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Geographical variation in seasonality and life history of pine sawyer beetles *Monochamus* spp: its relationship with phoresy by the pinewood nematode *Bursaphelenchus xylophilus*

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Abstract

1 *Bursaphelenchus xylophilus* (Steiner & Buhrer) (Nematoda: Aphelenchoididae), the pinewood nematode and the causal agent of the pine wilt disease, is a globally important invasive pathogen of pine forests. It is phoretic in woodborer beetles of the genus *Monochamus* (Megerle) (Coleoptera, Cerambycidae) and has been able to exploit novel indigenous species of *Monochamus* (but only *Monochamus*) in newly-invaded areas. North America (NA) is the continent of origin for the *B. xylophilus/Monochamus* spp. phoretic system. NA also contains the largest number of *Monochamus* species known to act as vectors for *B. xylophilus*. Understanding this phoretic system in its native geographical area helps to explain the evolutionary ecology of pine wilt disease.

2 In the present study, we measured the flight phenology, size, sex ratios and species identity of *Monochamus* species in five geographically distant forests in NA. We also measured phoresy by *B. xylophilus*.

3 We found the nematode to be abundant across eastern NA but rare or absent in western NA. In eastern forests, nematode phoresy was highest on the *Monochamus* species that flew earliest in the year. However, in the southeast, where *Monochamus* is most likely multivoltine with a long flight season, we found vectors with high nematode loads throughout the season, indicating that *B. xylophilus* can be transmitted to new hosts during most part of the year. The frequency distribution of nematode dauers on *Monochamus* was highly aggregated. *Bursaphelenchus xylophilus* in NA appears to be able to use all available *Monochamus* species as vectors.

4 In native NA pine forests, the pinewood nematode appears to have an ecology that is sufficiently flexible to exploit different species (and both genders) of *Monochamus*, and disperse at different times of the year. This flexibility may contribute to its recent success in invading Eurasian pine forests.

Keywords Biological invasions, flight phenology, North America, pine forests, plant parasitism, zero-inflated negative binomial distribution.

Introduction

Nematode–insect phoretic associations are very common, and have independently evolved in several nematode orders (Massey, 1974; Giblin-Davis *et al.*, 2003). In a very small subset of nematode lineages, exclusively within the orders Aphelenchida and Tylenchida, tritrophic associations involving both insects and plants have also evolved, which allowed the development of insect-vectored plant parasitism (Giblin-Davis *et al.*, 2003).

The pinewood nematode *Bursaphelenchus xylophilus* (Steiner & Buhrer) (Nematoda: Aphelenchoididae) is one of the few examples of a nematode species forming a tritrophic

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interaction with an insect acting as a dispersing vector and a plant host. This species is the causal agent of pine wilt disease. Originating from North America (NA), pinewood nematode is considered to be the most serious pine pest in Eurasia, where it is invasive (Yang & Wang, 1989; Togashi & Jikumaru, 2007; Vicente *et al.*, 2012). Most species within the genus *Bursaphelenchus*, including the closest relatives of *B. xylophilus*, are fungal feeders transmitted among dead and dying trees by insects (vectors) who are seeking the same resources for oviposition. This is apparently the ancestral life history from which the pinewood nematode is derived. The genome of *B. xylophilus* not only includes many sequences associated with mycophagy, but also a novel set of genes allowing the synthesis of enzymes for digesting plant tissues and detoxifying plant secondary metabolites (Kikuchi *et al.*, 2011). Thus, this species appears to be an unrefined non-obligate plant parasitic nematode that represents a relatively recent evolutionary origin of plant parasitism (Kikuchi *et al.*, 2011; Shinya *et al.*, 2013).

The pinewood nematode is almost exclusively associated with pine sawyer beetles, such as species of *Monochamus* (Megerle) (Coleoptera, Cerambycidae) (Linit, 1988; Akbulut & Stamps, 2012), although many species of *Monochamus* can function as vectors. In North America, where the pinewood nematode is native, six of eight native *Monochamus* species (Linsley & Chemsak, 1984) have been recorded as vectors: *Monochamus scutellatus* (Say), *Monochamus marmorator* (Kirby), *Monochamus mutator* (LeConte), *Monochamus notatus* (Drury), *Monochamus carolinensis* (Olivier) and *Monochamus titillator* (Fabricius) (Wingfield & Blanchette, 1983; Bergdahl *et al.*, 1991). Invasive populations of *B. xylophilus* in Asia and Europe have become associated with native species of *Monochamus*, *Monochamus alternatus* (Hope) (Togashi & Jikumaru, 2007) and *Monochamus galloprovinciallis* (Olivier) (Sousa *et al.*, 2001).

The genus *Monochamus* can be found throughout pine forests of the northern hemisphere. Adults feed on twigs of healthy pines and then oviposit into the bark of dying or recently dead pines. Larvae feed on phloem, and late larvae bore a U-shaped gallery into the xylem where they pupate before boring an almost circular exit hole (Linsley & Chemsak, 1984). These insects have not been well studied because, until the spread of pine wilt disease into Eurasia, they were at most a minor forest pest that rarely killed trees (Hughes & Hughes, 1982). Existing knowledge of *Monochamus* life history and population dynamics includes work from East Asia, which has the longest history of serious pine wilt disease (Togashi & Jikumaru, 2007), as well as North America (Linit, 1988; Bergdahl *et al.*, 1991; Togashi *et al.*, 2009) and Portugal (Sousa *et al.*, 2001). Our work contributes to knowledge partly by sampling *Monochamus* populations at a broader spatial scale than most previous papers, as well as by adding to very limited data from North America, where pinewood nematodes are native.

Phoretic tritrophic interactions are generally characterized by strong species specificity and life cycles that are synchronized among the three species (Krishnan *et al.*, 2010). Disentangling these interactions is essential for understanding the epidemiology of plant parasitism involving phoretic associations. The pinewood nematode and pinewood sawyers both subsist on fresh phloem of recently dead pine trees, which is a highly ephemeral resource. Thus, their life-cycles are necessarily synchronized within this rapidly degrading environment (Linit, 1988). With the Asian *M. notatus* and American *M. carolinensis*, degradation of the subcortical environment of host trees leads to a switch in *B. xylophilus* development from the reproductive form to a dispersal juvenile that tends to aggregate around *Monochamus* pupal chambers (Necibi & Linit, 1998; Sone *et al.*, 2011). It appears that genus-specific chemical substances produced during *Monochamus* pupation are the signal for *B. xylophilus* dispersal juveniles to molt into resistant dauers that climb onto callow adult sawyer beetles before they exit the tree (Necibi & Linit, 1998; Sone *et al.*, 2011).The pinewood nematode can develop associations with different species of *Monochamus*, including novel species in previously unoccupied areas, although it is not known how the extent and efficacy of phoresy is influenced by variation among pine sawyers in phenology, voltinism, developmental rates, behaviour, size and fecundity (Linsley & Chemsak, 1984; Akbulut & Stamps, 2012). Variation in the life-history traits of *Monochamus* could impact the population dynamics of pinewood nematodes and the epidemiology of pine wilt disease. For example, size and sex are generally related to dispersal in insects (Davis, 1984), which is important to epidemiology (Kay & Farrow, 2000). Furthermore, females are the sex that creates oviposition openings on pine trunks for *B. xylophilis* that are destined to be saprophytic, whereas both sexes carry out maturation feeding in the twigs where they could inoculate pathogenic nematodes into healthy trees (Wingfield & Blanchette 1983; Wingfield *et al.*, 1984). Size can also influence resource partitioning (Coulson *et al.*, 1980; Hughes & Hughes, 1982; Flamm *et al.*, 1989), which could influence the tree species, tree size class and/or portions of the tree that are most likely to be inoculated with nematodes. Phenology of adult flight and voltinism could also influence suitability as vectors for *B. xylophilus*, and therefore influence seasonal patterns of tree mortality (Togashi & Jikumaru, 2007). Different *Monochamus* species can occupy different climatic zones (Linsley & Chemsak, 1984) and climate is regarded as a major determinant in the occurrence of pine wilt disease (Rutherford & Webster, 1987).

Knowledge of the *Monochamus/B. xylophilus* system in NA native pine forests is informative in part because this was the area where the system initially evolved. Wingfield & Blanchette (1983, 1984) hypothesized that native NA pines gained resistance, leading *B. xylophilus* to depend upon a saprophytic life-style, at the same time as still retaining some pathogenicity for debilitated and non-native pine trees. In this view, populations of pinewood nematode that have invaded other continents may be most virulent because the relationship among *B. xylophilus*, *Monochamus* and *Pinus* is young in these regions. However, the capacity for virulence probably evolved on its native continent (Shinya *et al.*, 2013). Presumably, *B. xylophilus* has had time to occupy available niches in NA but remains to do so in Eurasia. One factor that could constrain the life history of *B. xylophilus* is the life history of the *Monochamus* species that are available as vectors. Consistent with this, NA is the continent with the largest recorded number of *Monochamus* species acting as vectors for *B. xylophilus*. Thus, NA provided the opportunity to characterize associations of *B. xylophilus* with *Monochamus* species in several widely separated regions that contained different communities of pine sawyer beetles.

In the present study, we explored: (i) the flight phenology of resident *Monochamus* species in five geographically distinct forests in NA and (ii) the prevalence and intensity of *B. xylophilus* across forests, seasons, species and sexes of *Monochamus*. Our objectives were to: (i) increase knowledge of the life-history, biogeography and phoretic associations of pine sawyer beetles and (ii) provide a baseline, against which newly-invaded forests can be compared, of the incidence of pinewood nematodes in forests where they are native.

Material and methods

Areas of study

The present study was made in five native pine forests in the eastern and western U.S.A., each with a distinctive pine species and climate: (i) a forest of the Northeast with mainly white pine *Pinus strobus* (L.) and some red pine *Pinus resinosa* (Aiton), located between New Hampshire and Vermont (VT: 43°42′N, 072°17′W, 162 m); (ii) the Pinelands in New Jersey (NJ: 39°57′N, 074°30′W, 27 m), with mainly pitch pine *Pinus rigida* (Mill.); (iii) the Kisatchie National Forest, in Central Louisiana (LA: 31°20′N, 92°24′W, 36 m), with longleaf pine, *Pinus palustris* (Mill.), loblolly pine *Pinus taeda* (L.), slash pine *Pinus elliottii* (Engelm.) and shortleaf pine *Pinus echinata* (Mill.); (iv) a forest near Flagstaff, Arizona, containing ponderosa pine *Pinus ponderosa* (Laws.) and pinion pine *Pinus edulis* (Engelm.) (AZ: 35°16.4′N, 111°50.3′W, 2251 m); and (v) the Lassen National Forest in California, with ponderosa and Jeffreypine *Pinus jeffreyi* (Balf.) (CA: 40°50.2′N, 121°26.5′W, 1400 m) (Fig. 1).

Field and laboratory study

In each forest, we deployed five Lindgren Multi-Funnel Traps with 12 black funnels (Lindgren, 1983), separated from each other by at least 3 km. We modified dry 5-L containers for these traps, and sprayed the inner part of the bottom funnel with Fluon® (Fisher Scientific, Pittsburgh, Paeensaylvania), which leaves a slippery surface and allows more efficient trapping of large insects (Morewood *et al.*, 2002; Graham *et al.*, 2010). Adult longhorn beetles are attracted to stressed trees or recently cut logs for mating and oviposition (Hughes & Hughes, 1982). They are attracted to a mix of bark beetle pheromones and the volatiles released from host trees (monoterpenes and ethanol; Morewood *et al.*, 2002; Miller *et al.*, 2013). Thus, we baited traps with α-pinene (170 g, releasing approximately 2 g/day) and Ipsenol (100 mg, releasing approximately 500 µg/day) (Synergy Semiochemicals Corp., Canada). Baits were replaced every 30 days. As much as possible, the traps were placed in areas with recently dead trees from storms, drought and bark beetles. *Bursaphelenchus xylophilus* has not been reported as a significant cause of tree mortality in any of our study areas. The traps were deployed sufficiently early in the season that there were always at least 2 weeks before we captured the first *Monochamus*. Traps were then checked weekly to collect live animals until at least 2 weeks after new captures ceased. On each sampling occasion, fresh pine shoots were placed inside the traps cups to provide substrate and food for insects until the next trap check. This worked in that our trapping produced large numbers of living *Monochamus* drawn from the population of free-flying adults in each study area. Flight traps offered the advantage of directly sampling the life stage of *Monochamus* that transports and transmits pinewood nematodes among host trees. An alternative strategy for estimating the frequency of phoresy is to capture *Monochamus* as they emerge from pine logs, although Kinn (1987) found a four-fold higher frequency of phoresy in *Monochamus* captured with flight traps compared with beetles emerging from field collected bolts. Furthermore, the flight traps were relatively easy to deploy and monitor, which made possible the study of geographical distant locations.

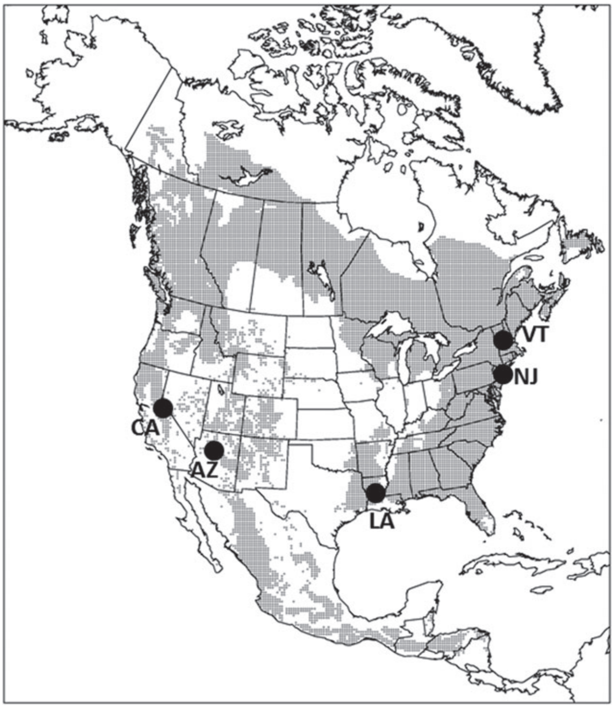

Figure 1 Distribution of pine forests in North America (grey area) with the location of our five study sites: CA, Lassen National Forest in California; AZ, Flagstaff in Arizona; LA, Kisatchie National Forest in Louisiana; NJ, Pinelands in New Jersey; VT, Upper Valley in Vermont/New Hampshire. Pine forest distribution was obtained from the U.S. Geological Survey web site (http://pubs.usgs.gov/pp/p1650-a/pages/conifers.html).

Studies were conducted during 2010 (AZ and NJ) and 2011 (CA, LA and VT). In AZ, LA and VT, *Monochamus* were monitored throughout the entire flight period, whereas NJ and CA were only sampled during 4 weeks of the early flight period.

All captured *Monochamus* were identified and sexed. In 2011 (CA, LA and VT), the body length of each beetle was also measured with a digital caliper (range 0–150 mm, resolution 0.01). To isolate phoretic nematodes from *Monochamus*, each beetle was wrapped in a Kimwipe (Kimberly-Clark, Irving, Texas), and placed in a small funnel filled with Milli-Q™ water (Millipore, Billerica, Massachusetts) for 24 h (for details on the methodology, see OEPP/EPPO, 2009). The number of dauers carried by each *Monochamus* was counted using a dissecting microscope. To confirm species identity, we cultured dauers from all the *Monochamus* vectors captured in NJ, CA and VT, and from 20% of the vectors captured in LA. Live dauers were introduced into fungal cultures [with *Botrytis cinerea* (De Bary) Whetzel or *Ophiostoma minus* (Hedgc.) Syd. & P. Syd] and kept in growth chambers at 25°C. After a few weeks, the cultures were extracted. We obtained adult nematodes in 70% of the cases, which were identified with the help of a phase contrast microscope. We identified *B. xylophilus* and *Diplogasteroides* sp. (Diplogasterida, Diplogasteroididae). In all samples from VT and LA, the dauers were also checked directly with phase microscopy, being easily assigned as Diplogasteroididae or Aphelenchoididae as a result of their body shape. Because *B. xylophilus* was the only Aphelencoides identified in the adults obtained from fungal cultures, we assumed that all dauers from this group were pinewood nematode.

Statistical analysis

For each site, we evaluated patterns in *Monochamus* body size with a general linear model that included species and gender as fixed effects and Julian date of trapping as a continuous variable (JMP® 8; SAS Institute Inc., Cary, North Carolina). Data satisfied assumptions of normality and equal variances.

To analyze the abundance of *B. xylophilus* dauers on *Monochamus*, we first compared the goodness of fit of four alternative frequency distributions for dauers/*Monochamus* via maximum likelihood estimation with the R package VGAM (Yee, 2008). The candidate distributions were: (i) Poisson; (ii) negative binomial; (iii) zero-inflated Poisson; and (iv) zero-inflated negative binomial. All describe a discrete distribution, which is a good phenomenological match with our system (Bolker, 2008). The negative binomial is favoured over the simpler Poisson if the inverse of the overdispersion parameter (k) is significantly different from zero (log 1) (Yee, 2008; Zeileis *et al.*, 2008). Models 3 and 4 are finite mixture models that add an additional parameter (phi) to allow for a surplus of zero counts relative to their simpler analogues.

Because it was strongly favoured in the model comparisons, we went on to use the zero-inflated negative binomial to compare levels of phoresy across regions, *Monochamus* species, vector size and vector gender. The model describes: (i) the probability that an individual *Monochamus* carries pinewood nematode and (ii) the frequency distribution of nematode dauers on those that carry it. We estimated and compared parameters of zero-inflated negative binomial regression models (ZINBR) with the R package PSCL (Jackman, 2008).

Results

Identity, life history and abundance of Monochamus spp.

As expected, different species of *Monochamus* were trapped from different regions of North America: In VT, *M. scutellatus* and *M. notatus*; in NJ and LA, *M. carolinensis* and *M. titillator*; in CA, *M. obtusus* (Casey) and *M. clamator* (LeConte); and, in AZ, only *M. clamator*. In western forests, AZ and CA, both located at high altitude, the flight period started relatively late, in mid-June and mid-July, respectively. Flights lasted approximately 3 months (until early September) in AZ (where we had data for the entire flight period). In the east, flights started in April in LA, May in NJ and June in VT. In LA, the flight period lasted 7 months (until November), whereas, in VT, it lasted 3 months (until September; Figs 2 and 3). We trapped the most *Monochamus* in LA (1906) and the fewest in AZ and VT (44 and 46, respectively).

Monochamus clamator was the only species found in AZ. Each of the other four regions contained two sympatric species of *Monochamus* that were both reasonably abundant. Average body sizes ranged from 16 to 24 mm across species and sexes. In LA, *M. notatus* adults averaged approximately 30% longer than *M. scutellatus* and, in VT, *M. titillator* averaged approximately 25% longer than *M. carolinensis*. In CA, size differences between *M. clamator*

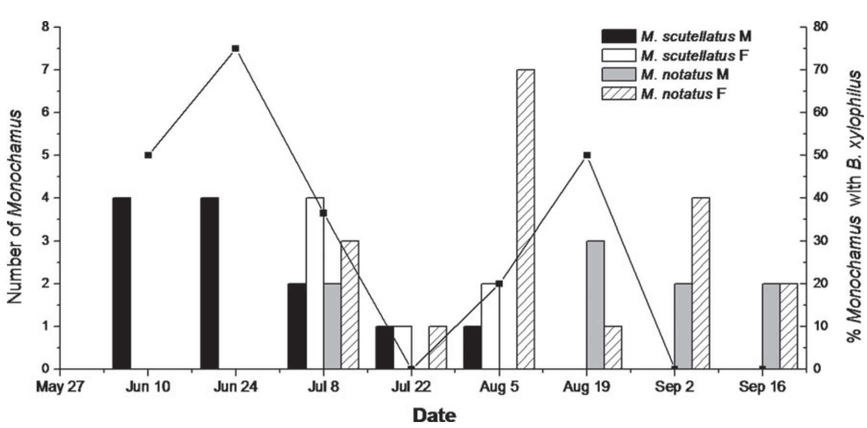

Figure 2 Captures of flying adult *Monochamus* in funnel traps during May to September 2011 in Vermont, according to species (*Monochamus scutellatus* and *Monochamus notatus*) and gender (M, male; F, female). Bars indicate number of captures (left y-axis) and the line indicates the proportion of *Monochamus* adults that were carrying pinewood nematode dauers (right y-axis).

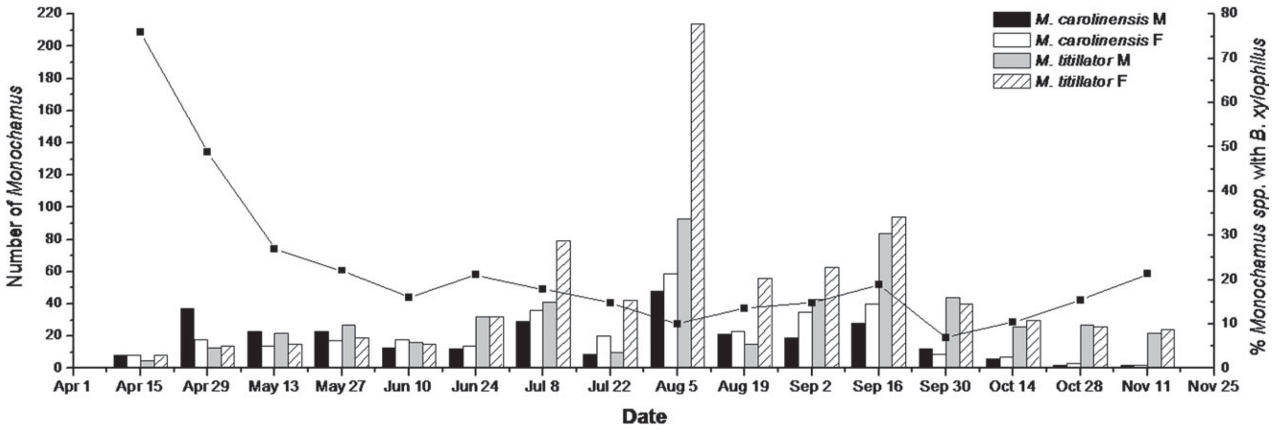

Figure 3 Captures of flying adult *Monochamus* in funnel traps during April to November 2011 in Louisiana, according to species (*Monochamus carolinensis* and *Monochamus titillator*) and gender (M, male; F, female). Bars indicate number of captures (left *y*-axis) and the line indicates the proportion of *Monochamus* adults that were carrying pinewood nematode dauers (right *y*-axis).

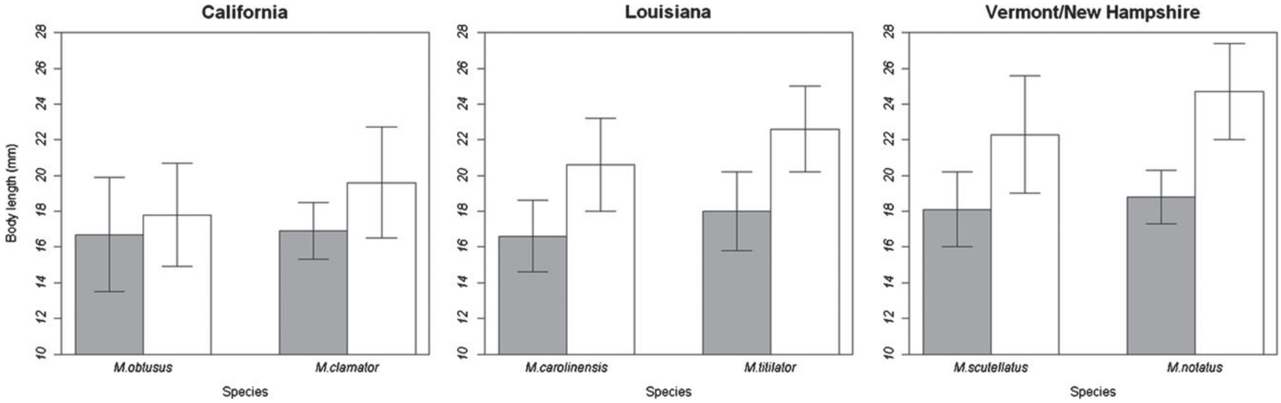

Figure 4 Mean ± SD body length (mm) of *Monochamus* adults in California, Louisiana and Vermont. Labels on the *x*-axis indicate *Monochamus* species and gender (M, male; F, female); closed bars refer to males and open bars refer to females.

and *M. obtusus* were not significant. Males were significantly smaller than females in LA and VT but not in CA (LA: Spp. $F_{1,1834} = 1286.2$, $P < 0.0001$, Gender $F_{1,1834} = 189.3$, $P < 0.0001$, Spp. × Gender $F_{1,1834} = 4.4$, $P = 0.036$; VT: Spp. $F_{1,45} = 40.1$, $P < 0.0001$, Gender $F_{1,45} = 3.8$, $P = 0.057$, Spp. × Gender $F_{1,45} = 1.2$, $P = 0.28$; CA: Spp. $F_{1,14} = 1.46$, $P = 0.25$, Gender $F_{2,14} = 0.38$, $P = 0.55$, Spp. × Gender $F_{1,14} = 0.3$, $P = 0.62$; Fig. 4). In LA and VT, where the complete flight period was monitored, the smaller *Monochamus* species tended to predominate at the beginning of the season (*M. carolinensis* in LA and *M. scutellatus* in VT; regression between the proportion of smaller *Monochamus* species and Julian date: LA % *M. carolinensis* = 77.8 to 0.21 × Date, $R^2 = 0.78$, $P < 0.0001$, $N = 20$; VT % *M. scutellatus* = −280.8 to 1.17 × Date, $R^2 = 0.64$, $P < 0.0001$, $N = 20$; Figs 2 and 3).

Males from the smaller *Monochamus* species fly earlier than females (*t*-test: VT *M. scutellatus* $t_{15} = 3.31$, $P = 0.0024$; LA *M. carolinensis* $t_{587} = 2.95$, $P = 0.0017$) but not males from the larger species (VT *M. notatus* $t_{14} = 0.52$, $P = 0.70$; LA *M. titillator* $t_{978} = 0.56$, $P = 0.71$) (Figs 2 and 3). Flight times of male and female *M. clamator* in AZ did not differ ($t_{41} = 0.69$, $P = 0.25$). The sex ratios were generally female biased (Figs 2 and 3), although less so and even favouring males in early flying species (sex ratio ♂/♀: AZ *M. clamator* 0.83; LA *M carolinensis* 0.90 and *M. titillator* 0.68; VT *M. scutellatus* 1.71 and *M. notatus* 0.50).

Nematode phoresy on Monochamus *spp.*

Phoresy by *B. xylophilus* on *Monochamus* was common in all three eastern forests that we sampled (LA, NJ and VT). We did not observe any *B. xylophilus* phoresy on *Monochamus* in the two western forests (AZ and CA). The distribution of *B. xylophilus* dauers on *Monochamus* individuals was highly gregarious, with many *Monochamus* having no nematodes but 20–30% having up to a few hundred dauers, and some individuals having several thousand, to a maximum of approximately 20 000 (Fig. 5). The zero-inflated negative binomial distribution (ZINB) provided a good fit to the observed distributions of dauers/*Monochamus*, and a much better fit (larger log-likelihood) than any of the three competing distributions in LA and VT, where we had data for the entire flight period (Table 1). In Lousiana, where we had the most captures, the estimated phi (representing the proportion of *Monochamus* that

Table 1 Parameter estimates and goodness of fit from four candidate probability density functions to describe phoresy of pinewood nematode dauer larvae on *Monochamus spp*.

			Phi			μ			*K*			
Data set	Distribution	*n*	Middle	Lower	Upper	Middle	Lower	Upper	Middle	Lower	Upper	Log-likelihood
LA	P	1549	–	–	–	173	172	178	–	–	–	−765757
	ZIP		0.81	0.79	0.83	921	917	924	–	–	–	−319199
	NB		–	–	–	172	125	238	0.02	0.02	0.03	−2900
	ZINB		0.79	0.76	0.81	811	650	1012	0.27	0.21	0.34	−2867
VT	P	46	–	–	–	474	468	481	–	–	–	−42614
	ZIP		0.72	0.57	0.83	1679	1657	1701	–	–	–	−15060
	NB		–	–	–	474	106	2116	0.04	0.02	0.07	−138
	ZINB		0.71	0.56	0.83	1639	766	3506	0.46	0.22	0.97	−134
NJ	P	22	–	–	–	415	407	424	–	–	–	−13919
	ZIP		0.23	0.10	0.44	538	527	549	–	–	–	−11574
	NB		–	–	–	416	155	1115	0.18	0.10	0.31	−121
	ZINB		0	0	1	416	155	1115	0.18	0.10	0.31	−121

Data from forests in Louisiana (LA) and Vermont (VT) refer to the entire flight period (7 and 3 months respectively), and data from New Jersey (NJ) refer only to the first 4 weeks of the flight period.P, poisson; ZIP, zero inflated poisson; NB, negative binomial; ZINB, zero inflated negative binomial.

Table 2 Comparison of models that modify estimates from a zero-inflated negative binomial of pinewood nematode dauers per *Monochamus* with parameters for Julian day (JDay), *Monochamus* species, *Monochamus* gender and/or *Monochamus* body length (BL)

		Count model coefficients		Zero-inflation model coefficients			
Area	Factors	*Z*-value	*P*	*Z*-value	*P*	Log-likelihood	d.f.
LA	JDay + Spp. + Sex	0.27, −0.07, −0.86	0.79, 0.95, 0.39	6.22, 2.70, −1.24	< 0.001***, < 0.01**, 0.22	−2836	9
	JDay + Spp.	0.17, 0.01	0.86, 0.99	6.15, 2.65	< 0.001***, < 0.01**	−2837	7
	JDay + BL	0.41, −1.21	0.68, 0.23	4.39, 1.24	< 0.001***, 0.21	−2512[a]	7
	JDay	0.19	0.85	7.07	< 0.001***	−2841	5
VT	JDay + Spp. + Sex	−6.27, 2.80, −2.23	< 0.001***, < 0.01**, < 0.05*	0.65, 1.49, 1.04	0.51, 0.14, 0.30	−120[a]	9
	JDay + Spp.	−5.38, 1.78, 0.50	< 0.001***, 0.08	0.98, 1.57	0.33, 0.12	−122	7
	JDay + BL	−5.32, 1.24	< 0.001***, 0.21	−2.19, 2.15	< 0.05*, 0.93	−124	7
	Jday	−4.70	< 0.001***	2.48	< 0.05*	−125	5

Asterisks indicate different levels of significance.

[a] Best model for each one of the two areas according to the maximum likelihood.

Shown are values for the *Z*-test and its probabilities for each parameter in the two states of the model. Data were obtained from 1549 *Monochamus* captures in Louisiana (LA) nand 46 in Vermont (VT).

lacked *B. xylophilus*) was 0.79 (approximately 95% confidence interval = 0.76–0.81), the estimated μ (a measure of central tendency of dauers/*Monochamus* when *Monochamus* were present) was 811 (650–1012) and the estimated *k* (inverse of overdispersion) was 0.27 (0.21–0.34). In VT, estimates were similar (Table 1) with a tendency for more *Monochamus* with dauers, more dauers per *Monochamus* when present, and somewhat weaker overdispersion. In NJ, where we only monitored the first 4 weeks of the flight period, 47% of *Monochamus* carried *B. xylophilus* (phi was close to 0) and the best fit was to the simple negative binomial distribution. In VT and LA, many zeros appeared later in the flight period. Our three study regions east of the Great Plains, although separated by 500–2500 km, were surprisingly similar with respect to phoresy by pinewood nematodes when comparing the first 4 weeks of the flight period; this was true both for the fraction of *Monochamus* with no phoretic nematodes and for the number of dauers per *Monochamus* when nematodes were present ($P > 0.30$ for comparisons of count coefficients and zero-inflation coefficients; 960 ± 1691 *B. xylophilus* dauers per *Monochamus* with at least some phoresy, N = 106 *Monochamus*).

For Louisiana, we were able to further test for possible effects on the ZINB models of Julian date, *Monochamus* species, *Monochamus* gender and/or *Monochamus* body length. The best model, as indicated by the log-likelihood, included Julian date and body length (Table 2). Examination of coefficients and *P* values indicated that the strongest relationship was in a seasonal increase in the proportion of *Monochamus* carrying no nematodes (Fig. 3). In VT, the best model included Julian date, vector species and gender. In this case, the patterns were in dauers/*Monochamus* given that there were dauers present: dauers per *Monochamus* decreased during the season, were higher on males than females (2049 ± 2437 and 846 ± 1310 respectively) and higher on *M. scutellatus* than *M. notatus* (1865 ± 2372 and 1059 ± 1510, respectively) (Fig. 2 and Table 2).

We also found occasional but widespread occurrences of phoresy on *Monochamus* of a different nematode that is nonpathogenic and only distantly related. In LA, 21 of 1549 *Monochamus* individuals carried three to 510 dauers of *Diplogasteroides* sp. (Diplogasterida, Diplogasteroididae). This distribution was also well fit by a zero inflated negative binomial:

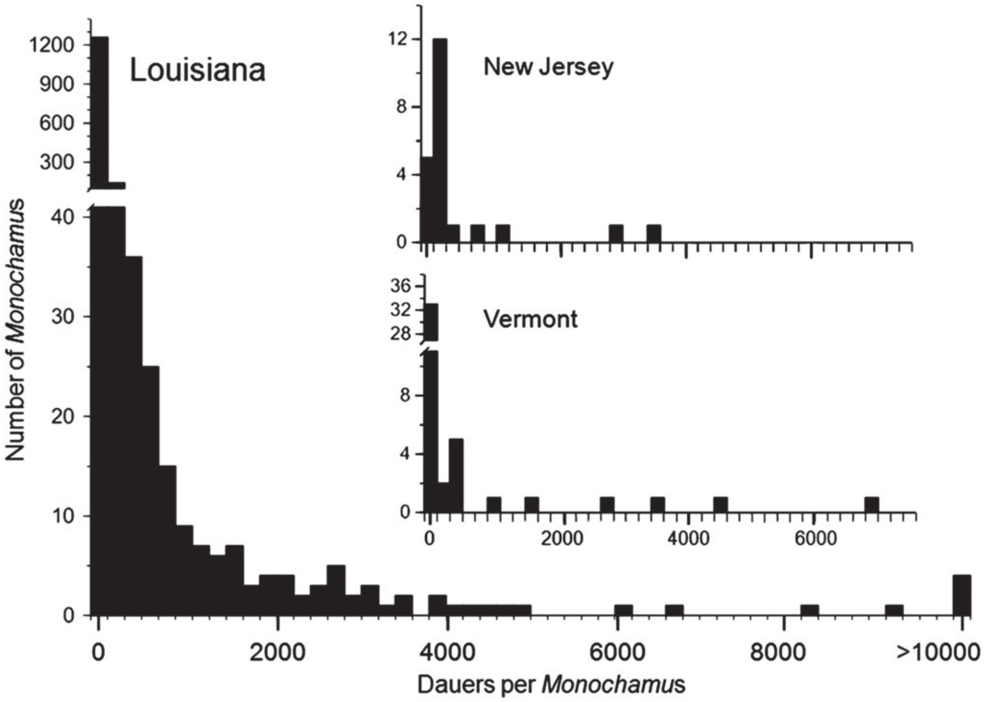

Figure 5 Histograms of the number of pinewood nematode dauers carried by individual *Monochamus* adults trapped in Louisiana, Vermont and New Jersey. The first bar in each histogram shows the number of *Monochamus* with no dauers; the size of all other bins is 200 units of the *x*-axis. Data from Louisiana and Vermont correspond to the complete flight period, whereas data from New Jersey correspond to the first 4 weeks of the flight period.

phi = 0.98 (0.97–0.99), $\mu = 94$ (44–199) and $k = 0.38$ (0.14–1.02); log-likelihood = −226 versus < −1600 for competing models). Elsewhere, numbers of *Monochamus* carrying *Diplogasteroides* sp. were one of 46 in VT, eight of 22 in NJ, six of 15 in CA and zero of 44 in AZ; when present, dauers per *Monochamus* were similar to that in LA.

Discussion

We found *B. xylophilus* to be ubiquitous in pine forests of eastern North America but rare and patchy in the west. The abundance of *B. xylophilus* per *Monochamus* was relatively stable in the eastern forests that we sampled from Louisiana to Vermont/New Hampshire. However, *Monochamus* were more abundant in the sub-tropical South than in the sub-boreal North, and therefore so was *B. xylophilus*. Furthermore, *B. xylophilus* was highly aggregated on its *Monochamus* vectors, and we found a clear pattern of higher incidence of phoresy early in the flight season.

Identity, life-history and abundance of Monochamus *in North America*

Our sampling in forests east of the Great Plains revealed two sympatric species of *Monochamus*: *M. scutellatus* and *M. notatus* at the Northern location in VT, and *M. carolinensis* and *M. titillator* in LA and NJ. Where we could monitor the complete flight period (VT and LA), the smaller species (*M. scutellatus* and *M. carolinensis*) flew earlier than larger species (*M. notatus* and *M. titillator*). Males tended to be smaller than females and to fly earlier in the season. There was a large difference in the length of the flight period between these two areas, which is presumably related to differences in temperature and the length of the plant growing season. If adult *Monochamus* live approximately 2 months (Togashi *et al.*, 2009), populations in VT are most likely univoltine and, in LA, are most likely multivoltine. In VT, there was less overlap between the flight period of both species, with only *M. scutellatus* emerging at the beginning of the season, and *M. notatus* at the end. In LA, both *Monochamus* species occurred throughout the flight period, although *M. carolinensis* accounted for most of captures at the beginning of the season and then became relatively less abundant as the season progressed. This pattern was also found in East Asia where the smaller *Monochamus saltuarius* emerge earlier than the larger *M. alternatus* (Togashi & Jikumaru, 2007).

A general relationship between body size and development time is predicted from life-history theory (Roff, 1992) and is broadly evident for insects (García-Barros, 2000). Thus, if two sympatric *Monochamus* species start larval development at the same time, and all things are equal, individuals from the smaller species will tend to emerge earlier. Divergence in body size between two sympatric *Monochamus* species might arise from interspecific competition within the phloem of infested pine trees (Coulson *et al.*, 1980; Flamm *et al.*, 1989). In this system, early differences in larval body size will lead to increasingly asymmetric competition, perhaps favouring early cessation of larval feeding (and therefore smaller adult size) in one of the species. The tendency for *Monochamus* males to be smaller than females reflects a common pattern in arthropods (Teder & Tammaru, 2005). This trend is generally attributed to fecundity selection favouring large females that can lay larger or more eggs than small females (Honěk, 1993). The tendency for *Monochamus* males to emerge earlier as adults and therefore predominate at the beginning of the flight season is also a common life-history pattern in arthropods (protandry, Wiklund & Fagerström, 1977).

Patterns were different in the western U.S.A. in that there were no difference in size and timing of flight for the two *Monochamus* species trapped in CA, and, in AZ, we found only one species, namely *M. clamator*. In this respect, AZ was similar to Portugal, where the only *Monochamus* species, and therefore only vector for *B. xylophilus*, is *M. galloprovinciallis* (Sousa *et al.*, 2001). With our traps, baited with pine volatiles and bark beetle pheromones, we captured not only *Monochamus* spp., but also seven to 10 other species of wood borers. These wood borers, together with bark beetles, form a rich community of pine phloem feeders with strong interspecific competition and some intraguild predation (Coulson *et al.*, 1980; Dodds *et al.*, 2002; Schoeller *et al.*, 2012). Understanding the ecological niche of *Monochamus* spp. and its interaction with other members of the guild is probably crucial for explaining the coexistence of species. Adaptation to local environmental conditions can also be important (Koutroumpa *et al.*, 2013).

Patterns of B. xylophilus *phoresy*

Previous information suggested that *M. carolinensis* was the preferred vector for *B. xylophilus* in NA (Linit, 1988; Akbulut & Stamps, 2012). However, there was no obvious explanation for this preference in relation to the other NA *Monochamus* species. *Monochamus carolinensis* occurs throughout eastern NA (Linsley & Chemsak, 1984) and is very common in the southeast, an area suitable for the development of the pine wilt disease as a result of its climate (Rutherford & Webster, 1987; Linit, 1988). However, in northern forests, *M. scutellatus* also appeared to be an important carrier for *B. xylophilus* (Wingfield & Blanchette, 1983; Bergdahl *et al.*, 1991). Our work confirmed that *M. carolinensis* and *M. scutellatus* are important vectors. We could not, however, determine whether there was preference by *B. xylophilus* for these particular species versus a tendency to differentially occur on *Monochamus* species that fly earlier.

A higher phoretic load in earlier flying vectors was found in another nematode/insect phoretic system (Cardoza *et al.*, 2006), as well as in phoretic mites, which are the best studied insect phoretic systems (Schwarz & Müller, 1992; Mori *et al.*, 2011). A potential explanation is a population build-up, and resource degradation of the phloem, during the autumn, winter and spring, until the first vectors start to emerge. This was illustrated in the case of VT, a cold area where nematodes and beetles have fewer degree days from one summer to the next. In this area, there is a short time overlap between both vector species, and the nematode load on infected animals is clearly higher on the earlier *M. scutellatus* males, with steadily decreasing phoresy as the season progresses, being much lower in the later *M. notatus*. The warmer climate in LA presumably allows for more rapid growth of the nematode population within a tree, although it probably also leads to faster degradation of the phloem resources. Warmer temperatures in LA are also related to increased rates of new host trees becoming available as a result of tree mortality from lightning, seasonal droughts and bark beetle activity (own observations; Kinn & Linit, 1992). In LA, as in VT, the frequency of dauers (on *M. carolinensis)* was highest early in the year but, unlike in VT, 10–20% of the *Monochamus* were still carrying dauers in the late season and the number of nematodes per infected vector was as high as earlier in the summer. This implies that, in LA (but not VT), there were new vectors with heavy loads of nematodes appearing throughout the flight season. It should be expected that older *Monochamus* adults, which can survive for several months, have fewer *B. xylophilous* per *Monochamus* because the nematodes exit their vector during maturation feeding and oviposition (Togashi, 1985; Linit, 1988, 1989). Presumably, the increasing proportion of *Monochamus* that lack nematodes as the summer progresses is partly the result of an increasing proportion of older *Monochamus* adults.

In Japan, there are also two sympatric *Monochamus* species, although the smaller and earlier species (*M. saltuarius*) appears to be a less important vector for *B. xylophilus* than the larger and later species (*M. alternatus*). Togashi and Jikumaru (2007) have proposed that this is because *B. xylophilus* is responsible for most of the tree mortality in this area, and therefore is the source of new hosts for *Monochamus*. Because the onset of the pine wilt disease takes time, a large number of dead trees are only available later in the season when *M. alternatus* is better able to exploit them than *M. saltuarius*. Thus, differences in *B. xylophilus* pathogenicity may contribute to differences between NA and East Asia in the seasonality of phoresy and the dominant vectors.

Because the pinewood nematode does not appear to be a plant pathogen in its native habitat Wingfield *et al.* (1984) suggested that *B. xylophilus* transmission in NA, is primarily through ovipositioning. In this case, *B. xylophilus* in NA pine forests would depend primarily on female vectors, and there should be selection for preferentially colonizing female *Monochamus*. By contrast to this expectation, we saw no clear gender preferences in *B. xylophilus* phoresy, and sometimes males even appeared to be preferred. In other insect/nematode phoretic systems, dauers are specifically attracted to the gender and life stages of vectors that provide the most reliable transport to new resource patches (Krishnan *et al.*, 2010; Yoshiga *et al.*, 2013). Apparently, *B. xylophilus* dauers can move from male to female *Monochamus* during mating (Togashi & Arakawa, 2003), although it still is probable that females would afford more efficient transport if oviposition was the major means of transmission. Transmission of dauers during maturation feeding of *Monochamus* has been confirmed to occur in NA (Wingfield & Blanchette, 1983; Luzzi *et al.*, 1984), and Kinn and Linit (1992), working in Louisiana, reported that *B. xylophilus* could survive in putatively resistant native pine species and may contribute to tree mortality (partly by facilitating attack by bark beetles). The frequency of *B. xylophilus* on male *Monochamus* adds to the evidence that opportunistic pathogenicity might be more important than is generally supposed in native NA pine forests.

We found a highly aggregated frequency distribution of *B. xylophilus* dauers on *Monochamus*, similar to that found in Japan and in plantations of Scots pine infested with pine wilt disease in the southern U.S.A. (Togashi, 1985; Linit, 1989; Sone *et al.*, 2011). A tendency for highly aggregated dispersion has been associated with establishment of the pine wilt disease because more nematodes per plant in the initial inoculation yield greater probability of disease (Togashi & Shigesada, 2006). Thus, our findings suggest that pathogenicity of pinewood nematode might be higher than previously assumed in NA. An alternative explanation for the aggregated dispersion of *B. xylophilus* is that aggregation enhances competitive success with the other nematodes (diplogasteroids) that are also vectored by *Monochamus*, which, according to our preliminary data, outcompete *B. xylophilus* when feeding in cultures of the blue-stain fungi *O. minus*. This is part of a larger community of phoretic nematode species vectored by wood borers and bark beetles in NA pine forests (Massey, 1974). Thus, *B. xylophilus* dauers, when inoculated into recently dead trees through female *Monochamus* oviposition activities, may benefit from high initial numbers to overcome interspecific competition during colony establishment.

The absence of *B. xylophilus* from the two western forests was a new result arising from the present study. Generally, the pinewood nematode is considered widespread throughout North America. However, previous information from the western U.S. states is rare and anecdotal. Indeed, a survey made on stressed trees in California could not confirm previous detection of *B. xylophilus* in the state (Bain & Hosking, 1988), and EPPO/CABI (1997) only lists two more western states (Oregon and Washington) where the occurrence of the pinewood nematode is stated as 'probable'. Thus, it appears that the occurrence of *B. xylophilus* in native western U.S. forests is rare and patchy. One possible explanation is that *B. xylophilus* evolved in eastern NA pine forests. Western and eastern NA native pine forests are geographically isolated by the Great Plains and western pine forests tend to be fragmented as a result of the mountainous terrain and associated climatic gradients (Fig. 1). If the pinewood nematode originated in eastern NA, its extension into western forests could be dispersal limited. Also, western and eastern forests differ in climate and ecology (e.g. different species of pine and *Monochamus*). Further studies of the occurrence and abundance of *B. xylophilus* in in western NA may improve our understanding of the ecology and evolution of this pathogen.

Sampling of flying adult *Monochamus* for quantification of phoretic *B. xylophilus* shows promise as a general tactic for obtaining an improved understanding of the epidemiology of pine wilt disease. The incidence of disease transmission must be related to the abundance of dispersing *Monochamus* adults and the frequency distribution of *B. xylophilus* per dispersing *Monochamus* adult. At present, we know of no comparable published data from the heavily impacted forests of east Asia, nor the newly-invaded forests of the Iberian Peninsula. One study limitation (including the present study) has been the lack of trap lures that are sufficiently attractive to capture *Monochamus* when they are at low to moderate abundance. This limitation is being alleviated by recent advancements (Allison *et al.*, 2012; Fierke *et al.*, 2012).

Conclusion

The present study shows that *B. xylophilus* can be widespread and common in native pine forests of North America that are not obviously afflicted with pine wilt disease, although it is relatively patchy in western pine forests. East of the Great Plains, the pinewood nematode can be found as phoretic on both sexes and all available species of *Monochamus* beetles, as well as across a climatic gradient from sub-tropical to sub-boreal. The abundance of nematodes on its vectors is clearly higher early in the flight season, indicating the importance of seasonality in sampling. However in warmer areas, where *Monochamus* have more than one generation and a long flight season, there were always some *Monochamus* with high nematode loads throughout the flight season, indicating that *B. xylophilus* can build populations and be transmitted to new hosts during a long season. The ecological flexibility of *B. xylophilus*, with respect to season, climate and species and gender of vectors, probably contributed to its success in invading pine forests of eastern Asia and, more recently, Western Europe.

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