 26.8 g of artificial silkworm diet (Silkmate 2M powder, Nosan Corporation, Kanagawa, Japan), 3.2 g of dried yeast (EBIOS, Asahi Group Foods, Ltd., Tokyo, Japan), and 62 ml of distilled water. Approximately 20 g of the diet was placed into 50-ml Erlenmeyer flasks. Each flask was plugged with a silicone-rubber stopper (Silicosen, Shin-Etsu Polymer Co., Ltd., Tokyo, Japan) and autoclaved at 121°C for 20 min. A hatched larva of *M. alternatus* was placed into each flask. Larvae were reared at 25°C in the dark for 3-4 months. When the larvae mature, they were incubated at 10°C in the dark for 5 months, removed from the flasks, and placed on wet filter paper with distilled water at 25°C in the dark to become pupae and then adults. *Monochamus alternatus* was reared for another generation in the same way. Mature larvae, after incubation at 10°C, were removed from the flasks, rinsed in distilled water, dipped in 70% ethanol for 5 s, and then rinsed again in distilled water. The larvae were placed on wet filter paper with distilled water at 25°C in the dark until they became pupae.

## NEMATODE CULTURES

Four isolates of *B. doui* were used: ones obtained from *Acalolepta fraudatorix* in Fukaura, Aomori, Japan (Kanzaki *et al*., 2013), *M. subfasciatus* found at the Tama Forest Science Garden, FFPRI, Hachioji, Tokyo, Japan (Kanzaki *et al*., 2008), *M. saltuarius* in Yamada, Iwate, Japan (Aikawa *et al*., 2020), and a dead tree of *P. densiflora* in Izu region, Shizuoka, Japan in 1995 by T. Kiyohara (Kanzaki *et al*., 2008). In addition, a virulent isolate (T-4) of *B. xylophilus*, which had been isolated from a dead *P. densiflora* tree in Ichinoseki, Iwate, Japan in 1992 by T. Kiyohara (Aikawa *et al*., 2003), was used for control.

Nematodes were reared on *Botrytis cinerea* Pers. grown on autoclaved barley grains at 20°C

142 in the dark for 10-11 days, and were isolated aseptically from the culture using the Baermann
143 funnel technique (Hooper, 1986). A nematode inoculum was prepared with 500 nematodes/30 µl
144 suspension.
145
146 LOADING BEETLES WITH NEMATODES ON FUNGAL PLATES
147 Mycelial disks (4 mm in diam.) of *Nectria viridescens* Booth, cut from fungal colonies growing
148 on malt extract agar (Difco) (5% agar), were placed on the same kind of medium in 9 cm diam.
149 Petri dishes. These dishes were incubated at 25°C in the dark for 20 days. A 30-µl nematode
150 suspension (= 500 individual mixed-stage nematodes) was inoculated into each dish, which was
151 incubated at 25°C in the dark for 14–23 days, and then at 10°C in the dark until the larvae of *M.*
152 *alternatus* pupated. After pupation, one pupa was placed onto each dish. Dishes were sealed with
153 Parafilm M® (Bemis Flexible Packaging, Wisconsin, USA) and incubated again at 25°C in the
154 dark.
155 The development of pupae was observed daily. Eight days after adult eclosion, adult beetles
156 were removed from the dishes. After removal, each beetle was rinsed with distilled water,
157 ground for 10 s using a blender with 40 ml of distilled water, and placed in a Baermann funnel
158 overnight to extract the nematodes from its body. To determine the number of nematodes that
159 were unable to enter beetle tracheae, *i.e.*, those retained on the culture plate and the surface of
160 the beetle's body, rinse water from the beetle and the agar medium were placed in another
161 Baermann funnel overnight. The harvested nematodes were then counted using a
162 stereomicroscope, and the numbers of $J_{IV}$ and PA (phoretic adults) were recorded for each beetle
163 sample, although the nematode stages were not distinguished and only the total numbers of
164 nematodes were recorded for each sample of the rinse water and the agar medium. When
165 nematodes were too abundant to count, the suspension was diluted, and the numbers of
166 nematodes were estimated.

167

168 ANALYTICAL METHODS

169 All analyses were performed using JMP® 11 (SAS Institute Inc., Cary, NC, USA). The total
170 numbers of nematodes represent those carried internally by a *Monochamus* beetle + those on the
171 surface of the beetle's body and remaining in the agar medium. One-way analysis of variance
172 (ANOVA) and Tukey-Kramer HSD test were used to analyse the differences in the total
173 numbers of nematodes, the numbers of $J_{IV}$, PA, and $J_{IV}$ + PA carried by a beetle, the percentages
174 of PA to $J_{IV}$ + PA, which were carried by a beetle, and those of $J_{IV}$ + PA carried by a beetle to
175 total nematodes among nematode treatments. For ANOVA, the numbers of nematodes were
176 $\log_{10}$-transformed, and the percentages of $J_{IV}$ + PA were arcsine transformed (Yonezawa *et al*.,
177 1988).

178

179 **Results**

180 Table 1 shows the transfer of four *B. doui* isolates and one *B. xylophilus* isolate to *M. alternatus*
181 adults. The total nematode numbers of four *B. doui* isolates in fungal plates with beetles were
182 significantly higher than the number of *B. xylophilus* in plates with beetles. The phoretic stages
183 of *B. xylophilus* and *B. doui* were $J_{IV}$ (Mamiya & Enda, 1972; Morimoto & Iwasaki, 1972) and
184 both $J_{IV}$ and PA (Kanzaki *et al*., 2013; Ekino *et al*., 2017), respectively. The numbers of *B. doui*
185 $J_{IV}$ carried by a *Monochamus* beetle were significantly greater in the isolates obtained from *M.*
186 *saltuarius* (531 on average) and *P. densiflora* (458) than in those from *A. fraudatrix* (6) and *M.*
187 *subfasciatus* (21). *Monochamus alternatus* adults, on the other hand, carried average 29 PA of *B.*
188 *doui* from *P. densiflora* and only small numbers of PA of the other three isolates. Regarding the
189 total phoretic stages, around 500 nematodes ($J_{IV}$ + PA) of *B. doui* from *M. saltuarius* and *P.*
190 *densiflora* transferred to *M. alternatus* adults, while only small numbers of nematodes from *A.*
191 *fraudatrix* and *M. subfasciatus* did. The percentages of $J_{IV}$ + PA carried by a *Monochamus* beetle

192 to the total numbers of nematodes showed a similar trend to the numbers of $J_{IV}$ + PA carried by a
193 beetle. The percentages of *B. doui* PA to $J_{IV}$ + PA carried by a beetle were significantly higher in
194 *A. fraudatrix* and *M. subfasciatus* isolates than in *M. saltuarius* and *P. densiflora* isolates, and in
195 particular, the percentage of the isolate from *M. saltuarius* was almost 0% and was not
196 significantly different from that of *B. xylophilus* (0%). The number of phoretic stages of *B.*
197 *xylophilus* carried by a beetle averaged 543 and was similar to the numbers of *B. doui* from *M.*
198 *saltuarius* (531) and *P. densiflora* (488). However, the percentage of phoretic stages of *B.*
199 *xylophilus* carried by a beetle to the total numbers of nematodes (2.3%) was significantly higher
200 than the percentages of the four *B. doui* isolates (0.004-0.70%).
201
202 **Discussion**
203 The total nematode numbers of four *B. doui* isolates in fungal plates with beetles (> 100000 on
204 average) were significantly higher than the number of *B. xylophilus* (ca. 20000) (Table 1). Because
205 Maehara *et al*. (2020) reported similar results, *B. doui* would grow better or faster on *N.*
206 *viridescens* growing on malt extract agar than *B. xylophilus*. Tanaka *et al*. (2017) demonstrated
207 that T-4 isolate of *B. xylophilus* used in the present study readily produced $J_{III}$ compared with other
208 conspecific isolates, and their growth was arrested at the stage. Maehara *et al*. (2018) also used T-
209 4 isolate and showed that the percentage of $J_{III}$ to total nematodes was high. A similar phenomenon
210 may have occurred under the present experimental condition.
211 *Acalolepta fraudatrix* and *M. subfasciatus* use both broad-leaved trees and conifers (Iwata,
212 1992; Makihara, 1992); accordingly, *B. doui* isolates obtained from these two cerambycid species
213 are associated with broad-leaved trees. In contrast, *M. saltuarius* inhabits only coniferous species
214 (Iwata, 1992). Therefore, two isolates of *B. doui* from the beetle and *P. densiflora* come from
215 conifers. The numbers and percentages of $J_{IV}$ + PA carried by a beetle were higher in *B. doui*
216 isolates from *M. saltuarius* (531; 0.70%) and *P. densiflora* (488; 0.44%) than in those from *A.*

217 *fraudatrix* (6; 0.004%) and *M. subfasciatus* (23; 0.02%) (Table 1). These results indicate that the
218 first two isolates of *B. doui* associated with conifers adapt to *M. alternatus* in conifers better than
219 the last two isolates associated with broad-leaved trees, and support the first half of Maehara *et*
220 *al*. (2020)'s hypothesis that "*B. doui*, or its ancestor, was transferred by *A. fraudatrix*, *A. sejuncta*,
221 and/or *M. subfasciatus* (or ancestral species of these beetles) from broad-leaved trees to conifers,
222 and switched vectors from these beetles to *Monochamus* beetles, *e.g*., *M. saltuarius*, in conifers".
223 Vector switching of *B. xylophilus* actually occurred from *Monochamus* beetles in North America
224 to *M. alternatus* in Japan, and then to *Monochamus galloprovincialis* (Olivier) in Portugal (Ryss
225 *et al*., 2011; Akbulut & Stamps, 2012).
226 In addition, the percentage of phoretic stages of *B. xylophilus* carried by a beetle to the total
227 numbers of nematodes (2.3%) was significantly higher than the percentages of *B. doui* from *M.*
228 *saltuarius* (0.70%) and *P. densiflora* (0.44%) (Table 1). Considering the propagation character of
229 T-4 isolate of *B. xylophilus*, *i.e*., easy production of $J_{III}$ and smaller population size, the values of
230 these *B. doui* isolates could be closer to those of the other *B. xylophilus* isolates. The percentage
231 of PA to $J_{IV}$ + PA of *B. doui* from *M. saltuarius* (0.008%) was lower than the percentages of *B.*
232 *doui* from *A. fraudatrix* (5.4%) and *M. subfasciatus* (13.2%), and was close to that of *B. xylophilus*
233 (0%) (Table 1). These results suggest that *B. doui* isolates that have adapted to beetles using
234 conifers produce less PA than those associated with beetles using broad-leaved trees, and support
235 the second half of our hypothesis (Maehara *et al*., 2020) that "the common ancestor of *B.*
236 *mucronatus* and *B. xylophilus* evolved from *B. doui* that switched vectors to *Monochamus* beetles
237 and completed its life cycle in conifers". The reason why PA, one of the phoretic stages,
238 disappeared during the evolution from *B. doui* to the common ancestor of *B. mucronatus* and *B.*
239 *xylophilus* is unclear.
240 Thus, the results of the present study using four isolates of *B. doui* reinforced the potentiality
241 of vector switching of the nematode species during the evolutionary history of the *B. xylophilus*

group, which was shown by Maehara *et al*. (2020). In the three above-mentioned nematode species, *i.e*., *B. xylophilus*, *B. mucronatus*, and *B. doui*, the first two species have pathogenicity to pine trees although *B. mucronatus* can kill pines only under severe stresses for them, such as severe drought, high temperature, and shading, and its virulence is low (Braasch, 2000; Kanzaki & Futai, 2006). In contrast, *B. doui* does not show pathogenicity to pines (Kanzaki *et al*., 2008; Maehara *et al*., 2011). Further studies are needed to reveal how the common ancestor of *B. mucronatus* and *B. xylophilus* acquired pathogenicity to pine trees during the latter part of the evolution of the *B. xylophilus* group.

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**Table 1.** Transfer of four isolates of *Bursaphelenchus doui* and one isolate of *B. xylophilus* to *Monochamus alternatus*

| Treatments | No. of observations | Total no. of nematodes | No. of $J_{IV}$ carried by a beetle | No. of PA carried by a beetle | No. of $J_{IV}$ + PA carried by a beetle |
|---|---|---|---|---|---|
| *B. doui* from *A. fraudatrix* | 13 | 142298 ± 33479 a | 6 ± 9 a | 0.2 ± 0.4 a | 6 ± 9 a |
| *B. doui* from *M. subfasciatus* | 13 | 106609 ± 19255 a | 21 ± 29 a | 1.4 ± 1.4 a | 23 ± 30 a |
| *B. doui* from *M. saltuarius* | 12 | 123238 ± 48878 a | 531 ± 459 b | 0.1 ± 0.3 a | 531 ± 458 b |
| *B. doui* from *P. densiflora* | 13 | 112954 ± 22116 a | 458 ± 431 b | 29 ± 43 b | 488 ± 467 b |
| *B. xylophilus* | 10 | 19532 ± 12439 b | 543 ± 577 b | 0 ± 0 a | 543 ± 577 b |

| Treatments | No. of Observations [1] | % PA to $J_{IV}$ + PA carried by a beetle [1] | No. of observations | % $J_{IV}$ + PA carried by a beetle to total nematodes |
|---|---|---|---|---|
| *B. doui* from *A. fraudatrix* | 11 | 5.4 ± 10.1 a | 13 | 0.004 ± 0.006 a |
| *B. doui* from *M. subfasciatus* | 12 | 13.2 ± 16.9 a | 13 | 0.02 ± 0.03 a |
| *B. doui* from *M. saltuarius* | 12 | 0.08 ± 0.26 b | 12 | 0.70 ± 1.04 b |
| *B. doui* from *P. densiflora* | 13 | 5.1 ± 4.1 c | 13 | 0.44 ± 0.40 b |
| *B. xylophilus* | 10 | 0 ± 0 b | 10 | 2.3 ± 1.0 c |

Values are means ± SD. Means followed by the same letter in a column are not significantly different at $P < 0.05$ (Tukey-Kramer HSD test). $J_{IV}$ and PA represent the fourth-stage dispersal juveniles and the phoretic adults, respectively.

[1] Two samples and one sample in *B. doui* from *A. fraudatrix* and *M. subfasciatus*, respectively, were discarded in the analysis because the percentages of PA to $J_{IV}$ + PA, which were carried by a beetle, were 0/0.