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SPECIAL ISSUE

Evolution in biological invasion

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Evolutionary change in a pine wilt system following the invasion of Japan by the pinewood nematode, *Bursaphelenchus xylophilus*

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Abstract Pinewood nematode (PWN), *Bursaphelenchus xylophilus*, is the causative agent of pine wilt disease (PWD) of pine trees and is transmitted by cerambycid beetles belonging to the genus *Monochamus*. PWN is believed to have been introduced into Japan from North America at the beginning of the 20th century. In this article, we first provide an outline of the PWD system and the range expansion of PWN in Japan and then review the literature, focusing on the virulence of PWN. Virulence is a heritable trait in PWN, with high virulence being closely related to a high rate of reproduction and within-tree dispersal. When two PWN isolates with different virulence levels are inoculated into pine seedlings, the more virulent nematodes always dominate in dead seedlings. In a laboratory setting, many more virulent nematodes board the insect vectors than avirulent ones. The age at which vectors transmit the most abundant PWNs to pine twigs changes during the course of a PWD epidemic. However, the relation between virulence and transmission of PWN remains as yet relatively unknown. Such information would enable ecologists to predict the evolution of the PWD system. In this review we also compare ecological traits between the PWN and the avirulent congener, *B. mucronatus*.

Keywords Biological invasion · *Monochamus* · Resistance · Transmission · Virulence

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Introduction

Biological invasion has been an important field of ecological study for the past several decades. Human activities, such as international travel and trade, have broken down the natural dispersal barriers of a large number of organisms, plants as well as animals (Kolar and Lodge 2001), often resulting in ecosystem disturbance by these organisms. In forest ecosystems, many of the most damaging insect and disease agents have been introduced from other continents (Liebhold et al. 1995). Chestnut blight, Dutch elm disease, and white pine blister rust are regarded as the three most serious forest diseases on a worldwide scale, and all are caused by invasive species. In the latter part of the 20th century, however, pine wilt disease (PWD) has become the most serious forest disease in East Asia and is currently considered to be one of the most serious threats to pine forests worldwide (Suzuki 2004).

PWD is an infectious disease of pine trees. The causative agent of PWD is the pinewood nematode (PWN), *Bursaphelenchus xylophilus* (Kiyohara and Tokushige 1971; Mamiya 1988), which is transmitted by cerambycid beetles belonging to the genus *Monochamus* (Linit 1988). The PWN is not native to Japan and is believed to have been introduced from North America at the beginning of the 20th century (Mamiya 1988; Tares et al. 1992). Since its introduction, it has spread throughout most of Japan despite massive control efforts, causing devastating damage to pine forests (Nakamura and Yoshida 2004). During the 1980s the nematode spread to China, Taiwan and South Korea, where it has subsequently caused serious damage to pine forests (Yang and Wang 1989; Kishi 1995). In 1999, the PWN invaded Portugal (Mota et al. 1999).

In this article, we review the PWD system, the range expansion of PWN in Japan, the intraspecific variation and changes in epidemiological traits, such as virulence and transmission, of the PWN, the impact of the PWN on the closely related, indigenous nematode, *Bursaphelenchus mucronatus*, the difference in ecological traits between the two *Bursaphelenchus* species and their interrelation. We then discuss the evolution of the PWD system. For other reviews that focus on the plant pathological, nematological, and/or entomological aspects of the PWD system, we refer the reader to Mamiya (1983), Kobayashi et al. (1984), Linit (1988), Fukuda (1997) and Giblin-Davis et al. (2003).

The PWD system

PWN is phytophagous and mycophagous, and reproduces in recently dead trees. It has two types of juveniles: propagative and dispersal (Mamiya 1984). The first-stage propagative juvenile molts to the second-stage propagative juvenile in an egg and then emerges. It then molts to the third-stage propagative or dispersal juvenile. The third-stage propagative juvenile develops to an adult via the fourth-stage propagative juvenile, whereas the third-stage dispersal juvenile (JIII) usually molts to the fourth-stage dispersal juvenile (JIV), the special stage for boarding the insect vectors. The JIVs are conveyed to new host trees and then molt to adults. Infection by the PWN causes a loss of hydraulic conductivity in healthy trees of susceptible *Pinus* species, resulting in rapid wilting (Ikeda and Kiyohara 1995). A specific number of PWNs is required for the development of PWD, and the speed of disease development is enhanced as the number of inoculated PWNs increases (Hashimoto and Sanui 1974). PWD-infected trees die within 1 year of the initial infection in most cases, and the PWN reproduces in such trees.

Monochamus alternatus is the primary vector of the PWN in East Asia (Mamiya and Enda 1972; Morimoto and Iwasaki 1972; Lee et al. 1990; Yang 2004). In Japan, most vector beetles have a 1-year life cycle, but there are some exceptions which require 2 years to complete the life cycle (Kishi 1995). The percentage of individuals with the 2-year life cycle is high in cool regions. The adults feed on the twig bark of healthy pine trees to mature reproductively. The adult females excavate the bark of dying or recently dead pine trees with their mandibles and then deposit the eggs under the bark through the wounds. The larvae feed on the inner bark and usually make a tunnel that includes a pupal chamber in the xylem before the winter. They pupate between May and July and eclose to adults in the pupal chambers. The adults emerge from dead trees after sclerotization of approximately 1 week.

M. alternatus adults carry the JIVs of the PWN in their tracheal system. The JIVs emerge from the spiracles, the opening of the tracheal system, and leave the beetle body. Both sexes of the beetle vectors transmit the PWN to healthy pine trees via the feeding wounds (Mamiya and Enda 1972), with the beetle females transmitting it to dying or recently dead trees via the oviposition wounds (Wingfield and Blanchette 1983; Edwards and Linit 1992). Recent studies indicate that the transmission pathway is complicated. The reproductively mature beetle males search for females on dying and recently dead pine trees and transmit PWNs to such trees via wounds on the bark (Arakawa and Togashi 2002). The PWN moves between both sexes of beetle vectors during the mating behavior, ultimately being transmitted to trees via the feeding and oviposition wounds (Edwards and Linit 1992; Togashi and Arakawa 2003). Some PWNs are harbored in the reproductive organs of *M. alternatus* females (Arakawa and Togashi 2004).

When PWNs and *Monochamus* larvae are within identical dead trees, the JIIIs aggregate around the pupal chambers during the winter and spring (Mamiya 1986). In the presence of a genus-specific substance(s) associated with *Monochamus* adult eclosion within the pupal chamber, they will molt to the JIVs (Necibi and Linit 1998). The JIVs enter the tracheae of the newly eclosed adult vectors in the pupal chambers. The adult beetles then emerge from the dead trees, and the cycle of PWD is repeated (Mamiya 1984).

Range expansion of the invasive PWN in Japan

The Japanese red pine, *Pinus densiflora*, the Japanese black pine, *P. thunbergii*, and the Ryukyu pine, *P. luchuensis*, have been heavily damaged by PWD since the PWN's invasion of Japan (Mamiya 1988).

In the absence of any control measures, an epidemic of PWD can continue in a pine stand for up to10 years (Kishi 1995). Empirical and theoretical studies have shown that the range of the vector's flight – and consequently the expansion rate of PWN – is several kilometers per year if no control effort is made (Takasu et al. 2000; Togashi and Shigesada 2006). The sudden incidence of PWD several hundreds of kilometers distant from the nearest PWD-infested area illustrates the significance of transporting pine logs infested with PWN and its vectors.

Early reports suggest that the first incidence of PWD was in Nagasaki City, Nagasaki Prefecture in 1905; this was followed by its appearance in 1921 in Aioi Town, Hyogo Prefecture, which lies 730 km east of Nagasaki City (Kishi 1995). The PWD epidemics subsequently spread steadily to the coastal areas of southwest Japan up to 1947. Between 1948 and 1958, the spread of PWD was stopped because the army that occupied Japan after World War II recommended and implemented exhaustive control measures which consisted of felling and burning in infected areas (Kishi 1995). After 1959, PWD spread inland to the cool summer areas of southwest Japan and to the coastal area of northern Japan. The number of newly invaded prefectures increased once again, reaching 45 in 1982, after which time the number of infected prefectures remained stable (Togashi and Shigesada 2006). It now remains for the two northernmost prefectures of Aomori and Hokkaido, with their cool summer climate, to be invaded.

Relationships of virulence to reproduction, within-tree dispersal, and affinity to vectors for the invasive nematode

Inoculation tests indicate a great variation in virulence among isolates of PWN collected throughout Japan, as assessed on the basis of pine seedling mortality, which ranges from 0 to 100% depending on the isolate (Kiyohara and Bolla 1990). The coexistence of virulent and avirulent isolates was observed even in a single pine stand (Kiyohara and Bolla 1990). However, no significant difference in virulence level among PWN isolates sampled from a single pine tree was found (Kiyohara and Bolla 1990). Kiyohara and Bolla (1990) also found no or very little difference in the virulence level among nine isofemale lines of PWNs established from each of three *M. alternatus* adults, suggesting little genetic difference in PWNs harbored in each vector. In contrast, a great difference in virulence was observed among PWN isolates sampled from five vectors in each of two pine stands (Kiyohara 1989).

The virulence of the PWN is a heritable trait. Iwahori et al. (1998) reported that the DNA sequence of ribosomal DNA was different between two groups of Japanese PWN isolates, the one virulent and the other avirulent. Mating occurs between isolates with different levels of virulence in the laboratory (Aikawa et al. 2003a). The crossing of virulent and avirulent isolates produces a virulent progeny in most cases (Kiyohara and Bolla 1990).

A virulent isolate of PWN was found to have a much higher reproduction rate than an avirulent one when both were inoculated on pine seedlings (Kiyohara and Bolla 1990). The same results were observed for two virulent isolates and two avirulent isolates inoculated onto a *Botrytis cinerea* fungal mat (Wang et al. 2005). However, Ibaraki et al. (1978) found considerable variations in reproduction rate on a fungal mat both among virulent isolates and among avirulent isolates. Inoculation tests of a virulent and an avirulent isolate on identical pine seedlings revealed that the proportion of virulent isolates in the PWN population is extremely large in PWD-killed seedlings irrespective of the sequence of inoculation (Aikawa et al. 2006).

A virulent isolate disperses in the xylem resin canals and cortical tissue more rapidly than an avirulent isolate when inoculated onto pine seedlings separately (Ichihara et al. 2000).

To determine the relationship between the boarding ability of the PWNs onto beetles and the virulence level, Aikawa et al. (2003b) placed *M. alternatus* larvae individually in holes of pine bolts onto which the fungus, *Ophiostoma minus*, had been inoculated as food as well as one of two PWN isolates with different virulence levels – in that order. The results of the experiment indicated that many more JIVs were isolated from beetle adults infected with the virulent isolate than those with the avirulent isolate. The results also showed that the productivities of JIIIs and JIVs were greater in the virulent isolate than the avirulent one, although there was no difference in the boarding probabilities of the JIVs between the two isolates.

Virulence and transmission of the invasive nematode

The number of nematodes transmitted per unit time from a single beetle to pine twigs via the feeding wounds changes depending on the number of nematodes carried by the beetle at emergence (initial nematode load) and the age of the beetle (Togashi 1985). The transmission curve of the PWN is defined for individual beetles as the change in the number of transmitted nematodes in relation to the age of beetle. It can be divided into L-shaped and unimodal types. The proportion of *M. alternatus* adults with a unimodal nematode transmission curve was found to vary greatly among the studied populations: 100% for the Mie and Nara populations (Shibata and Okuda 1989), 92% for the Ishikawa population (Togashi 1985), and a small percentage for the Ibaraki population (Kishi 1978).

Virulent PWNs that are transmitted to healthy, susceptible pine trees first kill the trees and then reproduce in them soon after the tree has died. Therefore, virulent PWNs that are transmitted early can initiate reproduction earlier than those transmitted late, possibly resulting in a greater fitness of the early-transmitted PWNs than the late-transmitted ones. Consequently, L-shaped transmission curves are expected to favor virulent PWNs. Conversely, avirulent PWNs are able to reproduce only when transmitted to dying and recently dead host trees (Wingfield and Blanchette 1983). *M. alternatus* adults mature reproductively a few weeks after the emergence and visit dying and recently dead host trees to copulate and oviposit (Togashi 1989). Avirulent PWNs are considered to gain a greater fitness when transmitted after the reproductive maturation of beetles than before their maturation. Therefore, a unimodal nematode transmission curve favors avirulent PWNs, although there have been no studies on the relationship between the virulence and transmission curves of the PWN.

When fewer than 30% of the trees survive a PWD epidemic in stands of *P. densiflora* and *P. thunbergii*, they include substantially resistant trees at low proportions (Toda and Kurinobu 2002). Thus, the mean level of tree resistance to PWD in a pine stand is expected to increase during the course of an epidemic. An increased mean level of tree resistance likely reduces the fitness of early-transmitted PWNs.

Observing *M. alternatus* adults that emerged from dead trees in a *P. densiflora* stand, Naka Town, Ibaraki Prefecture, Kishi (1995) reported that the averaged nematode transmission curve changed from an L-shaped type to a unimodal type and that the age of the beetle at which the peak of the transmission curve was observed increased during the first 4 years of the PWD epidemic

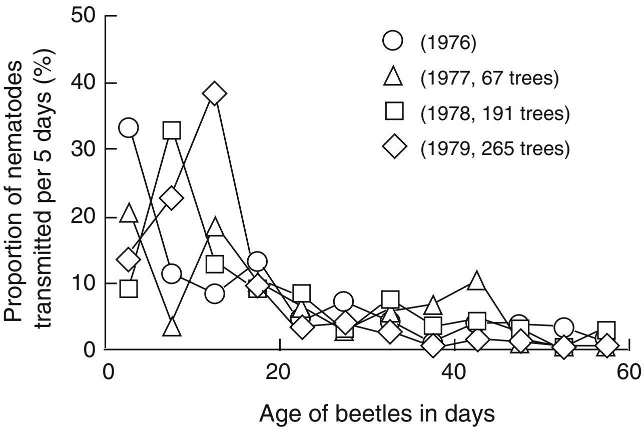

Fig. 1 Yearly changes in averaged transmission curves of *Bursaphelenchus xylophilus* into *Pinus densiflora* twigs by *Monochamus alternatus* from 1976 to 1979 (after Table 48 of Kishi 1995). Fifty *M. alternatus* adults, which emerged from dead trees in a *Pinus densiflora* stand in Naka, Ibaraki Prefecture, were examined in each year. The stand contained 667 living trees before the infection season of PWD in 1977. The *ordinate* represents the mean proportions of nematodes transmitted per 5-day period to the total number of nematodes transmitted. The *two numbers in parentheses* indicate the year and the number of PWD-killed trees in the pine stand in that year, respectively (after Table 54 of Kishi 1995)

(Fig. 1); however, the transmission curve returned to an L-shaped type for 2 years in the last half of the PWD epidemic. If a close relation between the virulence and transmission of the PWN is assumed, the change in the averaged nematode transmission curve during the early half of the epidemic may suggest a decreasing virulence of PWN to reach the maximum basic reproductive rate (Anderson and May 1982). The progress of the PWD epidemic would enhance the mean resistance level of the surviving trees. Higher resistance acts as a selective pressure on PWN, tending to select for PWNs with a higher virulence. This may have induced the change in the averaged nematode transmission curve during the second half of the epidemic.

Impact of the invasive nematode on insect vectors and the indigenous, avirulent nematode

A 4-year study of a *P. densiflora* stand before its invasion by PWN indicated that while there were always a few trees dying, this occurred in different months in different years and that the mean monthly tree mortality was low, fluctuating only slightly between April and November (Jikumaru and Togashi, unpublished). The primary factor of tree mortality at this time was the competition for sunlight, which resulted in the death of suppressed trees. Some of the larger trees in the adjacent pine stands were felled and broken by wind and snowfall in different seasons of different years. These facts indicate that it is difficult for beetle adults to predict when and where dying trees will occur in pine forests. *M. alternatus* seems to have been a rare species before the invasion of Japan by the PWN (Kishi 1995), which can be attributed to the scarcity and unpredictability of natural resources for the progeny – at that time. Thus, the long flight season of *M. alternatus*, from June through to September, and a highly tuned ability for locating dying trees would help improve its efficiency in providing resources for its progeny (Togashi 2002). A high survival rate of approximately 25% for the immature stages (Togashi 1990) also would have permitted the beetles to persist in the pine forest ecosystem.

The invasion of pine forests by the PWN results in a large production of dying trees between mid-summer and early autumn, when the oviposition activity of *M. alternatus* is at its peak (Mamiya 1984). Thereafter, an outbreak of *M. alternatus* occurs. Conversely, *M. saltuarius*, another vector of PWN in Japan (Sato et al. 1987), was found to have gone locally extinct in areas of an PWD epidemic and outbreak of *M. alternatus* (Makihara and Enda 2005). *M. saltuarius* adults emerge from dead trees between mid-April and early May in a hot summer area (Ochi 1969) and usually deposit their eggs on trees dying from a lack of sunlight, wind-broken trees, and boughs broken by snowfall (Jikumaru and Togashi, unpublished). Their oviposition season is between May and June (Ochi 1969). Thus, asynchronism between the oviposition of *M. saltuarius* and the occurrence of PWD-diseased trees is one of the plausible causes for preventing the population growth of *M. saltuarius*. In addition, PWD epidemics relax the competition of trees for sunlight and destroy big trees that supply snow-broken boughs, resulting in decreased oviposition resources for *M. saltuarius*.

Bursaphelenchus mucronatus is only slightly virulent towards *P. densiflora* and *P. thunbergii* in Japan (Mamiya and Enda 1979; Kishi 1995). It is also inferred to have had a wide distribution in Japan prior to the invasion by PWN (Kishi 1995; Mamiya 2006), whereas it is extremely difficult to find *B. mucronatus* in PWN-infested pine forests (Mamiya 2006). *B. mucronatus* seems to be replaced by the PWN as a PWD progresses. Such a replacement of the natives by the exotics is considered to be the worst-case scenario among the most common consequences of invasion reported (Crooks 2002).

Ecological traits of the indigenous, avirulent nematode in comparison to the invasive nematode

Both *B. mucronatus* and PWN have the same life cycle and are transported by cerambycid beetles. There is no difference in body size between the two species (Mamiya and Kiyohara 1972; Mamiya and Enda 1979). However, their virulence differs conspicuously. Therefore, it would seem likely that a comparison of *B. mucronatus* and PWN would provide some indication of the ecological traits associated with virulence.

When inoculated onto pine seedlings, *B. mucronatus* scarcely propagates while PWN propagates in most cases (Futai 1980b). The dispersal and multiplication of *B. mucronatus* is markedly restricted in the wood of living *P. densiflora* seedlings, whereas the Japanese isolates of PWN migrate freely and multiply (Odani et al. 1985). In laboratories, *B. mucronatus* has a smaller rate of population growth and a higher saturation density on a *B. cinerea* mycelial mat than PWN (Futai 1980a). The dispersal rate of a Japanese isolate of *B. mucronatus* in sections of living and boiled pine twigs does not differ from those of virulent and avirulent isolates of PWN (Togashi and Matsunaga 2003). The results of such studies indicate that both the reproduction and dispersal of PWN within living trees are much greater than those of *B. mucronatus*, although there is no difference in both traits within dead trees and under laboratory conditions.

There is a great variation in the number of PWNs carried by individual insect vectors at the time of emergence (initial nematode load) (Kobayashi et al. 1984; Linit 1988). A great difference is also observed in the initial load of *B. mucronatus* (Jikumaru and Togashi 2001). A heavy initial load of more than 10,000 PWNs reduces the lifespan of *M. alternatus* adults significantly (Togashi and Sekizuka 1982; Kishi 1995), whereas any reduction in *M. saltuarius* adult longevity due to a heavy initial load of *B. mucronatus* is insignificant, even though the elytral length of *M. alternatus* is 1.5-fold longer than that of *M. satuarius* (Jikumaru and Togashi 1995). It is still unknown whether *B. mucronatus* exerts an influence on the longevity of *M. alternatus* adults. In North America, a heavy load of PWN decreases the mean distance and duration time of flight by *M. carolinensis* (Akbulut and Linit 1999), although the impact of *B. mucronatus* on the flight performance of vectors has not yet been determined. A heavy load of PWN seems to have a greater deleterious effect on insect vectors than that of *B. mucronatus*, which may result in a low probability of transmission for individual PWNs compared with *B. mucronatus*.

The JIVs of *B. mucronatus* enter the tracheal systems of vectors and are transmitted to pine trees via feeding and oviposition wounds similar to those used by the PWN (Mamiya and Enda 1979; Schroeder and Magnusson 1992). The transmission curves of *B. mucronatus* via the feeding wounds by *M. saltuarius* adults are unimodal, and the peaks of the transmission curves occur 20–30 days after beetle emergence (Jikumaru and Togashi 2001). There are no L-shaped transmission curves, suggesting a close relationship between the virulence and transmission curve of nematodes.

Interrelation between invasive and indigenous nematodes and their crossing

Nagashima et al. (1975) found both the PWN and *B. mucronatus* in identical dead trees. The results of study in which both the PWN and *B. mucronatus* were inoculated simultaneously onto pine saplings indicated that the ratio of the two species does not change between 1 and 7 days immediately following the inoculation (Dozono and Kiyohara 1976). However, it is difficult to find *B. mucronatus* in wood samples taken from dead *P. densiflora* trees that were inoculated with the two nematode species (Jikumaru et al. 2000). This indicates the replacement of *B. mucronatus* by the PWN in pine forests through competition within trees, even though the presence of *B. mucronatus* deters the boarding of PWNs on *M. alternatus* (Jikumaru and Togashi 2004).

Both sexes of the PWN have sex pheromones (Kiyohara 1982). It is unlikely that the PWN and *B. mucronatus* mate in nature because the females of PWN or *B. mucronatus* do not attract heterospecific males (Riga and Webster 1992). Crossing experiments between three Japanese isolates of PWN and eight Japanese isolates of *B. mucronatus* resulted in the production of F_1 progenies, but the F_1 hybrids failed to produce the F_2 generation (Mamiya 1986). However, crossing between some North American and Japanese isolates of the PWN and European isolates of *B. mucronatus* did produce fertile progeny (de Guiran and Bruguier 1989; Bolla and Boschert 1993). It has been recently reported that fertile progeny were obtained by crossing Japanese isolates of PWN and those of *B. mucronatus* under laboratory conditions (Taga and Togashi 2006). The introgression of genes from *B. mucronatus* to PWN may occur in field.

Evolution of the PWD system

In the PWD system, two or more genotypes of PWN can infect the same tree – i.e. multiple infections – because of the transmission of PWN by two or more vectors to identical pine trees and the horizontal transmission of PWN between both sexes of vectors (Arakawa and Togashi 2002). Theoretically, multiple infections of the parasite lead to a higher mean virulence level than that expected to produce the maximum basic reproductive rate due to the competitive exclusion of less virulent strains by more virulent strains within hosts or to the infection of more virulent strains on hosts already infected by less virulent strains (Nowak and May 1994; May and Nowak 1995). Multiple infections also lead to an expanded variance in virulence.

The PWN is native to North America and does not induce the epidemics of PWD on the native pine forests in the original habitats due to the resistance of pine trees or to the cool summer temperatures that are unsuitable for PWD development (Rutherford et al. 1990). In Japan, *B. mucronatus* also does not induce epidemics on Japanese native pine forests because of no or little virulence. These facts suggest that no or less virulence of the nematode has been selected for in the PWD system.

Positive correlations between the virulence, reproduction rate, and dispersal of PWN within living pine trees lead to an increasing ratio of virulent individuals in trees. An increasing mean resistance of pine trees surviving the PWD epidemics may enhance the suppressive effect on the epidemics. However, as yet no studies have been carried out that elucidate the relation between virulence and the transmission of PWN. Hence, it is difficult to predict the evolution of the PWD system.

Few studies have clarified an evolutionary change in epidemiological and ecological traits of the component species in the PWD system. For better understanding of the evolution of the PWD system, spatio-temporal changes in the mean and variance of the virulence of PWN populations as well as in tree resistance and the vector's transmission should be studied during the progression of PWD epidemics within individual pine stands. In addition, if the number of loci related to the virulence of the PWN and the number of alleles on respective loci are determined, the dynamics of the relative frequencies of these alleles should be studied in pine stands.

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