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Is the emergence of *Dothistroma* needle blight of pine in France caused by the cryptic species Dothistroma pini?

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Abstract

Dothistroma needle blight (DNB) emerged in France in the past 15 years. This disease is induced by two closely related species *Dothistroma septosporum* and *D. pini*. While both species are nowadays present in France, only *D. septosporum* was reported in the past. We investigated whether a recent arrival of *D. pini* in France could be a cause of the DNB emergence. We analysed herbarium specimens of pine needle with DNB symptoms using PCR techniques to study the past frequency of *D. pini* in France. We also determined the present distribution within the country of *D. septosporum* and *D. pini* and compared it to the spatial pattern of DNB report in the DSF data base (french forest health monitoring agency). Although *D. pini* was detected on herbarium specimens from 1907 and 1965, it was not frequent in France in the past. It is nowadays frequent although not present throughout the country, being absent from the north and the east. There is no relationship between the *D. pini* distribution in France and the spatial pattern of DNB report in the DSF database. Thus, the emergence of DNB in France cannot be explained by a recent arrival of *D. pini*.

Key word: emergence, cryptic species, spatial pattern, *Pinus*, *Mycosphaerella pini*

Introduction

The emergence of plant diseases is an important concern throughout the world [1, 14]. Emergence can be defined by the increase in problems caused by a disease, whether the disease increases its range or its severity within the same range. The most important factors reported to influence disease emergence are climate change and the introduction of pathogens in new areas by trade [1, 9]. Assessing the relative importance of those two factors is a major scientific issue in epidemiology. Determining whether an emerging disease is caused by an invasive pathogen is not always easy. Indeed, many plant diseases are caused by complex of cryptic species. This is for example the case for many forest tree diseases such as *Sphaeropsis* shoot blight [10], *Dothistroma* needle blight [4] or oak powdery mildew [21]. When a disease caused by a complex of cryptic species emerges, it is often difficult to determine whether the pathogen responsible for the increase in disease severity or range has invaded the area; this is because knowledge about the past situation is often lacking. For example, it has been shown that oak powdery mildew is caused by several

Erysiphe species although it was previously thought to be caused only by *E. alphitoides* [21]. A new previously unreported species, *E. quercicola,* was shown to be frequent in the French oak forest [22]. However, it is not known since when this species is present in Europe. It might have been present since a long time remaining undetected due to inadequate means to distinguish the species.

The use of herbarium specimens is a good means to study the past geographic distribution of plant pathogens. Indeed, detection of *Silene* anther-smut disease in herbarium enabled Antonovic *et al.* [2] to document retrospectively the invasion of North America by *Microbotryum violaceum.* The development of molecular tools for species identification and quantification greatly improved the prospects of using herbarium specimens. For example, Shaw *et al.* [25] were able to study the relative frequency of two wheat pathogens, *Phaeosphaeria nodorum* and *Mycosphaerella graminicola*, in a 160-year long experiment. They showed that SO_2 pollution was the major factor that controls the relative frequency of the two pathogens, high SO_2 pollution levels strongly favouring P. *nodorum* while low levels favoured *M. graminicola*. Herbarium specimens were also used to determine if the ash pathogen *Chalara fraxinea* is a recent invader in Switzerland [24].

Dothistroma needle blight (DNB) has strongly impacted Laricio pine stands in France in the last 15 years [3]. Although the causal pathogen, *Dothistroma septosporum* had been present in France since at least the sixties, it was reported then as only a minor pathogen of pines [18]. The disease has emerged also in other European countries and in British Colombia in the recent years [29, 30]. The emergence of DNB in those areas has been related to recent climate evolution with an increase in the frequency of favourable warm and wet periods [31]. In the last years, it was shown that DNB can be caused by two closely related species with overlapping morphological features [4]. *D. septosporum* (teleomorph: *Mycosphaerella pini*) is the most important species throughout the world while *D. pini* (teleomorph unknown) was initially reported only in North-America. All isolates from present and past collections of Europe that were investigated using molecular tools were assigned to *D. septosporum* [4]. However, *D. pini* was recently found in Ukraine and south-western Russia where it has been associated with the emergence of DNB on *P. pallasiana* [5], and it was also recently showed that *D. pini* was present in France and Hungary [6, 15]. It is still not known if this species has recently arrived in Western Europe, what is its frequency in the pine forest and whether its arrival might have driven the emergence of DNB.

The aim of this work was to investigate whether the emergence of DNB in France could be linked to a recent introduction of *D. pini* in the country. For that purpose, a collection of *Pinus* needle herbarium samples were analyzed to determine if *D. pini* was frequent in France in the past. If a recent arrival of *D. pini* played a significant role in the DNB emergence, the disease severity should be related to the frequency of *D. pini* in the area. We thus compared the pattern of DNB severity within the country with the respective geographic distribution of the two *Dothistroma* species, taking advantage of the availability of data from the DSF (Département de la Santé des Forêts, french forest health survey system) on DNB occurrence in France in the last 20 years. A second aim of this study was to compare the response to climate of the two *Dothistroma* species.

Material and Methods

Dothistroma species distribution in France

A survey of the distribution of *D. septosporum* and *D. pini* was organised throughout France. Two different sets of sampling were done. A first set of samples (sample set 1) was collected in stands where a significant *Dothistroma* needle blight (DNB) impact was reported during the years 2007 to 2010 by the observers of the DSF. For approximatly 20% of the records, samples were collected and sent to the LNPV (Laboratoire National de la Protection des Végétaux) to confirm the presence of *Dothistroma* spp. Samples came from 80 different

Table 1. Herbarium specimens analyzed for presence of the two *Dothistroma* species

Species	Specimen	Host	Year	Location	Coordinate	Collector
D. pini	260[a]	*P. nigra sp. laricio*	1969	Les Barres (Loiret)	E2°43'47.6" N47°50'12.8"	M. Arbez
D. pini	NCY012997[b]	*P. sylvestris*	1907	Tourbière du Beillard (Vosges)	-	M. Joigny
ND	45[a]	*P. sylvestris*	1969	Garenne de Perthus (St Dizier)	E3°31'52.6" N46°51'16"	M. Morelet
ND a	1481[a]	*P. nigra sp. salzmanii*	1966	Amance	E3°40'46.1" N44°17'54.8"	M. Morelet
ND	NCY012993[b]	*P. incinata*	1862	Gérardmer (Vosges)	-	M. Godron
ND	NCY012995[b]	*P. pumilis*	1850	Le Belieu (Doubs)	-	M. Grenier
ND	NCY012996[b]	*P. pumilis*	1844	Le Belieu (Doubs)	-	M. Grenier
ND	NCY012998[b]	*P. sylvestris*	1831	Bouxwiller (Bas-Rhin)	-	M. Buchinger
ND	NCY012999[b]	*P. nigra sp. salzmanii*	1845	Saint Guilhem le désert	-	M. Touchy
D. septosporum	170[a]	*P. nigra sp. laricio*	1966	Gondreville	E5°51'31.5" N46°44'31.6"	M. Morelet
D. septosporum	173[a]	*P. nigra*	1966	Vitrey-sur-Mance	E4°56'43.2" N48°37'42.3"	M. Morelet
D. septosporum	266[a]	*P. nigra sp. laricio*	1968	Champvert	E2°45'2.4" N43°28'58.7"	De Perrier
D. septosporum	1262[a]	*Pinus sp.*	1968	Haguenau	E5°58'47.4" N48°40'55.6"	M. Morelet
D. septosporum	226[a]	*P. sylvestris*	1969	Les Barres (Loiret)	E5°45'39.7" N47°48'51.5"	M. Morelet
D. septosporum	227[a]	*P. sylvestris*	1969	Dijon	E4°56'43.2" N48°37'42.3"	L. Lanier
D. septosporum	257[a]	*P. ponderosa*	1969	Garenne de Perthius	E5°2'29.8" N47°19'59.7"	-
D. septosporum	261[a]	*P. sylvestris*	1969	Puy de Dôme	E2°43'47.6" N47°50'12.7"	C. Delatour
D. septosporum	262[a]	*P. nigra*	1969	Rainans	E3°7'43.5" N45°42'56"	C. Delatour
D. septosporum	263[a]	*P. nigra*	1969	Chassigny	E5°28'39" N47°9'26.9"	C. Delatour
D. septosporum	264[a]	*P. nigra*	1969	Liffol le Grand	E5°23'12.1" N47°43'8.1"	C. Delatour
D. septosporum	424[a]	*P. sylvestris*	1970	D64 entre Ranchal et Cours	E5°34'28.3" N48°18'55.8"	C. Delatour
D. septosporum	102[c]	*P. nigra*	1974	Col du Loumenot (Saint Julien d'Arpaon)	E4°24'7" N46°7'37.2"	A. Soutrenon
D. septosporum	143[c]	*P. nigra sp. laricio*	1974	Saint Pons	E1°25'54.4" N44°51'14.1"	A. Soutrenon
D. septosporum	438[c]	*P. nigra sp. laricio*	1982	Nadaillac de rouge	E2°6'32.3" N45°18'45.2"	J.F. Abgrall
D. septosporum	551[c]	*P. nigra sp. laricio*	1982	Vitray	E2°40'50" N46°36'54"	DDA Allier
D. septosporum	471[c]	*P. nigra sp. laricio*	1983	Saint-Hilaire-Foisson	E1°25'54.4" N44°51'14.1"	DDA Tulle
D. septosporum	569[c]	*P. nigra sp. austriaca*	1985	Nadaillac	E3°18'25" N47°15'5.4"	DDA Lot
D. septosporum	586[c]	*P. nigra sp. laricio*	1986	FD des Taillis de Dompierre (Saint Bonnet)	E5°33'38.2" N48°44'48.7"	ONF
D. septosporum	604[c]	*P. nigra sp. austriaca*	1986	FD Commercy	E7°49'12.7" N48°50'9.7"	ONF Commercy
D. septosporum	455[c]	*P. nigra sp. austriaca*	1982	Champagnole	E6°21'16.9" N48°43'53.9"	ONF Lons le Saulnier

Note. All specimens are samples of pine needles showing symptoms of DNB
[a] INRA-Nancy
[b] Jardin Botanique du Montet, Nancy
[c] CEMAGREF-Grenoble

stands. A second set of 136 samples (sample set 2) was collected based on a network of plots established over France for the survey of pine processionary moth, *Thaumetopoea*

pityocampa. All the *Pinus nigra* stands within this network were surveyed for presence of DNB in 2009 and for each stand with typical symptoms of DNB, samples were collected and sent for analysis to the LNPV. Finally, sample set 2 gathered samples collected in 136 stands where DNB was observed and for which the presence of *Dothistroma* spp. could be confirmed by the laboratory analysis. Sampling set 1 and 2 did not represent different areas as both came from all over France. However, while sample set 1 represented stands selected for significant DNB presence, sample set 2 mainly corresponded to stands with weak DNB impact. Disease prevalence, i.e. percent infected trees per stands, was only available for stands of sample set 2.

For each investigated pine stand (sample sets 1 and 2), a mix of needles showing typical symptoms of DNB (red bands) were collected from several affected trees, placed into an individual tightly closed paper bag and immediately sent for analysis. Typically, 5-30 needles were sampled on 2-3 trees, depending on the amount of disease found in the stand. For each individual sample, five to ten 5-mm length sections of different needles bearing red bands were excised aseptically and carefully transferred into a 2 ml sterile centrifuge tube. Total DNA was extracted and analyzed by a multiplex real-time PCR analysis following the protocol described by Ioos *et al.* [16] to determine the presence of *D. pini* and / or *D. septosporum*. Basically, 3 primer pairs were used, specific of either *D. pini*, *D. septosporum* or 18S ribosomal DNA. The 18S was used as an amplification control. Three replicates were done for each of the primer pairs. A *Dothistroma* species was considered to be present only if amplification was obtained with the relevant specific primer for at least 2 replicates. If no amplification was obtained with the 18S amplification control for 2 replicates or more, the detection was considered as not valid and no result was derived from the analysis.

Thirty herbarium specimens of pine needles showing symptoms of DNB were available (Table 1). The specimens came from eastern France, from the Mediterranean area to the NE german border. The majority of them came from natural ecosystems, mostly forests. The only exceptions are specimens from Amance (1) and Les Barres (3) which came from arboretum. As for fresh samples, herbarium specimens were analyzed by real-time PCR to determine the *Dothistroma* species present at that time in each locations. For each herbarium specimen, pieces bearing conidiomata were cut from different needles with a scalpel and put into a 2 ml sterile centrifuge tube (one to ten pieces by specimens). DNA extraction and real-time PCR analysis were done as described by Ioos *et al.* [16] for fresh samples, except for the specimens collected in the beginning of the nineties. For these very old samples, some steps of DNA extraction were adapted. Specimens were frozen in nitrogen before grinding and the incubation at +65°C with RNase was performed during one hour. Furthermore, all precipitation steps were done into a freezer instead of ice, and their time of incubation was three hours. The identification process, from the extraction to the PCR, was repeated twice.

Analysis of Dothistroma *spp. distribution in relation with climate*

For each stand, the nearest meteorological station of the Météo-France network with data available over a 15-year long period (1990-2005) was selected. The median distance between the plot and the meteorological station was 10 km (range 3-20 km). The climatic variables tested were the means over the 1990-2005 period of minimum, maximum and mean daily temperatures and sum of precipitations (in mm) for four periods, i.e. winter (December to February), spring (March to May), summer (June to August) and autumn (September to November). Precipitation and temperature in summer were not correlated for the set of studied stands (Spearman r of 0.016, P = 0.81);

The relationship between the presence of *D. pini* and climate in the 216 stands with confirmed DNB was investigated by logistic regression using the Genmod procedure of SAS (SAS/STAT 8.1, SAS Institute Inc., Cary, NC). The logistic function was used as link. The

model was built by a forward selection procedure and the model quality was checked (residual distribution, influential point).

Spatial distribution of Dothistroma needle blight impact in France

The presence of both *D. pini* and *D. septosporum* was compared with the spatial distribution of DNB severity in France. The Département de la Santé des Forêts (DSF) has implemented a database which stores records of health problems observed in France by a network of foresters trained for the diagnosis of all types of health affections, including abiotic, entomological or pathological damages. These observers devoted only part of their time to DSF activities, i.e. observation and report of forest health problems. Altogether, from 1989 to 2006, about 12 800 health problems on *Pinus* species were recorded in the database, 780 of which concerned DNB. The symptoms induced by *Dothistroma* are characteristic and a reliable diagnostic can often be established in the field. However, for about a third of the DNB report, a needle sample was taken in the affected stand and sent to the LNPV for a laboratory diagnostic. The test performed until 2007 did not enable to specify which *Dothistroma* species was involved in the reported DNB case and this information is thus not available for this dataset. After 2006, a change in the data collection procedure precluded the use of the data in the same analysis. Thus, records used in the previous section to study the distribution of *Dothistroma* species are not taken into account in this analysis.

The non-systematic and non-homogeneous way used for data collection and the fact that only occurrence is recorded impeded the data analysis and did not allow the estimation of infected hosts frequency. The local number of DNB records observed strongly depends on the local density of host and on the observation intensity. Indeed, more DNB records will be produced in area where the host (the so-called “at risk” population) is abundant and/or economically important and some observers will devoted more time to their DSF activity and therefore produce more records for a given problem intensity. Expected local number of DNB records were estimated to compare to the observed one in order to assess whether DNB is of significant local importance The methodology used was adapted from what is done in medical epidemiology [19]. Basically, the raw number of DNB records was compared to the raw number of records for a set of problems selected to be typical of the “at risk” population. The local density of reference records is considered to be proportional to the “at risk” population local importance and to the observation intensity by local observers and can thus be used to estimate an expected local number of DNB records. We used as reference records most health problems affecting pines trees other than DNB. However, a limited number of problems representing a very high proportion of the records, such as occurrence of *Thaumetopoea pityocampa,* were not used in order to avoid possible bias linked to heterogeneous distribution of records in time or space.

In a first step, time was not taken into account and all the records from 1989 to 2006 were pooled. To estimate the local density of DNB and reference records, a nonparametric kernel estimation method was used [13, 27]. This method estimates the record density on each point of a grid. Each record is weighted by the kernel function according to the distance to the grid point. The bandwidth is the important kernel parameter controlling the weight decay with the distance to the grid point. The records density for each point of a 10 x 10 km grid was computed. The quartic kernel with a bandwidth of 80 km was used for both DNB and reference records density. The least square cross-validation [28] was used to estimate the values of the bandwidth on several portions of the surveyed area, and to choose one which was large enough to avoid cases lacking record within the discus defined by the bandwidth.

A standardized record rate (SRR) was produced by dividing the observed DNB records density by the expected DNB records density, which was computed as the product of the reference records density and the average DNB record rate for France (total number of DNB records divided by total number of reference records). To assess the quality of SRR local

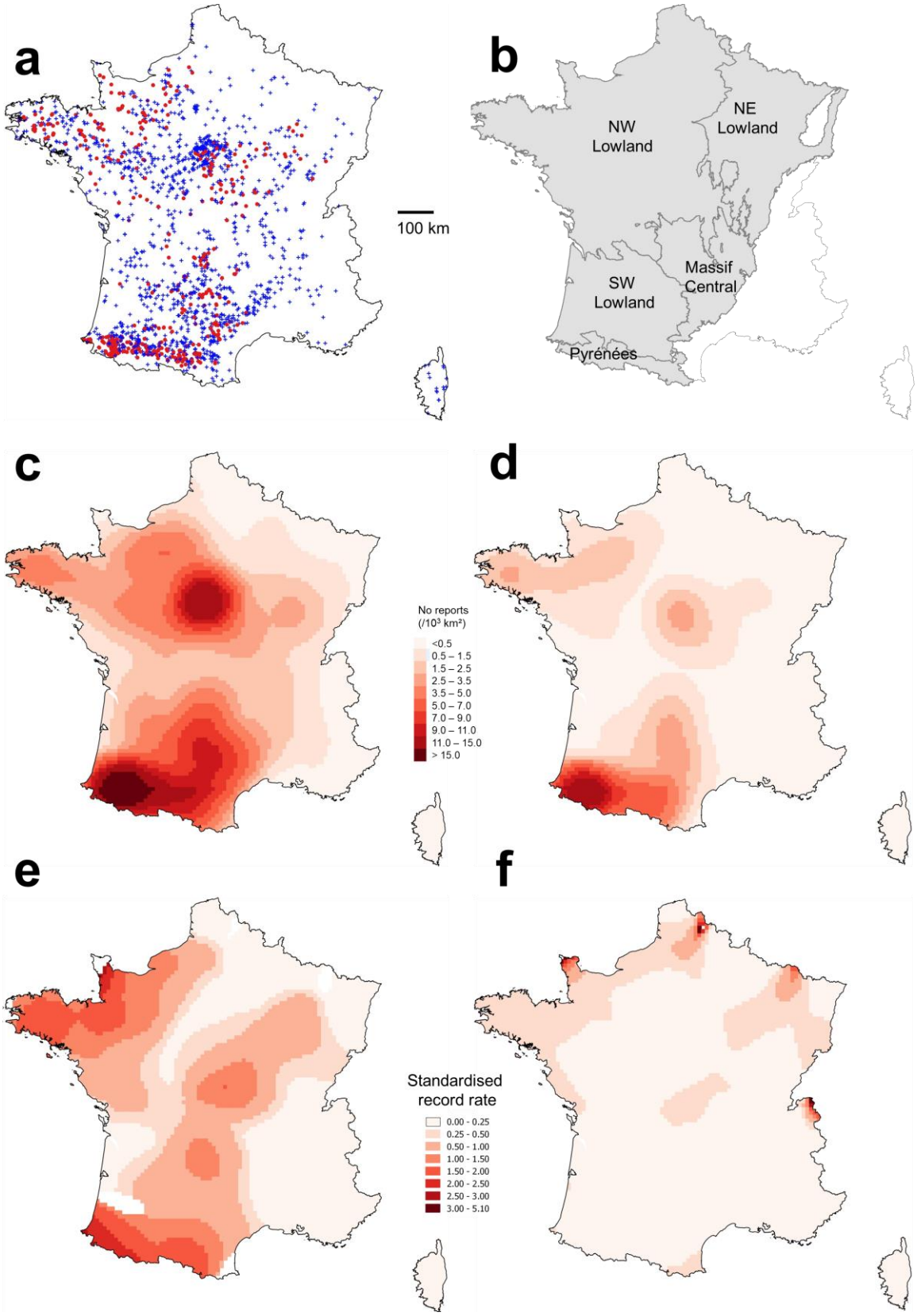

Fig.1. Distribution of the Dothistroma needle blight (DNB) in France. a. Records on laricio pine in the DSF database (●, DNB, +, others health problems used as reference); b. Areas for which the annual evolution of the DNB standardised record rate was computed; c. Density of DNB record; d. Density of reference problems records; e. DNB standardised record rate; f. Standard deviation in the DNB standardised record rate.

estimates, the potential bias (measure of the local potential departure from the mean) and the standard deviation were computed by boot-strap analyses for each grid points. New simulated data sets were automatically generated by assigning to each of the used records (DNB plus reference) a new value (either DNB or reference record) depending on the estimated local SRR. A thousand simulated data sets were generated by this method and used to produce 1000 SRR simulated estimates. The bias was estimated by comparing the observed SRR to the averaged simulated ones. The standard deviation of the SRR was estimated from the mean squared difference between the observed SRR and the averaged simulated ones. All these analyses were done using the R statistics software.

In a second step, the annual dynamic of DNB records was computed. This was done only for 5 areas of France with enough reference records (Fig. 1b). The set of reference diseases used in the previous analysis was also used to standardize the raw DNB record number. The annual number of reference records in an area was computed as the sum over all DSF observers present in the area of the average annual number of reference records per observers. The annual standardized records rate (SRR) was estimated as the annual observed number of DNB records divided by the annual expected observed number of DNB records (N_{ref} multiplied by the average DNB record rate for all France).

Disease severity in location with presence or absence of D. pini

To investigate the effect of the presence or absence of *D. pini* on disease severity, two different measurements of disease severity were used. First, the presence of the two *Dothistroma* species was compared with the regional DNB severity estimated from the DSF database (SRR, see above). Each location of the 2007-10 *Dothistroma* species survey was associated with the closest grid point estimate of SRR using a GIS device. The relationship between *D. pini* presence and SRR estimate was studied. Second, the disease prevalence associated with the record, *i.e.* the percentage of affected trees in the stand, was used as a measurement of the stand-level disease severity. This analysis was limited to the sampled stands concerning *P. nigra* ssp. *laricio* as there were not enough stands with other pine species to be included in the analysis. The disease prevalence at the stand level was available only for 128 *P. nigra* ssp. *laricio* stands of sample set 2.

Differences in disease prevalence and in SRR between sites infected or not infected by *D. pini* were statistically assessed by variance analysis. Prior to analysis, the data of disease prevalence was transformed by taking the arcsin of square root. To avoid any possible climate bias on the disease severity that could have been caused by the differences of distribution areas between the two *Dothistroma* species, the total rain in March to May and average temperature in June, July and August during the 1990-05 period were used as a covariate. These climate variables are known to be important for the epidemiology of the *Dothistroma* needle blight [23, 31].

Results

Dothistroma species distribution in France

Altogether, during the 2007-10 survey, *D. septosporum* and *D. pini* were detected respectively in 133 and 123 of the 216 studied stands. Both species were co-occurring in 40 stands. Most of the samples were collected on *P. nigra* ssp. *laricio*, whereas only 18 samples came from other pine species: 9 on *P. nigra* ssp. *austriaca,* 4 on *P. pinaster* and 5 on *P. sylvestris*. *D. septosporum* was found on all pines species while *D. pini* was found only on *P. nigra* (ssp. *laricio* and ssp. *austriaca*). *D. septosporum* was evenly distributed over France except in the Mediterranean area where it was scarcely found (Fig. 2). In spite of a specific survey of DNB, this disease was never found in Corsica which is the only place in France where *P. nigra* ssp. *laricio* is native. *D. pini* showed a more restricted distribution area, being very infrequent in the north and east of the country as well as in the mountainous areas (Massif Central and Pyrénées).

Detection and identification of a *Dothistroma* species were successfully achieved for 23 out of the 30 herbarium specimen available (Table 1). Most of the positive specimens were found to be infected by *D. septosporum*. *D. pini* was only detected in two occasions, one specimen collected in 1907 from Toubière du Beillard in NE France and the other one collected in 1965 in the Arboretum des Barres, in central France.

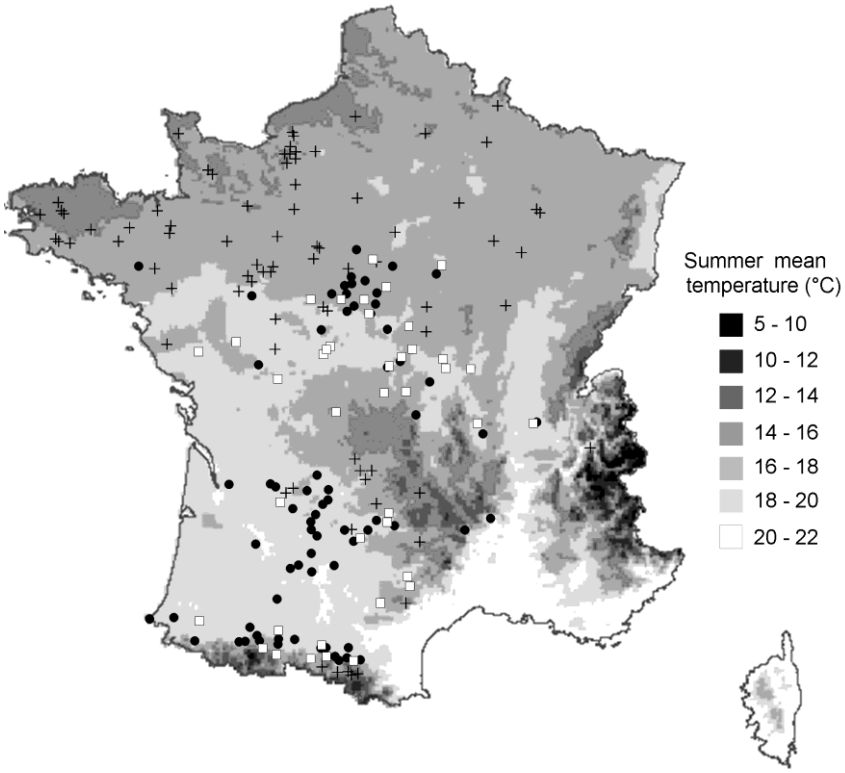

Fig.2. Presence of the two Dothistroma species in the 2007-10 survey (+, D. septosporum, ●, D. pini, □, both species). Mean summer temperature, from June to August (source Aurelhy, Météo-France, 1960-90).

Analysis of Dothistroma spp. distribution in relation with climate

The distribution of *D. pini* in France was strongly related to climate variables. In particular, 2 variables, *i.e.* mean temperature over June to August and total rainfall over June to August, explained a large part of the distribution of this species (Table 2). Indeed, high mean summer temperature strongly correlated with the presence of *D. pini* as the probability of this species presence was 4.1 fold higher for each additional 1°C increase in mean temperature of June-August (Fig. 3a, Table 2). This result explained the absence of this pathogen species in the north and east of France and at higher elevations, in areas with a mean summer temperature lower than to 16-17°C (Fig. 2). Summer rain had a minor effect on the frequency of *D. pini* presence with an odd ratio of only 1.2 (increase in presence probability for each 10 mm additional summer rain, Table 2). The influence of summer rain explained mainly the scarcity of *D. pini* in areas with low summer precipitations in north-west France (Fig. 3b).

Spatial distribution of Dothistroma needle blight severity in France

The ratio records number / forest surface area was significantly higher for *P. nigra* ssp. *laricio* than for other pine species. Indeed, from 1989 to 2006, 6.67 cases were recorded per 10^3 ha of *P. nigra* ssp. *laricio* forest compared to 0.00, 0.02, 0.07, 0.01 and 0.01 per 10^3 ha

forest of *P. halepensis*, *P. uncinata*, *P. nigra* ssp. *austriaca*, *P. pinaster* and *P. sylvestris* respectively (based on forest surfaces data for France provided by the Inventaire Forestier National). As *Pinus nigra* ssp. *laricio* represented 94 % of the available DNB records in the database, only records concerning that species were considered in the "at risk" population, *i.e.* a total of 687 DNB records and 2003 reference records (Fig. 1a).

Table 2. Relationship between climate and *D. pini* presence: results of the logistic regression analysis

Parameter	Df	Odd ratio (confidence interval)[a]	Wald Chisq (Pvalue)
Mean temperature (June to August)	1	4.1 (2.8 – 6.1)	48.8 (<.0001)
Rain in June to August	1	1.2 (1.1 – 1.4)	14.0 (<.0001)

NOTE. Model deviance was 193.8 for 213 df.

[a] measure the increase in the probability of *D. pini* presence per unit summer temperature (for each 1°C) and rainfall (for each 10 mm) increase, *i.e.* probability of *D. pini* presence 4.1 x higher for each additional 1°C increase.

Table 3. Relationship between *D. pini* presence and *Dothistroma* needle blight standardized record rate in the DSF database.

Source	df	Sum square	F	P>F
Model	3	8.81	10.45	<.0001
Error	212	56.86		
Presence of *D. pini*	1	0.01	0.00	0.961
Rain (June to August)	1	6.14	22.89	<.0001
Mean temperature (June to August)	1	0.46	0.70	0.194

Note. SRR, available for the 216 stands surveyed in 2007-10

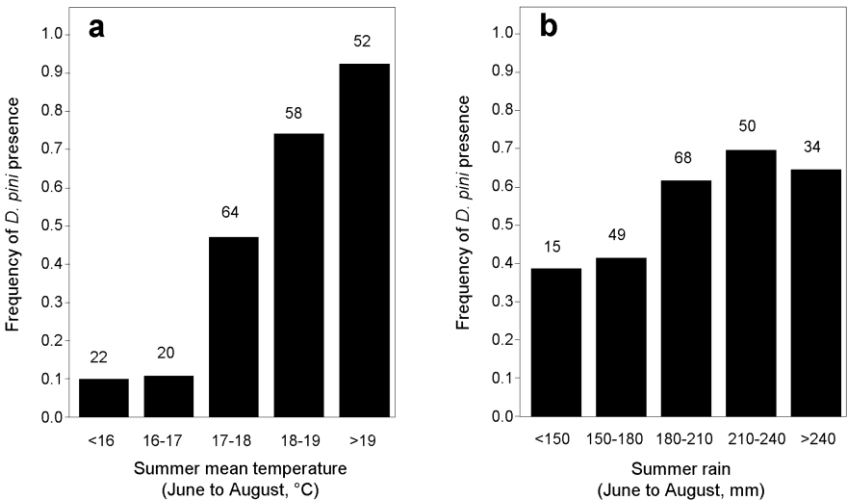

Fig. 3. Frequency of Dothistroma pini in needle blight affected pine stands during the 2008-2010 survey according to (a) mean summer temperature (June-August) and (b) mean summer rain (June-August).

DNB records were notably frequent in Western and Central France and in the Pyrenean Piedmont (Fig. 1c). A very limited number of DNB case were recorded for NE France, whereas none was registered for the Mediterranean area. In particular, no DNB case was recorded for Corsica, where this pine species is indigenous and represents an important proportion of the forest surface. The distribution of the references records was slightly different compared to DNB records, with a very high frequency in the Pyrenean piedmont and in central France (Fig. 1d). A low SRR standard deviation (ranging from 0.1 to 0.3) was observed throughout most of the country (Fig. 1f). However in some limited area, in particular in the Cotentin (NW France), the SRR standard deviation exceeded 2, which means that the SRR values for these locations should be interpreted with care. The bias in the SRR remained also low over most of the country (lower than 0.2) with area of high value in the same area as high value of SRR standard deviation (result not shown). Thus, the SRR could be safely interpreted for a major part of France, except part of the Cotentin, close to the channel. The SRR was high in Western France and in the Pyrenean Piedmont and, to a lesser degree in Central France, and notably low in Eastern France.

Table 4. Relationship between *D. pini* presence and disease prevalence in the 128 *P. nigra* ssp. *laricio* stands of 2007-10 (sample set 2) with prevalence data.

Source	df	Sum square	F	P>F
Model	3	4.33	17.76	<.0001
Error	124	23.05		
Presence of *D. pini*	1	0.28	1.49	0.224
Rain (June to August)	1	1.06	5.71	0.018
Mean temperature (June to August)	1	2.45	13.19	<.001

Overall, a continuous increase of *Dothistroma* needle blight SRR could be observed in France since 1992 (Fig. 4). A very limited number of DNB cases were recorded before 1992 and they remained scarce until 1998 when a dramatic increase in the SRR could be observed. The disease outbreak was observed first in SW France (Pyrenean piedmont and SW lowland) and later in Northern France, in particular in the NW lowland (in 2001-02). Since 1998, the disease has been consistently reported in the western half of France.

Comparison of disease severity versus presence of D. pini

The distribution of *D. pini* within France was compared to the distribution of DNB (see Fig. 1e and Fig. 2): no obvious relation was observed between areas of high disease record and the pattern of spatial distribution area of *D. pini*. For example, DNB SRR was equally high in the Pyrenean piedmont where *D. pini* was dominant and in Northern France close the channel, where only *D. septosporum* was found. Indeed, no significant relation was found between *D. pini* presence and needle blight SRR (Table 3). The mean SRR rate was 1.00 ± 0.09 when *D. pini* was absent and 1.01 ± 0.09 when it was present. By contrast, the *Dothistroma* needle blight SRR was significantly correlated to climate variables and in particular to rainfall in the June/August- period, with a disease record rate positively affected by increasing rainfall.

As for the DNB SRR, no significant relationship was observed between the presence of *D. pini* and the stand level disease prevalence (Table 4). The mean prevalence values in stands with or without *D. pini* were 45.3 % ± 6.5 and 48.8 % ± 8.9, respectively. Mean DNB

prevalence in stands also significantly increased with rainfall and mean temperature in June-August.

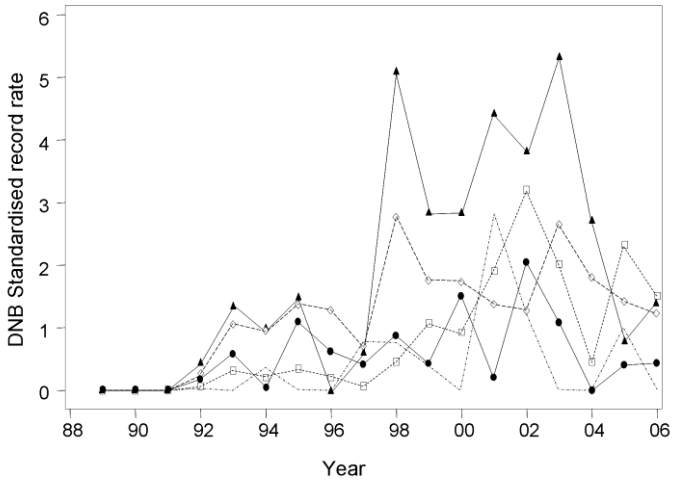

Fig. 4. Annual evolution of Dothistroma needle blight (DNB) in the DSF database. Pyrénées (—▲—), SW lowland (—◇—), NW lowland (-▫-), Massif Central (—●—) and NE lowland (▬▬).The annual SRR in a given area represent the ratio between observed and expected DNB record numbers.

Discussion

This work demonstrated that *Dothistroma pini*, previously thought to be absent from Western Europe and reported for the first time in France by Ioos *et al.* [15], is actually widespread in the southern part of the country, where this species was present in the majority of the sites affected by *Dothistroma* needle blight (DNB). *D. pini* was detected in old herbarium specimens collected in France. Although it is still not possible to determine precisely when this pathogen arrived in Western Europe, our results suggest that the emergence of DNB in France in the early 1990^{ies} was not caused by a recent introduction of this species. Indeed, there was no obvious relationship between the spatial patterns of disease severity and the presence of *D. pini*.

In this study, the presence of *D. pini* could be demonstrated in herbarium specimens collected back to 1907 and 1965, which suggests that this species is not a recent invader in France. However, *D. pini* was detected from only 2 herbarium specimens. Moreover, for the specimen of 1907 from the Tourbière du Beillard, the sample was too small to repeat the detection procedure. Thus, this result should be taken with caution.

The origin of *D. septosporum* has been controversial with proposed centre of origin in Central America or in the Himalaya [11, 16]. Morelet [20] proposed that this species had been present in France since the end of the 19th century. Indeed a fungus morphologically very similar to *D. septosporum* was described by Vuillemin [26] in 1896 on *P. nigra* and *P. sylvestris* in NE France under the name *Hypostonum flichianum*. Unfortunately, the herbarium of Vuillemin has not been kept and it thus cannot be confirmed that this description referred to either *D. septosporum* or *D. pini*. However, the identification of a *Dothistroma* species from a specimen collected in NE France in 1907 confirms that DNB has been present in France for at least a century and makes the exotic nature of these 2 species in Europe questionable.

It is worth noticing that DNB could not be found in Corsica although the very susceptible *Pinus nigra* ssp. *laricio* is abundant in the local forest where it is native in contrast to continental France where it is introduced. This is surprising as it may not be explained by

unfavourable climatic condition as in Corsica Laricio pine stands are mostly located in mountain areas where the climate with wet and warm springs should be suitable for the disease. We thus could not find support for the hypothesis that one of the *Dothistroma* species is native to Corsica, being present on the native host without inducing significant damages.

It may be hypothesized that *D. pini* was not frequent in France during the last century, since it was detected in only 9% of the *Dothistroma* positive herbarium specimens whereas this species was detected in 57% of the stands studied during the 2007-10 survey. However, many of the herbarium specimens came from areas where only *D. septosporum* can be found presently: only seven out of the 17 herbarium specimens from the sixties and seventies came from areas were *D. pini* can now be found. *D. pini* was detected from one out of those seven specimens, and was collected in the Arboretum des Barres (Loiret), an area where *D. pini* is now common. Alternatively, the frequency of *D. pini* might have strongly increased in France. This could be related to the recent climate warming, as this species is restricted to areas with warmer summers.

The pattern of emergence indicates a spread of the disease from the South-west of France up to northern areas. This could fit with a significant role in the disease emergence of a species that requires hotter condition such as *D. pini*. However, no relationship could be found between the spatial pattern of DNB report by the DSF and the distribution of *D. pini* in France. The distribution of *D. pini* remained so far mainly restricted to southern regions and *D. septosporum* was the only species present in severely affected areas close to the Channel. This is in agreement with the fact that DNB also emerged recently in Great Britain where only *D. septosporum* has been reported hitherto [7]. The clear pattern of disease emergence first in the south of the country with a later extension to northern areas could also be explained if climate change was an important factor in the disease emergence. In that case, climatic conditions would have been expected to be favourable earlier in the warmer SW part of France. Indeed, this study showed that disease prevalence at the stand level during the 1997-2010 survey was positively related to mean summer temperature. It has been reported that DNB requires periods of temperature in the 15-20°C range under continuous moisture to be severe [12, 23]. An increased frequency of such warm and wet periods during the vegetation season has been associated with the emergence of DNB in British Columbia [31]. The decade from 1990 to 2000 has been exceptionally warm in France with normal favourable rainfall and this could likely explain the emergence of DNB.

Temperature was the most important climatic factor to explain *D. pini* presence while it was less important for DNB severity. It is not unusual that temperature is the prominent factor in the climatic envelop of an organism (to explain it presence), while precipitation gets more important for explaining it local abundance [9]. Also, for the severity, we do no know the respective importance of *D. pini* and *D. septosporum* as causes of the symptoms and high temperature is mostly important for *D. pini*.

Invasion of plant pathogenic fungi closely related to indigenous species can strongly cloud the causes of disease emergence. An example of that is the *Chalara* induced ash decline in Europe. This disease is induced by *Hymenoscyphus pseudoalbidus*, a species closely related to an indigenous european species *Hymenoscyphus albidus* [24]. No quarantine regulations were originally taken against *Hymenoscyphus pseudoalbidus* because of confusion between the two species [17]. This study showed that the use of past herbarium specimens and the comparison of species distribution with the disease spatial pattern were powerful means to sort out the relation between *D. pini* and DNB emergence in France. However, this points out a lack of knowledge about plant pathogenic species communities even in well studied areas such as Europe. This is especially the case for pathogens that do not cause significant damages as for *Dothistroma* species in France in the past. A larger use of the recently developed molecular techniques such as mass sequencing of taxonomically relevant genes in environmental samples [8] would be a significant improvement. Indeed, as these techniques are not narrowed to a list of specific organisms, a

more comprehensive description of plant pathogens communities will probably be available in the near future.

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