Worldwide invasion routes of the pinewood nematode: What can we infer from population genetics analyses?

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Abstract Identifying the invasion routes and determining the origin of new outbreaks of invasive species are of crucial importance if we are to understand the invasion process, improve or establish regulatory measures and, potentially, limit the damage. We focused here on the invasion of Europe by the pinewood nematode (PWN), *Bursaphelenchus xylophilus* (Steiner & Buhrer, 1934; Nickle 1970; Nematoda: Aphelenchoididae), a major pest of forest ecosystems, native to North America and already invasive in Asia since the beginning of the twentieth century. We evaluated the genetic diversity and structure of worldwide field PWN samples by classical and Bayesian population genetics methods to determine the source of the European invasive populations and the number of introduction events in Europe. We found (1) a very strong spatial genetic structure in native PWN populations, (2) a very low level of polymorphism in each of the invaded areas and (3) contrasted results concerning the origin of European invasive populations. Our findings provide evidence for: (1) a large effect of genetic drift on the biological cycle of the PWN, due to intense demographic bottlenecks during tree infections, not compensated for by effective dispersal of its vector; (2) a single introduction event for each of the invaded areas in Japan and Europe and a small effective size for the introduced populations and (3) a mainland Portuguese origin for PWN populations from Madeira. However, more sophisticated methods of invasion route inference and broader sampling are required to conclusively determine the origin of the European outbreak.

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Introduction

Several studies have shown that it is more effective to fight biological invasions in their initial stages than to try to eradicate the invader after its establishment, in terms of cost, time and management efficiency (Allendorf and Lundquist 2003; Simberloff et al. 2013). Deciphering geographic pathways followed by invasive species is a key first step in this direction. Many studies carried out over the last decade have tried to identify the invasion routes of several invasive species, including insects (Papura et al. 2012; Pascual et al. 2007; Perdereau et al. 2013; Zepeda-Paulo et al. 2010), plants (Kelager et al. 2013), nematodes (Boucher et al. 2013) and fungi (Fontaine et al. 2013). Indeed, this approach can be used to address various questions of both practical and theoretical concern (Estoup and Guillemaud 2010). Specifically, from a practical point of view, it may facilitate the design of strategies for preventing new invasions, by highlighting weaknesses in control and phytosanitary measures. It may help to improve the control or eradication of detrimental invasive species, through the identification of natural enemies in the native area, better understanding of the biology of populations and definition of the ecological features of invasive populations (Mack et al. 2000; Strong and Pemberton 2000; Tsutsui et al. 2000; van Wilgen et al. 2013). From a more fundamental point of view, the inference of invasion routes may provide useful information about the invasion process. The testing of ecological or evolutionary hypotheses explaining the success of biological invasions requires relevant comparisons between native and invasive populations (Facon et al. 2006; Keller and Taylor 2008; Puth and Post 2005; Wilson et al. 2009) and, thus, basic knowledge of the invasion routes used.

Here, we focused on the invasions of Europe by the pinewood nematode (PWN), *Bursaphelenchus xylophilus* (Steiner and Buhrer 1934; Nickle, 1970; Nematoda: Aphelenchoididae), a microscopic worm that reproduces sexually (Futai 2013). Outside North America (widely recognized as the native range of this species, Dropkin et al. 1981; Kiritani and Morimoto 2004; Wingfield et al. 1982), PWN is the causal agent of pine wilt disease (Mamiya 1972, 1976, 1983) and it poses a serious threat to pine forests worldwide, due to ecological and economic consequences of infestation (Mamiya 1988; Soliman et al. 2012; Suzuki 2002; Vicente et al. 2011). It was first observed outside its native range in Japan, in 1905, near Nagasaki (Mamiya 1988), where 28 % of the 2.1 million ha of pine forest was found to be infested in 2000 (Mamiya 2004). It has since spread to other Asian countries, including China, Taiwan and South Korea (Moon et al. 2007). PWN was detected in the Setúbal Peninsula, close to Lisbon in Portugal, in 1999 (Mota et al. 1999) and new outbreaks have been identified since 2008 in the centre of mainland Portugal and on Madeira (Fonseca et al. 2012), as well as in Spain (Abelleira et al. 2011; Robertson et al. 2011). PWN was designated a quarantine organism by the European and Mediterranean Plant Protection Organization (OEPP/EPPO 1997) and the European Commission ruling (2006/133/CE) has imposed strict measures on the trade of wood, to limit the invasion. These measures have added significant costs to those already resulting from the destruction of pine forests.

Many studies have attempted to establish the origin of invasive outbreaks of PWN in different geographic areas (Cheng et al. 2008; Figueiredo et al. 2013; Fonseca et al. 2012; Metge and Burgermeister 2008; Pereira et al. 2013; Tares et al. 1992; Valadas et al. 2012a, b; Vieira et al. 2007; Zhang et al. 2008; Zhou et al. 2007). An Asian origin for European invasive populations of PWN was thus proposed (Figueiredo et al. 2013; Fonseca et al. 2012; Metge and Burgermeister 2008; Valadas et al. 2012b). However, these studies were subject to experimental limitations, such as the use of low-resolution markers (e.g. RAPD,

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RFLP), pools of individuals and/or collection samples. Thus, no study has as yet clearly investigated the worldwide invasion routes of PWN with relevant molecular markers, appropriate samples and the correct population genetics approaches as described by Estoup and Guillemaud (2010).

Therefore, the aim of this study was to perform population genetics analyses on natural PWN populations from around the world, with microsatellite markers developed previously (Mallez et al. 2013). In particular, we aimed to confirm the existence of the strong genetic structure in the native area of this species suggested by Mallez et al. (2013), through the use of a larger number of samples from different US states and hierarchical sampling. We also evaluated the genetic diversity of PWN populations from invaded areas, to obtain information about the number of introduction events. Finally, we investigated the relationships between populations in native and invaded areas, to clarify the invasion routes used by PWN. In particular, we investigated whether the European invasion resulted from an introduction independent of the Asian introduction or from successive introductions via Asia. We also analysed the relationships between invasive populations from mainland Portugal and Madeira.

Materials and methods

The biological system of the PWN and its generation time

The biological cycle of the PWN in natura is complex and involves at least two partners: the pine tree and the insect vector (Evans et al. 1996; Futai 2013 for a review). The PWN and its vector are closely associated and their life cycles closely match (Mamiya 1972). Thus, the PWN reproduce exclusively during summer once it invaded a susceptible pine tree (from June to September for the largest period). Its generation time is not precisely known in natura due to impossible direct observations of the PWN into the tree. However, from laboratory experiments, it was determined that the life cycle of the PWN depends mainly on the temperature, which determines its duration (Mamiya 1975). The generation time of the PWN may thus be very short and lasts from 4 5 days at 25 °C to about 12 days at 15 °C, with temperature

thresholds for development of 9.5 °C for the minimum and 33 °C for the maximum (Mamiya 1975). Consequently, by coupling this information, the generation time of the PWN was approximated to 30 generations per year (by averaging the summer temperature to 25 °C and by considering the June September period for PWN reproduction). This is about 450 generations since the PWN introduction in Portugal and more than 3,200 generations since its introduction in Japan.

Sampling

Nematode samples were obtained from three different geographic areas: a part of the native area, the USA and two invaded areas, Japan and Portugal/Madeira. Thirty-four locations were sampled and 770 individuals were analysed in total: 18 locations from the USA (391 individuals), nine from mainland Portugal and Madeira (169 individuals) and seven from Japan (210 individuals). The PWN samples are listed and described in Table 1 and the locations from which the samples were collected are shown in Fig. 1. All individuals were extracted from wood samples collected directly from field locations. Each location sample originated from a single tree and consisted of seven to 36 individuals, at various stages. Nematodes were extracted with a sieve or a Baermann funnel (Viglierchio and Schmitt 1983). No permission was required to collect samples of PWN from the infested areas and we obtained an official agreement from the French authorities (#2012060-0004) for the importation and manipulation of this quarantine organism at Institut Sophia Agrobiotech facilities. For some samples, extraction of individuals were carried out by our collaborators, who then sent them in DESS (Yoder et al. 2006). These samples were washed in distilled water before DNA extraction. For the other samples, DNA was extracted directly after the extraction of individuals from wood samples.

DNA extraction and genotyping of microsatellite loci

Single individuals were subjected to a thermal shock, as explained below, for DNA extraction (Castagnone et al. 2005). Each individual was hand-picked, transferred to 18 μl of lysis buffer (10× *Taq* buffer with MgCl$_2$, *Taq* Core Kits10, MP Biomedicals; 60 mg ml^{-1} proteinase K and sterile distilled H$_2$O) and placed at −80 °C for

Table 1 Characteristics and population genetics summary statistics of each sample of *B. xylophilus* used in this study

Sample code	No. of individuals	Origin			Mean Na	Max Na	He	Ho	Fis
MO1	31	USA	Missouri	Columbia	3.25	8	0.33	0.27	0.19[a]
MO2	23	USA	Missouri	Columbia	3.38	7	0.36	0.26	0.27[a]
NE1	16	USA	Nebraska	Davey	2.00	4	0.20	0.18	0.09
NE2	15	USA	Nebraska	Davey	1.81	4	0.21	0.16	0.24
NE5	14	USA	Nebraska	Pawnee Lake	1.94	5	0.21	0.18	0.14
NE6	21	USA	Nebraska	Pawnee Lake	1.38	2	0.18	0.10	0.48[a]
NE9	29	USA	Nebraska	Pawnee Lake	2.63	5	0.31	0.23	0.26[a]
NE10	26	USA	Nebraska	Pawnee Lake	1.75	4	0.19	0.15	0.19[a]
NE12	28	USA	Nebraska	Conestoga Lake	1.50	3	0.15	0.10	0.34[a]
NE13b	19	USA	Nebraska	Pioneers Park	2.38	5	0.28	0.20	0.31[a]
NE14	28	USA	Nebraska	Pioneers Park	1.69	3	0.24	0.16	0.34[a]
NE15	23	USA	Nebraska	Pioneers Park	2.25	5	0.22	0.14	0.38[a]
NE19	16	USA	Nebraska	UNL East Campus	1.81	3	0.23	0.20	0.15
NE22	17	USA	Nebraska	Lincoln	2.00	5	0.28	0.20	0.28[a]
NE23	25	USA	Nebraska	Lincoln	1.25	2	0.07	0.07	0.09
NE24	19	USA	Nebraska	Lincoln	1.31	2	0.07	0.06	0.15
VI9	22	USA	Virginia	Midlothian	1.50	3	0.16	0.14	0.14
MA1	19	USA	Massachusetts	Worchester	1.50	2	0.14	0.14	0.00
Jap120	23	Japan	Iwate	Shiwa	1	1			
Jap212	27	Japan	Iwate	Shiwa	1	1			
Jap308	25	Japan	Iwate	Shiwa	1	1			
Kasumig2	36	Japan	Ibaraki	Kasumigaura	1	1			
Kasumig3	29	Japan	Ibaraki	Kasumigaura	1.19	2	0.06	0.06	0.02
Kasumig5	35	Japan	Ibaraki	Kasumigaura	1	1			
Kosa	35	Japan	Kumamoto	Kosa	1.25	2	0.06	0.05	0.03
Mad23PC	12	Madeira Island	Porto da Cruz		1	1			
Mad24C	7	Madeira Island	Calheta		1	1			
128S	17	Portugal	Setubal	Grândola	1.06	2	0.03	0.01	0.62[a]
TR1	30	Portugal	Setubal	Troia	1	1			
TR2	27	Portugal	Setubal	Troia	1	1			
AM2	21	Portugal	Setubal	Aguas de Moura	1	1			
Comporta	28	Portugal	Setubal	Comporta	1	1			
E182	13	Portugal	Coimbra	Penela	1	1			
E1069	14	Portugal	Viseu	Castro Daire	1	1			

Mean Na is the mean number of alleles per sample over all loci, Max Na is the maximum number of alleles per locus in each sample, He is the expected heterozygosity and Ho is the observed heterozygosity. *FIS* was calculated as described by Weir and Cockerham (1984)

" " indicates that Ho, He and *FIS* were not calculated, for samples with only monomorphic markers

[a] indicates that the result of the HWE test was significant at the 5 % level after FDR correction (Benjamini and Hochberg 1995)

60 min. It was then immediately transferred to 60 °C for 60 min and, finally, to 95 °C for 15 min in a Biometra® T3-Thermoblock Thermocycler. We amplified 16 microsatellite loci in three multiplex PCRs: MA28 (5 microsatellite loci), MB28 (5 microsatellite loci) and MC33 (6 microsatellite loci), as described by Mallez et al. (2013). We excluded Bx07 from the MB28 multiplex reaction, because this marker is identical to
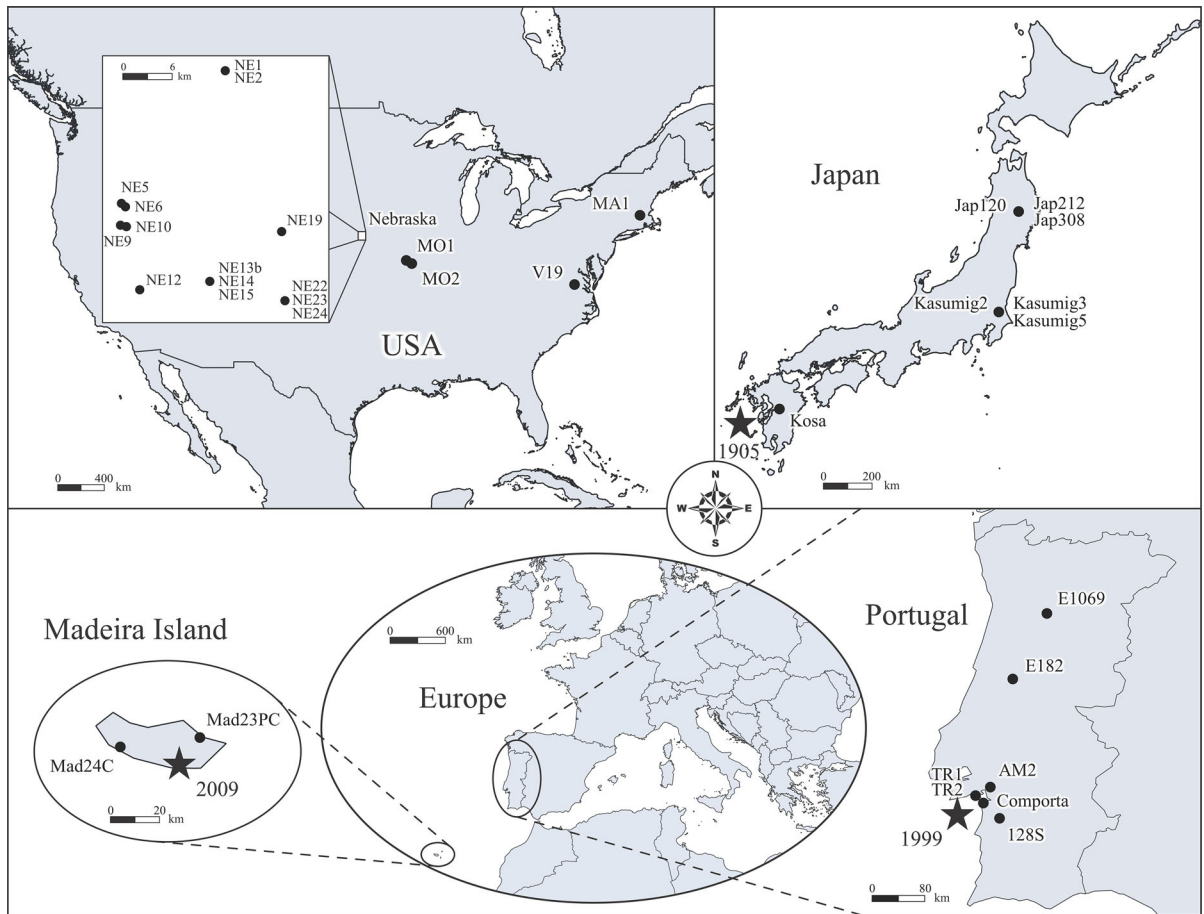
Fig. 1 Location of the sampling sites for *B. xylophilus* used in this study. Three different geographic areas are represented: the USA, a part of the native area, focusing on the Nebraska, and two different invaded areas, mainland Portugal and Madeira and Japan. The stars indicate the hypothetical entry points of PWN. The dates of first observation for each invaded area are indicated. The codes on the maps are the sample names. For more details, see Table 1

PWN 51 (Mallez et al. 2013). PCR amplifications were performed in 10 µl containing 1× QIAGEN Multiplex Master Mix, 2 µM of each primer, with forward primers labelled with a fluorescent dye (6-FAM, VIC, PET or NED) at the 5′ end, and 2 µl of genomic DNA extracted by thermal shock. The amplification reactions were performed in a Biometra® T3-Thermoblock Thermocycler and included a 15 min denaturation step at 95 °C, followed by 28 or 33 cycles (depending on the multiplex PCR) of 30 s at 94 °C, 1.5 min at 55 °C, and 1 min at 72 °C, followed by a final extension step of 30 min at 60 °C. Genotypes were determined with an ABI 3700 sequencer (Applied Biosystems), with the 500 LIZ™ GeneScan™ size standard (Applied Biosystems) and Genemarker™ version 1.75 software (SoftGenetics LLC).

Data analyses

Standard genetic analyses

For each sample, we determined the maximum number of alleles detected over all loci (Max. Na), the mean number of alleles (Mean Na) and the observed (Ho) and expected (He) heterozygosities per sample, with GENETIX version 4.05 (Belkhir et al. 1996 2004). We evaluated deviation from Hardy Weinberg equilibrium (HWE) with GENE-POP version 4.1.3 (Rousset 2008) and we quantified inferred deviations from HWE by calculating the Weir and Cockerham estimate of *FIS* (Weir and Cockerham 1984) with FSTAT version 2.9.3.2 (Goudet 2002). Linkage equilibrium between loci was assessed with

the log likelihood ratio test, in GENEPOP (Rousset 2008). We took multiple testing (HWE tests) and the non-independence of tests (linkage tests) into account by performing false discovery rate (FDR) correction (Benjamini and Hochberg 1995) and sequential Bonferroni adjustment (Sokal and Rohlf 1995), respectively. Null allele frequencies were estimated with FREENA (Chapuis and Estoup 2007).

Genetic structure analyses within areas

All the analyses described in this section were performed within each area, for precise characterization of the nematode samples from the native area and the two invaded areas. We first tested the hypothesis of uniform genotype frequencies between samples, by Fisher's exact test (Raymond and Rousset 1995) implemented in GENEPOP (Rousset 2008). As non-independent multiple tests were performed, sequential Bonferroni correction (Sokal and Rohlf 1995) was carried out to adjust significance levels. We also calculated Weir and Cockerham estimates of F_{ST} (Weir and Cockerham 1984) between samples, corrected for null alleles with FREENA (Chapuis and Estoup 2007). We then studied the structure of the populations from each area by a Bayesian clustering approach, as implemented in STRUCTURE version 2.3 (Pritchard et al. 2000). An admixture model with correlated allele frequencies was used (Falush et al. 2003). The numbers of clusters tested, K, varied from 1 to 18 for the USA, from 1 to 7 for Japan and from 1 to 4 for Portugal/Madeira. We carried out 20 independent runs for each value of K. Each run involved a Markov Chain Monte Carlo (MCMC) procedure with 10^6 iterations, following a burn-in period of 2×10^5 iterations. Default values were maintained for all the other parameters. The number of clusters was determined both as described by Evanno et al. (2005) and automated in STRUCTURE HARVESTER (http://taylor0.biology.ucla.edu/struct_harvest/index.php, Earl and vonHoldt 2012) and by checking all the bar plots of co-ancestry parameters for successive values of K. Using CLUMPP (Jakobsson and Rosenberg 2007), we identified the most frequent clustering patterns for each value of K among the 20 runs, which we plotted with DISTRUCT version 1.1 (Rosenberg 2004). We checked the adequacy of the clustering patterns chosen for successive values of K.

In the USA, the hierarchical sampling in Nebraska allowed us to investigate the possible occurrence of isolation by distance (IBD). This involved assessment of the correlation between genetic distance ($FST/(1 - FST)$) and the logarithm of geographic distance, for pairs of populations (Rousset 1997). We used the Mantel test in GENEPOP (Rousset 2008) and 20,000 permutations to assess the significance of the correlation.

Relationships between the different areas

We focused on the origin of the Portuguese mainland and island (Madeira) samples. The most probable source population, in North America or in Asia, for each Portuguese sample was investigated in several ways. More precisely, the aim of this part was to select the most probable scenario among the following scenarios: (1) a scenario with two independent introductions, in Asia and in Europe, from North America and (2) a scenario with two successive introductions, from North America to Asia and then from Asia to Europe.

We first analysed the FST values corrected for null alleles (Chapuis and Estoup 2007; Weir and Cockerham 1984) between each Portuguese sample and each American or Japanese sample. We then carried out an individual assignment likelihood analysis (Paetkau et al. 2004; Rannala and Mountain 1997), as in previous studies on invasion routes (Ciosi et al. 2008; Pascual et al. 2007) with GENECLASS2 software version 2.0 (Piry et al. 2004). This analysis involves calculating the mean individual assignment likelihood (denoted $L_{i \rightarrow s}$) of each Portuguese sample i, to each possible source population s (the American samples and the Japanese samples, in our case). The most probable source of a target invasive population sample i is considered to be the population with the lowest corrected FST values with i and the maximum assignment likelihood of i. We expect a lower corrected FST value between the USA and Portugal/Madeira Island than between Asia and Portugal/Madeira Island under the scenario of independent events of introduction and the opposite under the scenario of successive events of introduction.

We also plotted a neighbour joining (NJ) tree (Saitou and Nei 1987), based on Cavalli-Sforza and Edwards genetic distances (Cavalli-Sforza and Edwards 1967) with POPULATION software version 1.2.30

(http://bioinformatics.org/~tryphon/populations/). The robustness of the tree topology was evaluated by carrying out 2,000 bootstrap replicates over loci. A tree based on Cavalli-Sforza and Edwards genetic distances (Cavalli-Sforza and Edwards 1967) corrected for null alleles (Chapuis and Estoup 2007) was also built. The most probable source of a target invasive population sample i is considered to be the population from outside Europe whose sample is clustering closest to i in the tree.

Finally, we carried out Bayesian clustering analysis with STRUCTURE software (Pritchard et al. 2000) using all the samples from North America, Asia and Europe to determine the origin of the sampled Portuguese populations. The number of clusters tested, K, varied from 1 to 10. As before, an admixture model with correlated allele frequencies (Falush et al. 2003), 20 runs per K, 10^6 iterations for the MCMC and 2×10^5 iterations for the burn-in period were used. CLUMPP (Jakobsson and Rosenberg 2007) and DISTRUCT (Rosenberg 2004) was used to identify the most frequent clustering patterns for each value of K and to display the corresponding bar plots, respectively. This method identifies the most probable source of a target invasive population sample i as the population for which the sample(s) is (are) the last to cluster with i with increasing values of K. Thus, under the scenario of independent events of introduction, we expect that an American sample is the last one to still cluster with Portugal/Madeira Island with increasing values of K. Conversely, under the scenario of successive events of introduction, we expect an Asian sample to be the last one to still cluster with Portugal/Madeira Island with increasing values of K.

Results

Standard genetic analyses

Three markers were monomorphic (M3, M30 and M49), even in the native area (but see Mallez et al. 2013, who found polymorphism at these markers in the native area, in analyses of collection and field samples). Genetic diversity was low to moderate in the USA and low to extremely low in invaded areas (see Table 1). In the native area, we detected up to eight alleles per locus and per sample (and up to 13 alleles per locus over all samples), with a mean number of alleles per sample (Mean Na) of 1.31 3.38 and a mean

expected heterozygosity (He) of 0.07 0.36. Numerous monomorphic markers were detected in the invaded areas: 10 markers in Japan and 15 markers in Portugal/Madeira, of the 16 considered. No more than two alleles per locus and per sample (and no more than three per locus over all samples in Japan) were detected, with a mean Na of 1.25 and a mean He of 0.06 at most. Ten samples in the native area and one sample in Portugal deviated significantly from HWE because of a heterozygous deficit (see Table 1). Significant linkage disequilibrium was found in 10 of the 624 pairwise tests carried out (after sequential Bonferroni correction, Sokal and Rohlf 1995), for four pairs of loci: M62 and Bx08; M51 and M56; M35 and M56 and M35 and M51. Examination of the results of FREENA analysis (Chapuis and Estoup 2007) showed that most of the deviation from HWE (especially in Portugal) and most of the significant linkage tests were accounted for by the presence of null alleles at the loci involved (estimates of null allele frequencies from 7 to 24 %, data not shown). However, null alleles were not systematically observed for a given marker across all the samples or for a given sample across all the markers. In addition, none of the samples or markers had more than 10 % null alleles on average. We therefore used the entire dataset for further analyses.

Genetic structure analyses within geographic areas

In the USA, all samples displayed significant differentiation after correction for multiple testing (Fisher's exact tests, $p < 10^{-5}$). Pairwise corrected estimates of FST values were also very high: from 0.06 to 0.76 (for more details see Table S1). The number of clusters could not be clearly inferred from the Bayesian clustering analysis, because ΔK (Evanno et al. 2005) had several peaks, at different values of K (see Figure S1). A biologically meaningful genetic structure occurred for large values of K (see the examples of bar plots for several values of K in Fig. 2A and all bar plots in Figure S2). Most samples were progressively unambiguously assigned to different clusters, at least until $K = 14$. We also detected evidence of IBD (slope = 0.064, $p = 0.013$).

In Japan, the three samples from Iwate in the North were genetically identical, presenting one fixed allele for all the markers. All the remaining samples appeared to display significant differentiation (Fisher's exact tests, $p < 10^{-5}$), with extremely high
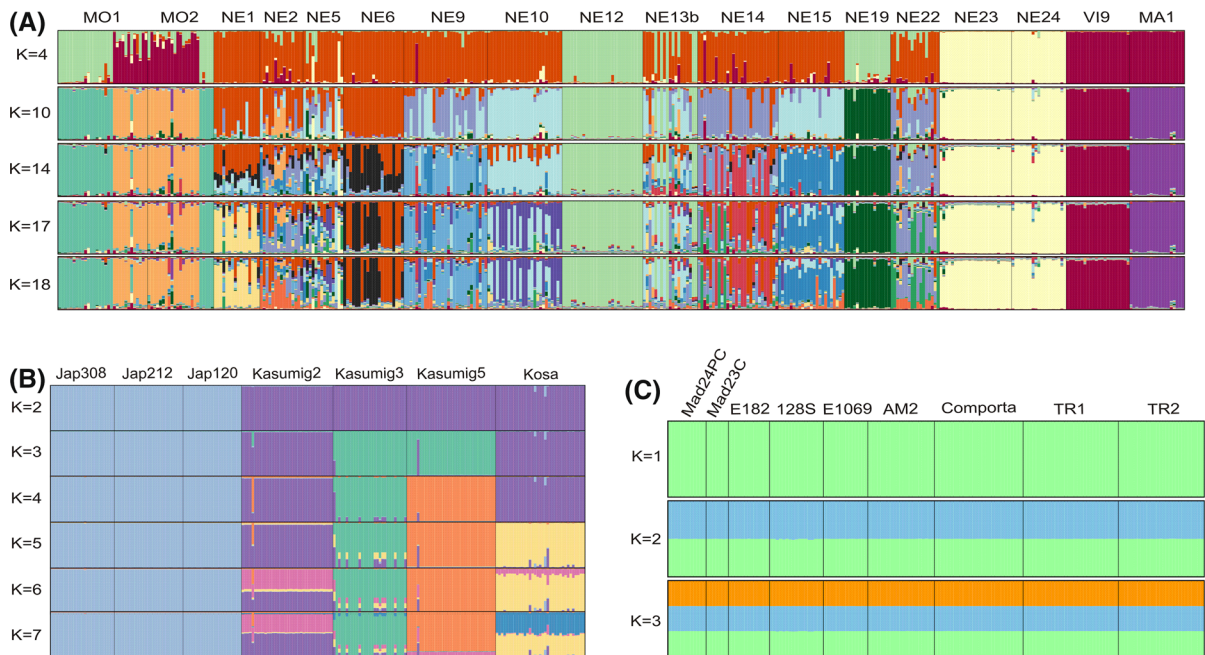
Fig. 2 Genetic structure of the PWN field samples within each area. Bar plots of the coefficients of co ancestry obtained in various STRUCTURE analyses with several values of K for (**A**) a par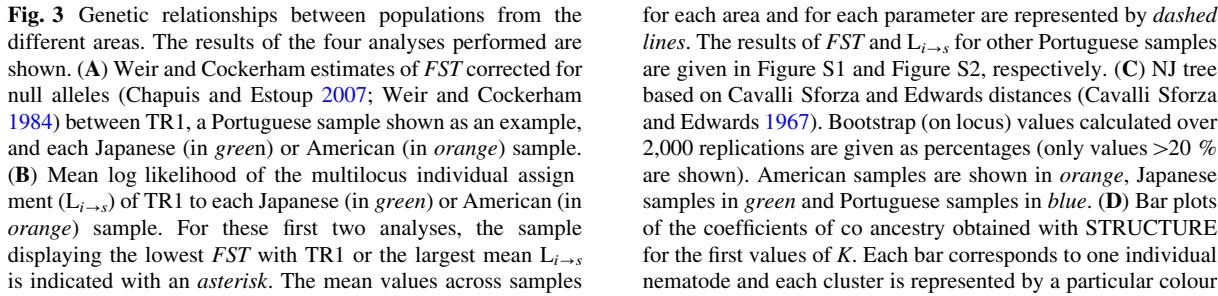t of the native area, the USA and for the two invaded areas studied here, (**B**) Japan and (**C**) Portugal/Madeira. Each bar corresponds to one individual nematode and each cluster is represented with a particular colour

corrected FST values, ranging from 0.63 to 0.99 (excluding the three identical samples, see Table S1 for more details). Strong genetic structure was also detected at the scale of the tree, with STRUCTURE. The ΔK method (Evanno et al. 2005) inferred the existence of three clusters (see Figure S1), but likelihood values reached a plateau at $K = 5$ and an examination of successive bar plots for co-ancestry suggested a meaningful structure for $K = 5$ (see Fig. 2B).

In Portugal, eight of the nine samples were genetically identical and 162 individuals (of the 169 sampled) had identical homozygous multi-locus genotypes. The remaining sample (128S) differed significantly from seven of the other eight samples after correction for multiple testing (Fisher's exact tests, $p < 0.017$). The result of one test was not significant (Mad24C vs. 128S, Fisher's exact test, $p = 0.065$), probably because of the small size (seven individuals) of the Mad24C sample. The clustering analysis inferred a single cluster, grouping together the samples from mainland Portugal and Madeira (see Fig. 2C, Figure S1).

Relationships between populations from the different geographic areas

The results of the various analyses performed to clarify the relationships between the populations in different geographic areas are visualized and summarized in Fig. 3. The lowest FST values obtained with Portuguese samples always corresponded to American samples (see the example of one Portuguese sample in Fig. 3A and all Portuguese samples in Figure S3). The mean FST value (across samples) between Portugal/Madeira and the USA was also lower than that between Portugal/Madeira and Japan, as shown by the dashed lines in Fig. 3A. Thus, the populations of Portugal/Madeira seem to be closer to the American populations than to the Japanese populations, on the basis of FST. Portuguese samples were assigned to American samples with the largest mean individual likelihood ($L_{i \to s}$, see the example of one Portuguese sample in Fig. 3B and all Portuguese samples in Figure S4). However, if we averaged across samples, Portugal was assigned to Japan with the largest mean individual likelihood (dashed lines on Fig. 3B). The

Fig. 3 Genetic relationships between populations from the different areas. The results of the four analyses performed are shown. (**A**) Weir and Cockerham estimates of *FST* corrected for null alleles (Chapuis and Estoup 2007; Weir and Cockerham 1984) between TR1, a Portuguese sample shown as an example, and each Japanese (in *green*) or American (in *orange*) sample. (**B**) Mean log likelihood of the multilocus individual assign ment ($L_{i \to s}$) of TR1 to each Japanese (in *green*) or American (in *orange*) sample. For these first two analyses, the sample displaying the lowest *FST* with TR1 or the largest mean $L_{i \to s}$ is indicated with an *asterisk*. The mean values across samples

for each area and for each parameter are represented by *dashed lines*. The results of *FST* and $L_{i \to s}$ for other Portuguese samples are given in Figure S1 and Figure S2, respectively. (**C**) NJ tree based on Cavalli Sforza and Edwards distances (Cavalli Sforza and Edwards 1967). Bootstrap (on locus) values calculated over 2,000 replications are given as percentages (only values >20 % are shown). American samples are shown in *orange*, Japanese samples in *green* and Portuguese samples in *blue*. (**D**) Bar plots of the coefficients of co ancestry obtained with STRUCTURE for the first values of *K*. Each bar corresponds to one individual nematode and each cluster is represented by a particular colour

origin of the Portuguese samples is thus not clearly inferred with this statistic. On the NJ tree, the Portuguese samples were closer to the Japanese samples than to the American samples, for trees generated with both uncorrected and corrected Cavalli-Sforza and Edwards distances (Cavalli-Sforza and Edwards 1967; Chapuis and Estoup 2007; Fig. 3C, Figure S5, respectively). This result was robust to the use of other genetic distances (data not shown). Finally, Bayesian clustering analysis (Fig. 3D) showed that the Portuguese samples clustered with the Japanese samples at the lowest level of structur ation ($K = 2$), with the Portuguese samples and three Japanese samples subsequently clustering together,

separately from the other Japanese samples, at $K = 3$. This result was confirmed by a clustering analysis of the samples from invaded areas only (Portugal/ Madeira and Japan, data not shown).

Discussion

In this study, we investigated the genetic diversity and structure of natural PWN populations from around the world and the relationships between populations from native and invaded areas. Three main results were obtained: (1) we confirmed here, with a much larger number of samples, the existence of the strong genetic

structure of PWN populations suggested by Mallez et al. (2013), consistent with notion that genetic drift has had a major effect on the genetic structure of PWN, (2) we found very low levels of polymorphism in the invaded areas, suggesting single introduction events, introduced populations with small effective sizes and clarifying the relationships between invasive populations within Europe and (3) we observed that classical and Bayesian population genetics methods were inconclusive concerning the invasion routes followed by PWN, and that more powerful inference methods are therefore required.

Strong genetic structure in PWN populations

We observed a strong spatial genetic structure of native PWN populations. A very low genetic diversity within samples from the native area compared to that between populations, highly significant differentiation tests, Bayesian clustering analysis and the extremely high values of pairwise FST were indicative of this structure. The very low within sample variability may have artificially increased FST values.

Such a genetic structure has already been reported in the native ranges of invasive species (e.g. for a Cuban lizard, by Kolbe et al. 2004). Our findings confirm those reported by Mallez et al. (2013), for a larger number of samples with hierarchical sampling in Nebraska. They confirm that the migration-drift equilibrium is highly biased towards significant genetic drift without compensation by efficient dispersal, even over short distances. The limited dispersal of the PWN may reflect the complexity of its dispersal process, which is principally dependent on an insect vector, *Monochamus* species (Akbulut and Stamps 2012; Linit 1988; Mamiya and Enda 1972; Sousa et al. 2001). This insect probably has a weak dispersal capacity, resulting in dispersal over short distances (Shibata 1986; Togashi 1990), particularly when the vector is heavily loaded with nematodes, potentially affecting its ability to fly (Akbulut and Linit 1999). The evidence of genetic IBD is also consistent with the probably spatially limited dispersal capacity of the PWN.

PWN must also overcome several potential obstacles to its dispersal. It must aggregate around pupal chambers, entering the beetles just before emergence, facilitating its entry into the tree via the maturation feeding sites of the beetles, for effective reproduction within the tree (reviewed in Futai 2013; Vicente et al. 2011). These steps may also contribute to the strong genetic drift observed in this system, because (1) the population of nematodes in an infested tree is aggregated into a small number of beetles that emerge from the tree (Akbulut and Linit 1999), (2) most beetles carry only a bit of nematodes (Kobayashi et al. 1984; Linit 1988) and (3) not all nematodes successfully invade pine trees (Togashi 1985), resulting in relatively low transmission rates (10 20 %, Kobayashi et al. 1984; Togashi 1985).

Thus, the initial PWN population in a tree may be small, subsequently increasing exponentially in size. This creates considerable genetic drift, coupled with rates of dispersal between trees too low to homogenise genetic diversity.

Very low levels of polymorphism in invaded areas

Another finding in this study was the very low level or even complete absence of genetic diversity in the invaded areas, as observed in previous reports on the same species (Fonseca et al. 2012; Pereira et al. 2013; Vieira et al. 2007; Zhang et al. 2008; Zhou et al. 2007). However, this finding is remarkable in comparison with other invading species in which a loss of genetic diversity during invasion is not as common as previously expected (Bossdorf et al. 2005 for a review in plant invasive species; Roman and Darling 2007 for a review in aquatic invasions). The large number of monomorphic markers and the similarity between them across individuals (fixed alleles) are surprising at first glance for a sexual species (Futai 2013) and for microsatellite markers. However, these findings may be accounted for by the low level of intra-sample genetic diversity observed in the native area, together with the genetic bottlenecks and founder events often occurring during the introduction of species in new areas (Allendorf and Lundquist 2003; Sakai et al. 2001). Moreover, biological invasions tend to occur over short timescales, so mutational processes have very little effect on the genetic structure of invasive populations in the short term. Consequently, genetic structure is shaped mostly by demographic processes, such as intense demographic bottlenecks resulting in intense genetic bottlenecks, as in this study.

This very low level of genetic diversity provides information about the most probable number of introduction events. In cases of multiple introductions

in a restricted area, such as mainland Portugal, Madeira or Japan, significant genetic diversity would be expected, due to admixture of the various introduced populations, particularly given the highly structured nature of native populations. The almost complete absence of polymorphism in mainland Portugal, Madeira and Japan therefore strongly suggests that a single introduction occurred in each of these areas, with each introduced population having small effective size. In the European context, these findings firmly suggest that the second outbreak detected in the centre of mainland Portugal in 2008 resulted from expansion of the first outbreak detected close to Lisbon in 1999. This conclusion contrasts with the findings of Valadas et al. (2012a), who suggested that multiple introductions had occurred. Our findings also suggest that the PWN populations on Madeira originated from mainland Portugal, given the near identity of the populations from Madeira and mainland Portugal and the first detection of PWN outbreak on Madeira 10 years after the first outbreak in mainland Portugal.

Intra-population genetic diversity is widely considered to be a prerequisite for adaptation to changing conditions and/or environment (Reed and Frankham 2003; Willi et al. 2006). Biological invasions have thus brought to light a genetic paradox (Allendorf and Lundquist 2003; Frankham 2005): the occurrence of successful invasions with low levels of genetic diversity. The case studied here provides a good example of this paradox, because PWN populations with low levels of diversity have managed to invade several regions around the world. There are several ecological mechanisms that might account for this paradox in the case of PWN: (1) the presence of appropriate insect vectors in each of the countries invaded (Akbulut and Stamps 2012; Mamiya and Enda 1972; Naves et al. 2007; Sousa et al. 2001), (2) the presence of susceptible hosts (Evans et al. 1996) and (3) the greater competitiveness of PWN than of its closely relative resident in the area, *B. mucronatus* (Cheng et al. 2009; Vincent et al. 2008). There is also some published evidence that a loss of genetic diversity (demonstrated with neutral markers) does not hamper the adaptive phenotypic variation of fitness-related traits (Dlugosch and Parker 2008) and that measurements of neutral genetic diversity are of only limited value for the prediction of quantitative genetic variability (Reed and Frankham 2001). Further

studies are therefore required to determine whether invading PWN populations display significant adaptive genetic variability and whether this variability contributes to the success of PWN.

Worldwide invasion routes of PWN: the need for more powerful methods

The third main finding of this study was the difficulty elucidating the worldwide invasion routes of PWN. Depending on the analysis and the method used, we alternatively inferred two possible origins, North America and Asia, for the Portuguese outbreaks. The F_{ST} and mean F_{ST} values across samples suggested an American origin for all the Portuguese samples, whereas the NJ tree and the Bayesian clustering analysis suggested a Japanese origin for these samples. A discrepancy was also found in the mean individual assignment likelihood analysis, which gave inconsistent results, as the sample with the minimum mean individual assignment likelihood suggested an American origin and the mean individual assignment likelihood between samples suggested an Asian origin. This discrepancy partly results from large inter-sample variance of allelic frequency in North America. Previous studies have proposed an Asian origin for the European populations of PWN (Fonseca et al. 2012; Metge and Burgermeister 2008; Valadas et al. 2012b), essentially on the basis of tree analyses. Our finding with the NJ tree and Bayesian clustering is consistent with this conclusion. One key point here is that the conclusions drawn from these previous studies are no more robust than ours because (1) the methods used are included among those used here and (2) these previous studies did not use other methods possibly leading to alternative conclusions.

These inconclusive results highlight a major problem with traditional methods: a lack of statistical confidence evaluation for inferences of the source population of invasion. No statistical tests are carried out and no probabilities or type I or type II errors are calculated for classical and Bayesian clustering or distance methods. This makes it difficult to determine which result is the most likely when several alternatives are proposed (Estoup and Guillemaud 2010). However, these classical and Bayesian methods have proved both useful and conclusive in other cases of invasion (Ciosi et al. 2008; Facon et al. 2003; Kolbe et al. 2004; Papura et al. 2012; Perdereau et al. 2013; Wan et al. 2012). We can therefore put forward several

hypotheses to account for the conflicting results obtained here: (1) the low level of diversity may have resulted in a lack of power to discriminate between the two possible alternatives, (2) the strong spatial genetic structure observed in the USA, requiring a very large sampling scheme to embrace most genetic variation of the native and (3) the lack of samples from some of the existing invaded areas, such as China or South Korea, potentially serving as sources for the invasion of Europe, as suggested by Figueiredo et al. (2013) on the basis of analyses of collection samples for PWN.

Given the limitations of classical and Bayesian methods, the use of recent model-based methods, such as the approximate Bayesian computation (ABC, Beaumont et al. 2002; Bertorelle et al. 2010; Guillemaud et al. 2010) may prove useful. The enthusiasm linked to the use of genetic data for reconstructing the history of invasive species was restricted by failures or technical limitations (Barun et al. 2013; Fitzpatrick et al. 2012). However, ABC make possible to perform extensive simulations of various/alternative hypothesis, which is needed to make reliable biological interpretations of invasion (Barun et al. 2013). Moreover, ABC offers several advantages (described by Estoup and Guillemaud 2010) that may be crucial in studies of PWN: (1) it takes complex scenarii into account, (2) it manages incomplete sampling by providing the possibility of considering unsampled "ghost" populations and, most importantly, (3) it makes it possible to evaluate quantitatively and to compare statistically the various competing scenarii, through the calculation of posterior probabilities. Finally, efforts should be made in future studies to obtain more representative samples. These new samples should provide a better representation of the genetic diversity existing around the world, more precisely describing the populations of the native area and all the main invaded areas.

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