Spatial distribution and basic ecology of *Heterodera schachtii* and *H. betae* wild populations developing on sea beet, *Beta vulgaris* ssp. *maritima*

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Summary – Most populations of crop pathogens have wild relative populations from which they can originate but for which basic knowledge of their ecological requirements *in natura* is scarce. However, the study of spatial distribution and ecology of wild pathogen populations may help control them in crops through a better understanding of the environmental factors driving population dynamics. Here, we focused on *Heterodera schachtii* and *H. betae*, two cyst nematodes that cause severe damage to sugar beet (*Beta vulgaris* ssp. *vulgaris*) crops and can develop on a wild beet relative, the sea beet (*B. vulgaris* ssp. *maritima*). We investigated the occurrence of both nematode species in the wild and explored some environmental factors that may influence their geographical distribution. To do so, we sampled the wild host *B. v.* ssp. *maritima* along the European Atlantic and North Sea coastlines. Results showed that *H. schachtii* mainly occurred in the colder environments of northern Europe, whereas *H. betae* was preferentially distributed in the warm environments of southern Europe. It was previously established that *H. betae* only recently appeared in The Netherlands, which are in the north of Europe. Thus, our results do not support this hypothesis. Overall, this study accurately documents the geographical occurrence of two nematode crop pest species in the wild and helps identify the main environmental factors affecting their distribution range.

Keywords – cyst nematode, environmental factors, geographical distribution, wild beet, wild crop pathogen populations.

Yield reduction due to crop plant pests is a worldwide concern causing several billion euros of economic losses annually, and can sometimes even lead to famine. Pest management has thus been a challenge for agronomic research for several decades (Matthews, 1984). Recently, increasing restrictions on the use of pesticides has led to the development of new environmentally friendly control methods, among which resistant varieties often appear as one of the most cost-effective alternatives. In most cases, resistance comes from the wild relatives of cultivated species and results from long co-evolutionary interactions between wild host plants and wild pathogen populations (Cook, 2004). Also, field pathogen populations can be assumed to originate more or less recently from wild pathogen populations and to exchange some individuals with wild reservoirs (according to their dispersal features). As a result, the efficiency of introduced resistance in crop plants depends on the similarity of genetic characteristics between field and wild pathogen populations. Thus, any knowledge about the spatial distribution, ecology and genetics of wild pathogen populations will give us a better understanding of the dynamics of those species and may shed light on the management of field pathogen populations by improving crop resistance deployment strategies (van der Putten *et al.*, 2006).

There is little information currently available on the distribution and the basic ecology of wild populations of most plant-parasitic nematodes associated with major crops. This is particularly true for *Heterodera schachtii* and *H. betae*, two species of cyst nematodes that cause

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severe annual damage to sugar beet (*Beta vulgaris* ssp. *vulgaris*). In sugar beet fields, the use of resistant varieties developed from the wild beet relative, the sea beet (*B. vulgaris* ssp. *maritima*), has recently become the only way to control *H. schachtii* populations or to minimise their effects on yield. This nematode is an amphimictic species that is widely distributed throughout the world and has a lot of alternative host species. Resistance to *H. schachtii* bred in certain sugar beet varieties has also helped to control *H. betae* populations, although to a lesser extent. *Heterodera betae* is considered as a true species (Wouts *et al.*, 2001) and has been found in Europe and Morocco (Amiri *et al.*, 2002; Subbotin *et al.*, 2010), although its reproductive mode and host range remain to be confirmed.

Heterodera schachtii and *H. betae* can reproduce on *B. v.* ssp. *maritima* in the laboratory (Maas & Heijbroek, 1982; Steele *et al.*, 1983). This wild host plant is widely distributed all along the European Atlantic and Mediterranean coastlines (Biancardi *et al.*, 2012) on a narrow area, very close to the sea. Ecosystems where sea beet occurs may thus be considered as potential natural reservoirs of field populations of both nematodes species. *Beta vulgaris* ssp. *maritima* is an ideal candidate for a study on the geographical occurrence and basic ecology of wild populations of *H. schachtii* and *H. betae* in Europe. Here, we carried out a large-scale study to define the geographical distribution of both nematodes, to test the influence of environmental factors on their respective distributions, and to give a description of the favourable wild habitats for two major nematode crop pests.

Materials and methods

BIOLOGICAL MATERIAL AND SAMPLING PROCEDURES

Sampling was designed according to previous population genetic analyses on *H. schachtii* field populations, which demonstrated that gene flow can occur among populations of nematodes occurring in *B. vulgaris* ssp. *vulgaris* fields separated by distances of up to about 100 km (Plantard & Porte, 2004). To select distinct populations, we sampled 33 different locations separated by distances of 150 km. They were distributed along the Atlantic coastline, from northern Morocco to southern Sweden (Fig. 1). At each location, a minimum of three different sites were sampled along 20 km of the coastline. At each site, one population of *B. v.* ssp. *maritima* was characterised by selecting at least ten live plants from which fresh leaves were collected for molecular identification.
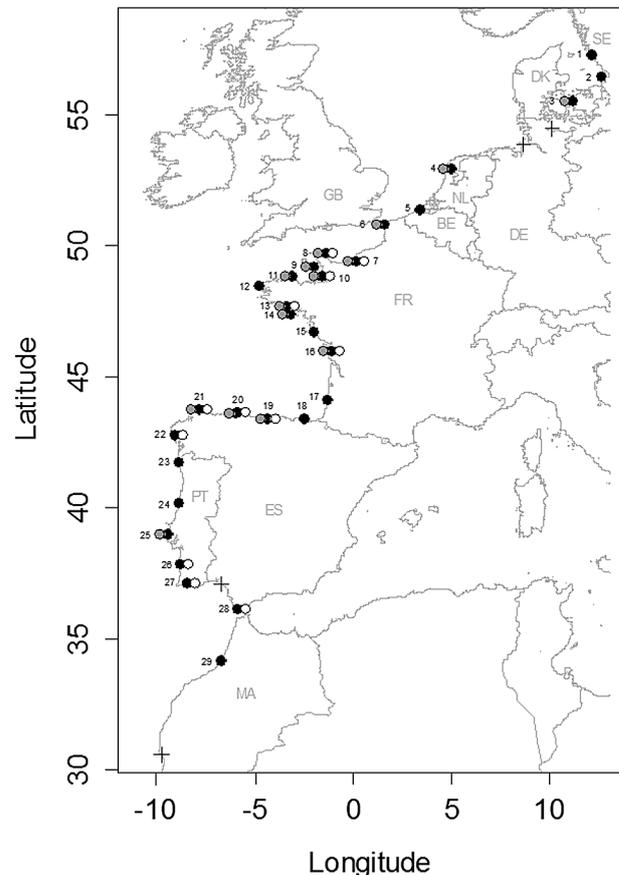

Fig. 1. Geographical distribution of *Heterodera schachtii*, *H. betae*, and their common host, *Beta vulgaris* ssp. *maritima*, along the Atlantic coastline. Black circles represent locations where *B. v.* ssp. *maritima* plants were found. Grey and white circles correspond to locations where *H. schachtii* and *H. betae*, respectively, were observed. Black crosses correspond to locations where *B. v.* ssp. *maritima* was not found. Numbers refer to the location code used in Table 1.

Soil samples surrounding the roots of each plant in these populations were collected, pooled and stored in a single plastic bag. Each bulk soil sample taken from a site was then considered as a population of *H. schachtii* and/or *H. betae* if nematodes were present. Cysts of *Heterodera* spp. were manually collected in every soil sample after extraction using a Kort elutriator. Cysts were then stored in moistened sand at 4°C before molecular characterisation.

MOLECULAR CHARACTERISATION OF NEMATODES

Heteroderidae species (*H. schachtii*, *H. betae*, *H. ciceri*, *H. diverti*, *H. galeopsidis*, *H. glycines*, *H. medicaginis* and *H. trifolii*) can be discriminated by RFLP polymorphism
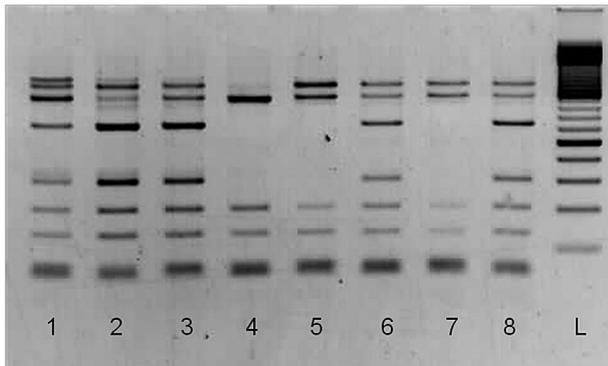
Fig. 2. Restriction patterns of ITS amplified fragments. The amplification and the digestion of the ITS region were used to discriminate *Heterodera schachtii* and *H. betae* individuals. Lanes 1, 2, 3, 6, 8: *H. schachtii*; Lanes 4, 5, 7: *H. betae*; L: 100 bp DNA ladder (Promega).

of ribosomal DNA (Subbotin *et al.*, 2000, 2001). In our case study, the digestion of PCR products by *Mva*I (2 h at 37°C) leads to specific restriction profiles for the *H. schachtii* and the *H. betae* ITS sequence, allowing accurate identification of these nematodes (Amiri *et al.*, 2002) (Fig. 2).

For each population, and for a minimum of 20 cysts, cysts were manually crushed and one juvenile from each cyst was transferred to a tube containing 20 μl NaOH (0.25 M) overnight. Samples were then incubated at 99°C for 2 min. A buffer (0.25 M HCl, 0.5 M Tris HCl (pH 8), 2% Triton X-100) was added to samples that were then incubated for 2 min at 99°C. Proteinase K (5×) was subsequently added and samples were held at 55°C for 1 h. Two primers (5′-TTGATTACGTCCCTGCCCTTT-3′ and 5′-TTTCACTCGCCGTTATAAGG-3′) targeting the nematode ITS region were used for PCR amplification. The cycling procedure for PCR amplification was as follows: 1 min at 94°C, 35 cycles with 1 min at 94°C, 50 s at 60°C, 1 min at 72°C, and a final step at 72°C for 5 min.

GENETIC CHARACTERISATION OF *BETA VULGARIS* SSP. *MARITIMA*

To avoid any possible confusion with other species of the *Beta* genus, the multilocus genotypes of plants collected in sampled locations were characterised individually to ensure subspecies identity and reveal another potential host plant species, the closely related self-fertilising species, *Beta macrocarpa*. This genotyping is described elsewhere (Leys *et al.*, 2014).

HABITAT ANALYSIS

The overall degree of differentiation or exclusion of the two nematode species was evaluated with the kappa statistic κ (Cohen, 1960), estimated as described in Guelat *et al.* (2008). This statistic is an analogue of the correlation coefficient for categorical variables. In our study, this index was used to evaluate whether the spatial distributions of *H. schachtii* and *H. betae* were correlated, *i.e.*, whether they preferentially co-occurred. κ was considered to differ from random expectations if the 95% confidence interval of the bootstrap (1000 replicates) distribution did not include zero.

Generalised linear models (GLM) were also used to test for the effects of habitat variables on the geographical distributions of the two nematode species. The habitat variables we considered included abiotic and biotic factors (see Table 2 for details). GLM are an extension of classical multiple regression and are particularly suitable for analysing spatial distribution of species because they can handle binomial presence-absence data and non-normal response variables (Guisan *et al.*, 2002). For *H. schachtii* (or *H. betae*), GLM were fitted considering *H. betae* (or *H. schachtii*) as an explanatory variable.

Correlation tests were performed to remove redundant covariables and simplify models. All analyses were performed with R software version 2.15.2 (R Development Core Team, 2012).

Results

Molecular characterisation of the sampled sea beet plants ensured that all soil samples were taken from *B. v.* ssp. *maritima* or its closely related species, *B. macrocarpa* (Leys *et al.*, 2014). *Beta v.* ssp. *maritima* was present in all locations surveyed along the Atlantic and North Sea coastlines, except in Germany and one Moroccan population composed of *B. macrocarpa*, totalling 90 populations of *B. v.* ssp. *maritima* from 29 locations (Fig. 1; Table 1). ITS characterisation revealed 27 populations of *H. schachtii* distributed in 15 (52%) of the sampled locations. *Heterodera schachtii* was mainly observed in northern Spain and in northwestern France. It also occurred sporadically in Portugal, Denmark and The Netherlands. ITS characterisation also revealed 21 populations of *H. betae* in 12 (41%) locations. Ten populations of *H. betae* were found in association with *H. schachtii* (Table 1). *Heterodera betae* populations were mainly observed in France,

Table 1. Location and number codes of nematode populations. The presence (x) of *Heterodera schachtii* and/or *H. betae* was determined by ITS characterisation.

Location code	Country	Region	Site	Population code	Presence of *H. betae*	Presence of *H. schachtii*
1	Sweden	Skalderviken	Torekov	Su1-1		
			Torekov	Su1-2		
2		Batfjorden	Venner	Su2-1		
3	Denmark	Kerteminde	Nordskov	Dan1-1		
			Nordskov	Dan1-2		
		Kalundborg	Reerso	Dan1-3		x
4	The Netherlands	Den Helder	Den Helder	Hol2-1		
		Wieringen	Den Oever	Hol2-2		x
5	Belgium	West-Vlaanderen	Zwin	Hol1-1		
6	France	Nord-Pas de Calais	Groffiers	Fra10-1		
			Etaples	Fra10-2		x
			Wimereux/Slack	Fra10-3		x
			Audresselles	Fra10-4		x
7		Haute Normandie	Honfleur	Fra9-1		x
			Pennedepie	Fra9-2		
			Pennedepie	Fra9-3	x	
8			Fermenville	Fra8-1		x
			Cosqueville	Fra8-2	x	
			Gatteville le Phare	Fra8-3		
			Montfarville	Fra8-4	x	x
9	United Kingdom	Jersey	Gouray	Gb1-1		x
10	France	Basse Normandie	Granville	Fra7-1	x	x
			Granville	Fra7-1No	x	x
			Carolles	Fra7-2		x
			Genêts	Fra7-3		
			Saint Léonard	Fra7-4	x	x
11			Paimpol	Fra6-1		
			Loguivy	Fra6-2		
			Ile à bois	Fra6-3		
			Île à bois	Fra6-4		x
12			Aber Ildut	Fra5-1		
			Pors Gored	Fra5-2		
			Pors Gored	Fra5-3		
13		Bretagne	Gâvres	Fra4-1		x
			Gâvres	Fra4-2		x
			Maguero	Fra4-3	x	
			Barre d'Etel	Fra4-4		x
			Nestadio	Fra4-5		
14			Port Jean	Fra4-bis1		x
15		Pays de Loire	Saint Gilles Croix de Vie	Fra3-1		
			Saint Gilles Croix de Vie	Fra3-2		
			La Sauzaie	Fra3-3		
			La Sauzaie	Fra3-4		
16		Poitou-Charentes	Baie d'Yves	Fra2-1		
			Fouras	Fra2-2		x
			Île Madame	Fra2-3	x	x
			Port des Barques	Fra2-4		
			Marennes	Fra2-5	x	

Table 1. (Continued.)

Location code	Country	Region	Site	Population code	Presence of *H. betae*	Presence of *H. schachtii*
17		Aquitaine	Contis plage	Fra1-1		
18	Spain	Euskadi	Lekeitio	Esp1-1		
			Laga	Esp1-2		
			Kanala	Esp1-3		
19		Cantabria	Oyembre	Esp2-1	x	x
			San Vicente de la Barquera	Esp2-2		
			San Vicente de la Barquera	Esp2-3	x	x
			San Vicente de la Barquera	Esp2-4	x	x
			San Vicente de la Barquera	Esp2-5	x	x
			San Vicente de la Barquera	Esp2-6		
			San Vicente de la Barquera	Esp2-7		
20		Asturias	Verdicio	Esp3-1		
			Verdicio	Esp3-2	x	
			Xago	Esp3-3		x
21		Galicia	Celtigos	Esp4-1		
			Celtigos	Esp4-2	x	x
			Espasante	Esp4-3		
22			Texueiro	Esp5-1		
			San Francisco	Esp5-2		
			San Francisco	Esp5-3	x	
23	Portugal	Viana do Castelo	Paço	Por1-1		
			Areosa	Por1-2		
			Areosa	Por1-3		
24		Coimbra	Praia da Tocha	Por2-1		
			Quiaios	Por2-2		
			Cabedelo	Por2-3		
25		Lisboa	Ericeira	Por3-1		x
			Ericeira	Por3-2		
			Lizandro	Por3-3		
26		Setúbal	Samoqueira	Por4-1		
			Porto Covo	Por4-2		
			Pessegueiro	Por4-3	x	
27		Faro	Carvoeiro	Por5-1		
			Carvoeiro	Por5-2	x	
			Marinha	Por5-3	x	
			Armacao de Pera	Por5-4		
28	Spain	Andalucía	Chiclana de la Frontera	Esp6-1		
			Sancti Petri	Esp6-2		
			Roche	Esp6-3		
			Barbate	Esp7-1	x	
			Zahara	Esp7-2	x	
			Zahara	Esp7-3		
29	Morocco		Sidi Boukdanel	Mar1-1		

Table 2. Description of environmental variables used in the habitat use analysis.

Habitat variable	Justification
Latitude	Proxy of temperatures, which is known to influence juvenile development in laboratory conditions
Distance to the nearest cultivated field	Possible exchanges between fields and sea beet plants
Distance to the nearest river estuary	Possible facilitation of the surface runoff carrying cysts from inland zones to the coast by the presence of river drainage basins
Presence of alternative hosts	Probability of having alternative host-plant species for both nematode species coded as a binary variable (0 or 1) based on the observation in the immediate vicinity of sampled plants. 0: no contact between sea beet and potential alternative host plants (Goodey *et al.*, 1965); 1: presence of potential alternative host plants close to sea beet

All habitat variables were estimated with the measure tool of Google Earth version 7.1.1.

Spain and Portugal and were absent from northern Europe (Fig. 1).

The two species occurred together significantly more frequently than expected by chance, but the low κ value suggests that distributions of both species do not completely overlap ($\kappa = 0.29$; 95% CI (0.07, 0.49)). Results of GLM showed that *H. schachtii* was preferentially distributed at high latitude values (northern Europe) ($P < 0.001$) and where *H. betae* was present ($P < 0.01$). Occurrence of *H. betae* occurred preferentially at low latitudes (southern Europe) where *H. schachtii* was present ($P < 0.001$) and when the probability of presence of alternative hosts plants was high ($P < 0.05$). There were no significant effects of the distance to the nearest cultivated fields or the nearest river estuary for either species.

Discussion

This study investigated environmental factors influencing the spatial distribution of wild populations of two crop nematodes along the Atlantic and North Sea coastlines. Results showed that nematodes are widely distributed in Europe, with a large area of overlapping distribution, and are influenced by the latitude, a proxy of temperature, and the presence of other host species for *H. betae*.

GEOGRAPHICAL DISTRIBUTION OF NEMATODES IN WILD POPULATIONS OF THE SEA BEET

Heterodera schachtii was found in 52% of its wild host locations. Its presence was previously reported in cultivated fields in all European countries involved in this study (for a review, see Subbotin *et al.*, 2010). This is the first work that documents its geographical occurrence throughout Europe on one of its wild hosts. *Heterodera schachtii* seemed to be relatively rare in southern Spain and in Portugal, whereas it was more frequent on the northern coast of Spain, in France, in The Netherlands and in Denmark. These observations were supported by the analysis of the effect of habitat variables, showing that *H. schachtii* occurred preferentially in northern Europe, therefore in cooler habitats. These results were in accordance with several studies on the impact of temperature on the biology of *H. schachtii*. Indeed, cyst survival, maximal reproduction, root penetration and juvenile development occur preferentially between 10 and 28°C, and the development of juveniles and females is inhibited at temperatures greater than 30°C (Wallace, 1955; Thomason & Fife, 1962; Griffin, 1981; Maas & Heijbroek, 1982). *Heterodera schachtii* is thus better adapted to temperate and cold climatic conditions, explaining its presence only in the irrigated cultivated fields of the warmest regions of the southern Iberian Peninsula.

Heterodera betae can also develop successfully on sugar beets in the field and on wild beets in laboratory experiments (Steele *et al.*, 1983; Wouts *et al.*, 2001). This species was found in 41% of the sampled locations. It has recently been documented in cultivated fields in Sweden, The Netherlands, Belgium, Germany, Lithuania, Switzerland, France and Italy (Subbotin *et al.*, 2010), but our study is the first that describes its distribution in wild habitats. Moreover, its occurrence in Spain and Portugal has never been reported before. In several locations, *H. betae* co-occurred with populations of *H. schachtii*. Previous studies have indicated high similarities in the host ranges of *H. betae* and *H. schachtii* (Maas *et al.*, 1982; Steele *et al.*, 1983), but this is the first time, to our knowledge, that mixed populations have been described. The univariate κ statistic was significant, suggesting that

it is more probable to find both species co-occurring than either species alone. This suggests that, while both species were found alone in areas matching their typical habitat requirements (*i.e.*, cold conditions for *H. schachtii* and warmer conditions for *H. betae*), they preferentially co-occur in natural areas with favourable environmental conditions for both species. As *B. v.* ssp. *maritima* is a particularly suitable host for both species, they occur in the same host populations in a given location.

The distribution of *H. schachtii* was not influenced by the presence of other host species in areas surrounding sampled sites. This nematode has a wide host range including several species with a wide geographical distribution in disturbed habitats (Goodey *et al.*, 1965), such as urban areas. By contrast, the distribution of *H. betae* was influenced by the presence of other host species in the surrounding area. According to the literature, this nematode has a host range that is as large as the host range of *H. schachtii* (Maas & Heijbroek, 1982; Andersson, 1984). However, understanding clearly how the presence of alternative hosts influences the distribution of *H. betae* and *H. schachtii* requires further investigation.

Finally, the distribution of these nematode species was not related to the distance to the nearest estuary or cultivated fields. This suggests that the presence of nematodes on wild sea beet is independent of their potential occurrence in cultivated fields. The detection of effective exchanges of individuals among wild and field populations needs further population genetics investigations.

THE NATURAL HISTORY OF *HETERODERA BETAE* REVISITED

Our results challenge what was previously known for *H. betae*. This species was discovered in Dutch beet fields in 1975 and was first considered as a biotype of the clover cyst nematode, *H. trifolii*, able to parasitise a non-usual host, *B. v.* ssp. *vulgaris* (Maas *et al.*, 1982). More recently, molecular and morphological characterisation established that *H. betae* was distinct from the *H. trifolii* complex and constituted a true species (Wouts *et al.*, 2001). Interestingly, *H. betae* has 35, possibly 36, chromosomes and reproduces by mitotic parthenogenesis indicating that this species is potentially tetraploid (Steele & Whitehand, 1984). More specifically, it was suggested early on that *H. betae* may have evolved very recently from *H. schachtii* or *H. glycines* because both species have nine chromosomes (Mulvey, 1957, 1958, 1959; Triantaphyllou & Hirschmann, 1964; Steele & Whitehand, 1984; Subbotin *et al.*, 2010). However, *H. glycines* was

absent from Europe until 2008, which brings into question a common ancestry with *H. betae* (Steele & Whitehand, 1984; Greco & Inserra, 2008). Comparative morphological studies show that *H. betae* and *H. schachtii* are similar but have minor differences in size. Eggs and juveniles of *H. betae* are longer than those of *H. schachtii* (Steele & Whitehand, 1984; Wouts *et al.*, 2001; Amiri *et al.*, 2003). In nematodes, differences in ploidy are often associated with differences in individual size (Hirschmann, 1956; Hirschmann & Triantaphyllou, 1979). Thus, the differences and similarities between those two species could be the result of speciation by polyploidy, whereby *H. betae* derives from *H. schachtii*.

After its discovery, *H. betae* was documented only in Dutch regions for 10 years. It was thus assumed that its native area was The Netherlands (Steele & Whitehand, 1984). However, we did not find any occurrence of this nematode on *B. v.* ssp. *maritima* in northern European countries (Germany, Sweden or Denmark), whereas it was detected in cultivated fields of the same countries (Subbotin *et al.*, 2010). Moreover, our habitat use analysis showed that *H. betae* was preferentially located in warm habitats of southern Europe (Spain, Portugal and France). These results may be due to our sampling design: the 20 km coastline segments were randomly defined and it is possible that locations of *H. betae* were missed. Nevertheless, the systematic presence of the nematode in western France and on the Iberian Peninsula does not suggest that there was any sampling bias. Moreover, the observed *H. betae* distribution was consistent with the biology of this nematode: the hatching rate of juveniles from cysts is higher at warm temperatures (between 25 and 30°C) and close to zero for temperatures below 15°C (Maas & Heijbroek, 1982). Unlike *H. schachtii*, *H. betae* may thus be better adapted to warm climatic conditions and its presence in northern Europe may result from the cultivation of sugar beet crops in favourable climatic conditions. Finally, our results do not support the assumption of a very recent appearance of this species and suggest a more complex evolutionary history in natural environment, unless *H. betae* arises recurrently from its amphimictic relative *H. schachtii*. Thus, The Netherlands, which are located in northern Europe, are probably not the centre of origin of *H. betae*.

CONCLUSIONS

Our study demonstrated that *H. schachtii* and *H. betae* are widely distributed along the Atlantic coastline, with a large area of overlapped distribution. Temperature ap-

peared to influence their relative spatial distribution. Our results raise many questions on the origin and evolution of those nematodes and on their coexistence in *B. v.* ssp. *maritima*. The centre of origin of both nematode species is still unknown. Determining its location could provide interesting clues for breeders selecting new resistant beet varieties. The relationship between *B. v.* ssp. *maritima* and both nematode species is also an interesting issue. The wild beet shows a south-to-north phylogeographical pattern attesting to post-glacial recolonisation (Leys *et al.*, 2014). Both nematode species should thus exhibit the same recolonisation history, unless alternative hosts may have played a major role in their evolutionary history. This hypothesis requires further phylogeographic analyses. Finally, the significant presence of both nematodes species in natural environment also raises questions as to the contamination risk of cultivated fields.

Acknowledgements

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