



**HAL**  
open science

## Evidence of strong gene flow among French populations of the carrot cyst nematode *Heterodera carotae*

Magali Esquibet, Camille Gautier, Christophe Piriou, Eric Grenier, Sylvain Fournet, Josselin Montarry

► **To cite this version:**

Magali Esquibet, Camille Gautier, Christophe Piriou, Eric Grenier, Sylvain Fournet, et al.. Evidence of strong gene flow among French populations of the carrot cyst nematode *Heterodera carotae*. *Plant Pathology*, 2020, 69 (1), pp.168-176. 10.1111/ppa.13102 . hal-02457188

**HAL Id: hal-02457188**

**<https://hal.science/hal-02457188>**

Submitted on 8 Sep 2022

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

1 **Evidence of strong gene flow among French populations of the**  
2 **carrot cyst nematode *Heterodera carotae***

3

4 M. ESQUIBET<sup>1</sup>, C. GAUTIER<sup>1,2</sup>, C. PIRIOU<sup>1</sup>, E. GRENIER<sup>1</sup>, S. FOURNET<sup>1§</sup> and J.  
5 MONTARRY<sup>1§\*</sup>

6

7 <sup>1</sup> IGEPP, INRA, Agrocampus-Ouest, Université de Rennes 1, 35650, Le Rheu, France

8 <sup>2</sup> Centre Mondial de l'Innovation-Laboratoire de Nutrition Végétale Pôle Biocontrôle, Groupe  
9 Roullier, Saint Malo, France

10 \* Corresponding author: josselin.montarry@inra.fr

11 § These authors contributed equally to this work

12

13 Short title: Strong gene flow in *Heterodera carotae*

14

15 *Keywords:* AMOVA, Genetic structure, Heterozygote deficit, Isolation by distance,  
16 Microsatellite markers, Migration.

17

18 **Abstract** The recent ban of the most efficient chemical nematicides lets growers without  
19 method to control the carrot cyst nematode *Heterodera carotae*. This phytoparasitic nematode  
20 species has a very narrow host range and causes severe crop losses in the main carrot-growing  
21 regions worldwide. The development of alternative means of management of *H. carotae* is thus  
22 essential and need knowledge about the adaptive abilities of *H. carotae*, which mainly depend  
23 on gene flow among populations. The goal of this study was to describe the genetic structure  
24 of *H. carotae* populations at the spatial scale of the main infested French carrot-producing  
25 region, i.e. Lower Normandy, and to disentangle the causes of the heterozygote deficit in this  
26 polyvoltine species. Our results, obtained through the microsatellite genotyping of populations  
27 collected at the plant and at the field scales, showed i) that the heterozygote deficit is mainly  
28 due to sub-structure, and ii) strong gene flow among populations, leading to low  $F_{ST}$  and to no  
29 clear genetic structure at the explored spatial scale. Soil transport through both agricultural  
30 machinery and the transport of leek seedlings is probably responsible of the very strong *H.*  
31 *carotae* migration among fields and among production areas. Measures should be considered  
32 to limit the passive spread of *H. carotae*.

33

## 34 **Introduction**

35 Plant-parasitic nematodes are distributed worldwide and pose a major threat for numerous crops  
36 of high agricultural interest. They are obligatory parasites and feed only on their host plants  
37 leading to poor plant growth and severe crop losses (Nicol *et al.*, 2011). In Europe, the ban of  
38 the chemical nematicide 1,3 dichloropropene (CE 2076/2002), which was used thanks to  
39 dispensations (CE 1107/2009) until 2018 in France, gives a strong opportunity to the use of  
40 new control solutions, such as plant resistances and biocontrol products. However, these new  
41 control methods may be more dependent on the evolutionary potential of nematode population  
42 (i.e. the ability for a population to adapt and evolve in its environment), which mainly depends  
43 on gene flow among populations (McDonald & Linde, 2002). In cyst nematode species,  
44 population genetic structure are mainly the result of the active dispersal of free-living stages in  
45 the soil (i.e. second-stage juveniles and males) at short distances, leading to strong heterozygote  
46 deficits (e.g. Montarry *et al.*, 2015), and of the passive dispersal of cysts at long distances.  
47 Indeed, massive gene flow were revealed for the potato cyst nematode *Globodera pallida*  
48 (Picard *et al.*, 2004), the beet cyst nematode *Heterodera schachtii* (Plantard & Porte, 2004) and  
49 the tobacco cyst nematode *G. tabacum* (Alenda *et al.*, 2014). The migration of cysts was  
50 subsequent to commercial trades at large geographical scales, resulting in a strong geographical  
51 structure (Plantard *et al.*, 2008; Boucher *et al.*, 2013; Alenda *et al.*, 2014) and also to agricultural  
52 activities resulting in a lack of structure at finer geographical scales (e.g. Alenda *et al.*, 2014).  
53 As a new recent example, the study of the genetic structure of seventeen European populations  
54 of the carrot cyst nematode *Heterodera carotae* revealed that i) *H. carotae* populations were  
55 characterised by a strong heterozygote deficit, ii) they were grouped into two genetic clusters  
56 geographically structured and iii) gene flow were low among populations in each cluster  
57 (Gautier *et al.*, 2019). However, there is a lack of knowledge of the genetic structure of the  
58 populations of this nematode at a more restricted spatial scale.

59           *Heterodera carotae* had a very narrow host range constituted of different subspecies of  
60 *Daucus carota* L. and a wild Apiaceae, *Torilis leptophylla* L. (Mugniery & Bossis, 1988). It is  
61 a polyvoltine species able to perform up to four generations during a growing season in  
62 favourable conditions (Subbotin *et al.*, 2010). As a result, infestations in the field correspond to  
63 irregular areas of stunted plants, characterised by small, abnormally developed and  
64 unmarketable carrots. *Heterodera carotae* is present in most of carrot-growing regions  
65 worldwide: in the state of Michigan in the USA (Berney & Bird, 1992), in the province of  
66 Ontario in Canada (Yu *et al.*, 2017), in an important Mexican carrot producing area (Escobar-  
67 Avila *et al.*, 2018), in sixteen countries of Europe including France (Osborne, 1971; Mugniéry  
68 & Bossis 1988; Gautier *et al.*, 2019) and in South-Africa (Subbotin *et al.*, 2010). Ranges of  
69 estimated carrot crop-losses related to *H. carotae* were estimated from 20 to 90% in Italy (Greco  
70 *et al.*, 1993) and more than 35% in Ontario (Yu *et al.*, 2017).

71           Till now, the French carrot production system relied on the dispensations granted for  
72 the chemical nematicide 1,3 dichloropropene. The development of alternative means of  
73 management of *H. carotae*, economically and environmentally safe, is thus essential in carrot-  
74 growing regions. Since 10 years, several alternatives to current chemical nematicides have been  
75 explored: soil solarisation, biofumigation, biological control and plant genetic resistance (e.g.  
76 Grevsen, 2012; D'Addabbo *et al.*, 2013). Whatever the alternative control measure, the  
77 knowledge of the adaptive abilities of *H. carotae* populations constitutes a key factor in the  
78 development of sustainable strategies, i.e. strategies that reduce the probability of adaptive  
79 events.

80           The first goal of the present study was thus to investigate the genetic diversity and  
81 structure of *H. carotae* populations using microsatellite genotyping of individuals collected  
82 within Lower Normandy, the major carrot producing region infested by *H. carotae* in France,  
83 which includes three distinct production areas: Créances, Val-de-Saire and Mont St-Michel.

84 The second goal was to estimate the relative contribution of inbreeding and Wahlund effect to  
85 the heterozygote deficit highlighted in this polyvoltine species (Gautier *et al.*, 2019), using *H.*  
86 *carotae* populations sampled at the plant scale.

87

## 88 **Materials and methods**

### 89 **Sampling of nematode cysts**

90 Cysts of the carrot cyst nematode, *Heterodera carotae*, were collected in December 2016 from  
91 commercial carrot fields, all located in Lower Normandy, one of the two major French regions  
92 producing carrots. In contrast with Aquitaine (the second major French region producing  
93 carrots), the extensive occurrence of *H. carotae* in Lower Normandy lead to important yield  
94 losses. Three distinct production areas were investigated in Lower Normandy: Créances, Val-  
95 de-Saire and Mont St-Michel. A sampling strategy was conducted in order to study two types  
96 of populations: populations collected at the field scale and populations collected at the plant  
97 scale. The GPS coordinates of each field were recorded (Table 1).

98 Twenty-two populations were collected at the field spatial scale: two fields in the Mont  
99 St-Michel area, five fields in the Val-de-Saire area and 15 in the Créances area. According to  
100 Garcia *et al.* (2018), ten sample points were defined alongside the longest diagonal of each  
101 field. The ten points (depth of 15 cm using a manual auger (diam. 2.5 cm)) were pooled,  
102 resulting in one sample per field. Geographical distances between the three production areas  
103 varied between 65-74 km (from Mont St-Michel to Créances) to 120 km (from Mont St-Michel  
104 to Val-de-Saire) and fields in each area were separated from each other by 0.1 to 14 km.

105 For the investigations at the plant scale, soil samples were collected around roots of  
106 individual carrot plants *Daucus carota* subsp. *sativus* alongside the diagonal of two fields  
107 located in the Créances area, named Cre4 and Den3. Nine and 10 distinct *H. carotae* populations

108 were collected from distinct plants in Cre4 and Den3, respectively. Within each field, sampled  
109 populations were separated from each other by 10 m.

110 Nematode cysts were extracted from 100 g of soil by a Kort elutriator. The number of  
111 cysts in 100 g of soil ranged from 15 to 280 and the estimated number of second-stage juveniles  
112 per gram of soil ranged from 3.4 to 64.3. To obtain at least 40 cysts per population, up to 500 g  
113 of soil were extracted for some populations.

114

### 115 **Microsatellite genotyping**

116 In order to assess the genetic diversity of the carrot cyst nematode populations, we used a set  
117 of 13 microsatellite markers developed by Gautier *et al.* (2019). For each population, 40 second-  
118 stage juveniles (J2) from 40 distinct cysts, randomly chosen, were genotyped. DNA extractions  
119 were performed as described in Gautier *et al.* (2019). DNAs were diluted with a 1:2 dilution  
120 ratio, and 2  $\mu$ L was used for the microsatellite genotyping. PCR multiplex was performed in 5  
121  $\mu$ L of working volume, containing 1X of Type-it Microsatellite PCR kit (Qiagen) and 0.2  $\mu$ M  
122 of each primer. Cycling conditions are the same as those described by Gautier *et al.* (2019), i.e.  
123 an initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 30  
124 s, annealing at 57 °C for 90 s and extension at 72 °C for 30 s, followed by a final extension at  
125 60 °C for 30 min. PCR products were then diluted to 1:40 in sterile water, and 3  $\mu$ L of this  
126 dilution was mixed with 0.05  $\mu$ L of GeneScan 500 LIZ Size Standard (Applied Biosystems)  
127 and 5  $\mu$ L of formamide (Applied Biosystems). Analyses of PCR products were conducted on  
128 an ABI Prism®3130xl sequencer (Applied Biosystems). Allele sizes were identified using the  
129 automatic calling and binning procedure of GeneMapper v4.1 (Applied Biosystems) and  
130 completed by a manual examination of irregular results.

131

### 132 **Dataset cleaning**

133 Among the 13 microsatellite loci (Hc07, Hc29, Hc35, Hc40, Hc49, Hc55, Hc59, Hc63, Hc72,  
134 Hc76, Hc87, Hc91 and Hc94), 12 markers were retained. Hc91 was discarded because the  
135 amplification of this locus failed for 55 % of our individuals. This marker was also the one  
136 showing the highest frequency of potential null alleles (i.e. 28.7%) in the study of Gautier *et al.*  
137 (2019), which comforted us in the decision to remove it. For each collected population, 33 to  
138 40 J2 from distinct cysts were successfully genotyped.

139 A preliminary factorial correspondence analysis (FCA) was performed using GENETIX  
140 4.05.2 (Belkhir *et al.*, 2004) on all individuals from the cysts collected at the field scale (i.e.  
141 822 individuals) and showed that all individuals were closely grouped, except for 114  
142 individuals from four populations from the Val-de-Saire area (all individuals from populations  
143 VDS3 and VDS6, 33/39 individuals from SIL1 and 11/33 individuals from VDS4) which were  
144 grouped separately. The high genetic distance between the two groups suggested the presence  
145 of the cabbage cyst nematode *Heterodera cruciferae* which resembles *H. carotae*. In order to  
146 check the possible membership of those 114 individuals to the *H. cruciferae* species, 32 J2 from  
147 32 distinct cysts of two *H. cruciferae* populations, maintained in our lab collection (and  
148 multiplied on cauliflower), were genotyped and included to the dataset before rerunning the  
149 FCA. Moreover, to confirm the membership of our populations to the *H. carotae* species, two  
150 reference *H. carotae* populations (i.e. 36 individuals) from Gautier *et al.* (2019), both multiplied  
151 on carrot, were added to this analysis. The FCA revealed two groups: the 114 J2 were clearly  
152 grouped with all *H. cruciferae* individuals from the reference populations and all the other  
153 individuals were clearly grouped with the reference *H. carotae* populations (Fig. S1, Supporting  
154 information). Consequently, the *H. cruciferae* individuals were excluded from the final dataset  
155 as well as the six *H. carotae* remaining individuals of the population SIL1, insufficient to  
156 constitute a population.



157 We finally analysed 19 *H. carotae* populations (i.e. two from the Mont St-Michel area,  
158 two from the Val-de-Saire area and 15 from the Créances area) at the field scale (i.e. 702  
159 individuals) and 19 *H. carotae* populations (i.e. 768 individuals) at the plant scale, which were  
160 genotyped using a set of 12 polymorphic microsatellite markers.

161

## 162 **Data analysis**

### 163 *Analyses at the field scale*

164 Genotypic linkage disequilibrium among all pairs of loci was assessed using a Markov Chain  
165 approximation of the Fisher's exact test, as implemented in GENEPOP 4.0.7 (Raymond &  
166 Rousset, 1995). A Bonferroni adjustment was applied to take into account multiple testing, i.e.  
167  $\alpha = 0.05$  was lowered to  $\alpha = 0.00076$  for 66 comparisons.

168 Null allele frequencies were estimated for each locus across all populations using the  
169 likelihood-based method of Chybicki & Burczyk (2009) implemented in the INEst program.  
170 Moreover, the relationship between the percentage of missing data for each locus and  $F_{IS}$  was  
171 examined (Beaumont *et al.*, 2001) with the Spearman's rank correlation coefficient rho using  
172 the statistical software R version 3.6.1. A positive relationship between  $F_{IS}$  and missing data  
173 would indicate that amplification failure is due to individuals bearing a null allele at the  
174 homozygous state.

175 Allelic richness ( $A_r$ ) was estimated using the rarefaction method implemented in  
176 POPULATIONS 1.2.32 (Langella, 1999), which estimated the mean number of alleles per locus  
177 for a reduced sample size. An unbiased estimate of gene diversity ( $H_{nb}$  according to Nei (1978))  
178 and deviation from random mating ( $F_{IS}$ ) were computed using GENETIX 4.05.2 (Belkhir *et al.*,  
179 2004). The statistical significances of  $F_{IS}$  were estimated using the allelic permutation method  
180 (10,000 permutations) implemented in GENETIX.

181 To explore the genetic structure of the 19 *H. carotae* populations at the regional spatial  
182 scale, the Bayesian clustering algorithms implemented in STRUCTURE 2.3.4 (Pritchard *et al.*,  
183 2000) was run on a reduced dataset free of any missing data (i.e. 595 individuals). According  
184 to Gautier *et al.* (2019), which followed the recommendations of Wang (2017), default priors  
185 were chosen except for alpha, for which the value was set to 0.0526 (i.e.  $1 / p$ ,  $p$  being the  
186 number of populations) and the uncorrelated allele frequency model was used. The initial burn-  
187 in period consisted of 1,000,000 iterations and the number of Markov chain Monte Carlo  
188 (MCMC) repetitions was 3,000,000. The K value was set from 1 to 20 (i.e.  $p + 1$ ) and thirty  
189 different runs were executed for each K. We applied Structure Harvester Web ver.0.6.94 (Earl  
190 & vonHoldt, 2012) to determine the most likely number of clusters statistically determined  
191 using the ad-hoc Evanno statistic  $\Delta K$  (Evanno *et al.*, 2005).

192 The hierarchical structuring was assessed with an AMOVA model using ARLEQUIN  
193 v.3.5 (Excoffier & Lischer, 2010) and considering three hierarchical levels: among production  
194 areas (Créances, Val-de-Saire and Mont St-Michel), among populations within each production  
195 area and within populations.

196 The differentiation coefficients between each pair of populations ( $F_{ST}$ ) were computed  
197 using GENEPOP 4.5.1 according to Weir & Cockerham (1984), and their statistical  
198 significances were estimated by 5,000 random permutations of individuals among populations.  
199 A Bonferroni adjustment was applied to take into account multiple testing, i.e.  $\alpha = 0.05$  was  
200 lowered to  $\alpha = 0.00029$  for 171 comparisons. A pattern of isolation by distance (IBD) was tested  
201 by calculating the correlation between the matrices of pairwise genetic distances ( $F_{ST} / (1 -$   
202  $F_{ST})$ ) and the natural logarithm of geographic distance for each pair of populations (Rousset,  
203 1997). The statistical significance of the correlation was assessed through a Mantel test (10,000  
204 permutations) using XLSTAT 2019.3.1 (Addinsoft SARL).

205

206 *Analyses at the plant scale*

207 The unbiased estimate of gene diversity ( $H_{nb}$ ) and the  $F_{IS}$  were computed for each of the 19 *H.*  
208 *carotae* populations collected at the plant scale as described above. The statistical significance  
209 of  $F_{IS}$  values for each population was tested using the allelic permutation method (10,000  
210 permutations) implemented in GENETIX.

211 Because heterozygote deficits in cyst nematodes could be due to a Wahlund effect (i.e.  
212 sub-structure) and/or to consanguinity (Montarry *et al.*, 2015), we used the method of Overall  
213 & Nichols (2001) in order to calculate a likelihood surface for the genetic correlation due to  
214 population subdivision ( $\theta$ ) and the proportion of the population practicing consanguinity (C).  
215 The method, which is based on the argument that consanguinity and sub-structure generate  
216 distinctive patterns of homozygosity in multilocus data, was applied assuming a degree of  
217 relatedness of 1/4 (see Montarry *et al.*, 2015) to all *H. carotae* populations showing significant  
218 heterozygote deficits. Likelihood estimates were obtained by searching for the maximum of  
219 the likelihood function over a grid of 10,000 combinations of  $\theta$  and C values. Moreover, graphs  
220 of the likelihood surface were obtained for each nematode population using the statistical  
221 software R version 3.6.1.

222

## 223 **Results**

### 224 **Genetic features of *H. carotae* field populations**

225 At the fields scale, using our set of 12 microsatellites, we identified 54 alleles among the 19 *H.*  
226 *carotae* populations (i.e. 702 individuals) genotyped, with two (for Hca-07) to 10 (for Hca-63)  
227 alleles per locus. Using the Bonferroni's adjustment no significant linkage disequilibrium was  
228 detected among the 66 pairs of loci.

229 The percentage of null allele estimated by INEst averaged 6.23% among our 12  
230 microsatellite markers. The percentage of potential null allele ranged from 0% (locus Hca-29)

231 to 15% (locus Hca-63) and there was no significant correlation between the percentage of  
232 missing data and  $F_{IS}$  ( $\rho = 0.253$ ;  $P = 0.297$ ). Consequently, we assumed that null alleles would  
233 have no strong effect on the population genetics analyses performed hereafter.

234 The genetic diversity was quite similar among the different *H. carotae* populations: the  
235 allelic richness ( $A_r$ ), estimated on a reduced sample of 16 individuals, ranged from 2.28 to 2.77  
236 alleles per locus and the unbiased expected heterozygosity ( $H_{nb}$ ) ranged from 0.30 to 0.45 (Table  
237 1). Among the 19 populations, only four populations were at the Hardy-Weinberg equilibrium  
238 ( $F_{IS}$  not significantly different to zero). All the 15 remaining populations showed strong  
239 heterozygote deficits ( $F_{IS}$  ranging from 0.088 to 0.198 - Table 1).

240

#### 241 **Genetic structure among *H. carotae* field populations**

242 Although the Bayesian clustering analysis identified  $K = 3$  as the optimal number of genetic  
243 clusters, there was no clear genetic structure. Indeed, only a small proportion of individuals  
244 were well assigned to one or the other clusters: only 10.6% of individuals were assigned to a  
245 cluster with a percentage of assignation higher than 95% (6.9% to cluster 1, 1.7% to cluster 2  
246 and 2.0% to cluster 3). Moreover, the three clusters were represented in each of the 19  
247 populations (Fig. 1), even if Bre1 was mainly assigned to cluster 1, StM4 and Sil5 were mainly  
248 assigned to cluster 2 and Den3 was mainly assigned to cluster 3 (the average percentages of  
249 assignation of those populations to the corresponding clusters being 75.1%, 72.4%, 65.7% and  
250 60.2%, respectively). Anyway, the three genetic clusters did not correspond to the three  
251 production areas (Fig. 1).

252 The AMOVA performed on *H. carotae* populations partitioned according to the three  
253 carrot production areas revealed that only 3.24% of the genetic diversity was explained by the  
254 productions areas and only 3.93% by the populations within each production area, while the  
255 diversity within populations explained 92.83% of the total variation.

256

### 257 **Genetic differentiation between *H. carotae* populations**

258 At the field scale,  $F_{ST}$  values were low, ranging from 0 to 0.18 (Fig. 2). Among the 171 pairwise  
259 comparisons, only 96 were significant after Bonferroni adjustment. The lowest pairwise  $F_{ST}$   
260 values were observed between populations from the Créances production area, except for the  
261 population Bre1 (Fig. 2). Bre1 displayed the highest  $F_{ST}$  values observed (from 0.05 to 0.18),  
262 significant for all of the pairwise comparisons, indicating some degree of differentiation  
263 between Bre1 and all other populations. High  $F_{ST}$  values were also observed for populations  
264 Sil5 and StM3 from Val-de-Saire and Mont-St-Michel, respectively, while VDS4 and StM4,  
265 collected also from those production areas, showed low  $F_{ST}$  values in most comparisons (Fig.  
266 2). Altogether, this suggests that important gene flow occurred, even between the different  
267 production areas in Lower Normandy.

268 Moreover, the positive correlation between genetic and geographic distances (Fig. 3)  
269 highlighted an IBD pattern, showing that gene flow occurred among *H. carotae* populations  
270 and that their intensity was higher between the closest fields.

271

### 272 **The heterozygote deficit in *H. carotae* populations**

273 Among the 19 *H. carotae* populations sampled at the plant scale, 12 showed a significant  
274 heterozygote deficit, eight in the field Cre4 and four in the field Den3, the remaining  
275 populations being at the Hardy-Weinberg equilibrium (Table 2).

276 Among those 12 populations, the heterozygote deficit was attributed only to  
277 consanguineous mating for Cre4.1 and Cre4.7 populations (Table 2 and Fig. S2 supporting  
278 information). For all other populations, the most likely parameter combination for explaining  
279 the heterozygote deficit included a high level of sub-structure (a Wahlund effect), and there was

280 even no evidence of consanguinity for three populations, Cre4.3, Cre4.4 and Den3.3 (Table 2  
281 and Fig. S2 supporting information).

282

## 283 **Discussion**

284 The goal of this study was to describe the genetic structure of *H. carotae* populations at a spatial  
285 scale corresponding to the main infested French carrot-producing region, i.e. Lower Normandy,  
286 and to disentangle the causes of the heterozygote deficit in this polyvoltine species. Through  
287 the microsatellite genotyping of populations collected at the plant and at the field scales, we  
288 showed i) that the heterozygote deficit is mainly due to sub-structure, and ii) strong gene flow  
289 among populations, leading to low  $F_{ST}$  and to no clear genetic structure at the explored spatial  
290 scale.

291 To determine the extent of *H. carotae* distribution and to better control this nematode in  
292 carrot-producing regions, the accurate detection and identification of the carrot cyst nematode  
293 is a fundamental prerequisite. *H. carotae* belongs to the *Goettingiana* group which includes *H.*  
294 *goettingiana*, *H. carotae*, *H. urticae*, and *H. cruciferae* (Subbotin *et al.*, 2010). *H. carotae* is  
295 morphologically and genetically very close to the cabbage cyst nematode, *H. cruciferae* and the  
296 correct diagnosis is difficult and time consuming. rRNA and mitochondrial molecular markers  
297 such as ITS, cytochrome c oxidase 1 subunit (coxI) region (Vovlas *et al.*, 2017, Escobar-Avila  
298 *et al.*, 2018) and coxI derived primers set (Madani *et al.*, 2018) can help in the identification of  
299 members of the *Goettingiana* group, but none are able to provide an unequivocal distinction  
300 between *H. carotae* and *H. cruciferae*. As a result, only a host range test using carrot allowed  
301 to accurately distinguish these two species (Escobar Avila *et al.*, 2018). The microsatellite  
302 markers used in the present study successfully amplified both *H. carotae* and *H. cruciferae*, and  
303 the high degree of genetic differentiation observed between reference *H. carotae* populations

304 and reference *H. cruciferae* populations allowed us to well distinguish *H. carotae* and *H.*  
305 *cruciferae* individuals in our dataset. Due to the conservation of microsatellite loci among taxa,  
306 the set of microsatellite markers initially developed for *H. carotae* (Gautier *et al.*, 2019) could  
307 be used i) to explore the genetic diversity of *H. cruciferae*, but also ii) to develop a diagnostic  
308 tool able to differentiate *H. carotae* from *H. cruciferae*. Indeed, combined into a single real-  
309 time PCR assay, diagnostic markers for three cyst nematode species, *G. pallida*, *G.*  
310 *rostochiensis* and *H. schachtii*, were identified and developed from microsatellite loci (Gamel  
311 *et al.*, 2017). We thus propose to investigate *H. carotae* microsatellite loci, including the large  
312 number of discarded loci (monomorphic loci or loci that contain few repeats of the  
313 microsatellite motif), to develop a molecular tool for the diagnosis of *H. carotae* and *H.*  
314 *cruciferae*.

315         If an accurate diagnosis is the first step in selecting appropriate control measures, a fine  
316 knowledge of the active and passive dispersal capabilities of the nematode is essential to  
317 promote the best and sustainable management strategies. Our results showed that 15/19 *H.*  
318 *carotae* populations sampled at the field scale and 12/19 populations sampled at the plant scale  
319 deviate from the Hardy-Weinberg equilibrium and show significant positive  $F_{IS}$  values. Such  
320 heterozygote deficit was previously highlighted for several cyst nematode species at the field  
321 scale (for *Globodera pallida* – Picard *et al.*, 2004, *Heterodera schachtii* – Plantard & Porte,  
322 2004, *G. tabacum* – Alenda *et al.*, 2014, *H. glycines* – Wang *et al.*, 2015, *H. avenae* – Wang *et*  
323 *al.*, 2018 and *H. carotae* – Gautier *et al.*, 2019) and at the plant scale (for *G. pallida*, *G. tabacum*  
324 and *H. schachtii* - Montarry *et al.*, 2015). As for *G. tabacum* and *H. schachtii*, which performed  
325 also several generations per growing season, the heterozygote deficit of *H. carotae* populations  
326 was mainly attributed to substructure (Wahlund effect) at the spatial scale of the carrot's  
327 rhizosphere. The fact that footprints of consanguineous mating were also detected supports the  
328 hypothesis that from an isolated cyst, the restricted active dispersal capabilities of second-stage

329 juveniles lead in a first time to consanguineous mating and then to the differentiation of sub-  
330 populations during the next generations (Montarry *et al.*, 2015).

331         The analysis of partitioning of molecular variability (AMOVA) showed that a very  
332 small part of the genetic variability was observed among the three carrot production areas  
333 (Créances, Val-de-Saire and Mont St-Michel) and among populations within each production  
334 area. This result combined with the absence of genetic structure within Lower Normandy  
335 revealed the occurrence of strong gene flow among *H. carotae* populations. While *H. carotae*  
336 juveniles exhibit limited active dispersion capabilities in the soil, dormant eggs are able to  
337 disperse over long distances within the cysts through natural means and human activities. In the  
338 present case, we can for instance easily imagine that sea winds can lift up both sandy soil and  
339 cysts. However, human activities and the resulting soil movements play most certainly an  
340 important role in the *H. carotae* dispersion. Extensive gene flow among populations were also  
341 highlighted at comparable spatial scales for other cyst nematodes, such as the pale potato cyst  
342 nematode (e.g. Picard *et al.*, 2004) and the beet cyst nematode (Plantard & Porte, 2004). It is  
343 most probable that such extensive gene flow occur thanks to soil transport through agricultural  
344 machinery, especially during the harvest process, and to the exchange of plant material  
345 (Goeminne *et al.*, 2015). During a carrot-growing season, only few cultural practices lead to  
346 soil transports. Cysts can be transported from fields to fields in the soil stuck on farm  
347 implement, but there is no exchange of carrot plants (and soil) as in the case of seed potato  
348 tubers. However, fields in Lower Normandy are cultivated with leeks and carrots and unlike  
349 the carrot that is sown directly, the young leek plants (two months old) are traditionally  
350 transplanted into the field. Thus, passive spread of *H. carotae* by the transport of infected soil  
351 bound to leek seedlings is probably responsible of the extensive gene flow observed among  
352 production areas, and soil transport through both agricultural machinery and the transport of



353 leek seedlings are probably responsible of the very strong *H. carotae* migration among fields  
354 within each production area.

355         Considering the geographical localization of each field, a significant isolation by  
356 distance pattern was highlighted, showing that the intensity of gene flow was higher among  
357 close fields than among distant fields. This pattern is congruent with the different means of soil  
358 transport identified, i.e. agricultural machinery and leek seedlings within a production area and  
359 only leek seedlings among the different production areas. Moreover, the variability in the  
360 intensities of gene flow is true at the scale of the Lower Normandy, with  $F_{ST}$  higher between  
361 fields of different production areas than between fields of the same production area, but also at  
362 a finer spatial scale. Indeed, within the Créances production area,  $F_{ST}$  values observed between  
363 the Bre1 population and the others ones were the highest of the  $F_{ST}$  matrix. In this production  
364 area, all *H. carotae* populations were collected in infested fields located around the Créances  
365 town or along the main south-north road, while Bre1 was sampled in a more isolated field,  
366 located near the sea (about 1 km from the road).

367         In lower Normandy, the lack of strong genetic structure and thus the genetic  
368 homogeneity observed in *H. carotae* populations are both factors suggesting that the use of  
369 control means based on plant resistance may be efficient in all fields. Conversely, the strong  
370 gene flow highlighted among fields and among the three studied areas show that any adaptive  
371 events, even in a single field, is associated with a very high risk of spreading. As a result,  
372 measures should be considered to limit the passive spread of *H. carotae*: and therefore further  
373 investigations are required to identify the main dissemination vectors used by *H. carotae*. In  
374 the current context, i.e. the recent total withdrawal of 1,3 dichloropropene which lets growers  
375 without effective method to control *H. carotae* in Lower Normandy, growers will be tempted  
376 to exploit nematode-free fields in the coming years. But, without strong prophylactic measures,  
377 the risk to see these fields becoming also quickly infested remains high.

378

379 **Acknowledgments** We gratefully acknowledge the SILEBAN for their help in the sampling  
380 of *Heterodera carotae* populations and for useful discussions about the transport of leek  
381 seedlings. Camille Gautier is supported by PhD grant from Groupe Roullier and ANRT  
382 (Association Nationale Recherche Technologie).

383

384 **References**

- 385 Alenda C, Montarry J, Grenier E, 2014. Human influence on the dispersal and genetic structure  
386 of French *Globodera tabacum* populations. *Infection, Genetics and Evolution* **27**, 309-17.
- 387 Beaumont M, Barratt EM, Gottelli D *et al.*, 2001. Genetic diversity and introgression in the  
388 Scottish wildcat. *Molecular Ecology* **10**, 319-36.
- 389 Belkhir K, Borsa P, Chikhi L, Raufaste N, Bonhomme F, 2004. GENETIX 4.05, logiciel sous  
390 Windows TM pour la génétique des populations. Laboratoire Génome, Populations,  
391 Interactions, CNRS UMR 5000, Université de Montpellier II, Montpellier (France).
- 392 Berney M, Bird G, 1992. Distribution of *Heterodera carotae* and *Meloidogyne hapla* in  
393 Michigan carrot production. *Journal of Nematology* **24**, 776-8.
- 394 Boucher AC, Mimee B, Montarry J *et al.*, 2013. Genetic diversity of the golden potato cyst  
395 nematode *Globodera rostochiensis* and determination of the origin of populations in  
396 Quebec, Canada. *Molecular Phylogenetics and Evolution* **69**, 75-82.
- 397 Chybicki IJ, Burczyk J, 2009. Simultaneous estimation of null alleles and inbreeding  
398 coefficients. *Journal of Heredity* **100**, 106-13.
- 399 D'Addabbo T, Carbonara T, Argentieri MP *et al.*, 2013. Nematicidal potential of *Artemisia*  
400 *annua* and its main metabolites. *European Journal of Plant Pathology* **137**, 295-304.
- 401 Earl DA, vonHoldt BM, 2012. STRUCTURE HARVESTER: a website and program for  
402 visualizing STRUCTURE output and implementing the Evanno method. *Conservation*  
403 *Genetics Resources* **4**, 359-61.
- 404 Escobar-Avila IM, Óliver E, Subbotin SA, Tovar-Soto A, 2018. First report of carrot cyst  
405 nematode *Heterodera carotae* in Mexico: morphological, molecular characterization and  
406 host range study. *Journal of Nematology* **50**, 229-42.
- 407 Evanno G, Regnaut S, Goudet J, 2005. Detecting the number of clusters of individuals using  
408 the software STRUCTURE: a simulation study. *Molecular Ecology* **14**, 2611-20.

409 Excoffier L, Lischer HEL, 2010. Arlequin suite ver 3.5: a new series of programs to perform  
410 population genetics analyses under Linux and Windows. *Molecular Ecology Resources*  
411 **10**, 564-7.

412 Gamel S, Letort A, Fouville D, Folcher L, Grenier E, 2017. Development and validation of real-  
413 time PCR assays based on novel molecular markers for the simultaneous detection and  
414 identification of *Globodera pallida*, *G. rostochiensis* and *Heterodera schachtii*.  
415 *Nematology* **19**, 789-804.

416 Garcia N, Folcher L, Biju-Duval L, Maupetit A, Ricci B, Grenier E, 2018. Impact of agricultural  
417 practices and environmental variables on plant-parasitic nematode communities in fields  
418 at a landscape scale. *Nematology* **20**, 211-33.

419 Gautier C, Esquibet M, Fournet S *et al.*, 2019. Microsatellite markers reveal two genetic groups  
420 in European populations of the carrot cyst nematode *Heterodera carotae*. *Infection,*  
421 *Genetics and Evolution* **73**, 81-92.

422 Goeminne M, Demeulemeester K, Lanterbecq D, De Proft M, Viaene N, 2015. Detection of  
423 field infestations of potato cyst nematodes (PCN) by sampling soil from harvested  
424 potatoes. *Aspect of Applied Biology* **130**: 105-10.

425 Greco N, D'Addabbo T, Brandonisio A, Elia F, 1993. Damage to Italian crops caused by cyst-  
426 forming nematodes. *Journal of Nematology* **25**: 836-42.

427 Grevsen K, 2012. Biofumigation with *Brassica juncea* pellets and leek material in carrot crop  
428 rotations. *Acta Horticulturae* **933**, 427-31.

429 Langella O, 1999. Populations 1.2.31. Population Genetic Software (Individuals or Populations  
430 Distances, Phylogenetic Trees) 2012. [<http://bioinformatics.org/~tryphon/populations/>].

431 Madani M, Palomares-Rius JE, Vovlas N, Castillo P, Tenuta M, 2018. Integrative diagnosis of  
432 carrot cyst nematode (*Heterodera carotae*) using morphology and several molecular

433 markers for an accurate identification. *European Journal of Plant Pathology* **150**, 1023-  
434 39.

435 McDonald BA, Linde C, 2002. Pathogen population genetics, evolutionary potential, and  
436 durable resistance. *Annual Review of Phytopathology* **40**, 349-79.

437 Montarry J, Jan PL, Gracianne C *et al.*, 2015. Heterozygote deficits in cyst plant-parasitic  
438 nematodes: possible causes and consequences. *Molecular Ecology* **24**, 1654-67.

439 Mugniery D, Bossis M, 1988. *Heterodera carotae* Jones, 1950 - Gamme d'hôtes, vitesse de  
440 développement, cycle. *Revue de Nématologie* **11**, 307-13.

441 Nei, M, 1978. Estimation of average heterozygosity and genetic distance from a small number  
442 of individuals. *Genetics* **89**, 583-590.

443 Nicol JM, Turner SJ, Coyne D, Den Nijs L, Hockland S, Maafi ZT, 2011. Current nematode  
444 threats to world agriculture. In: Jones J, Gheysen G, Fenoll C, eds. *Genomics and*  
445 *Molecular Genetics of Plant-Nematode Interactions*. Dordrecht, The Netherlands:  
446 Springer Science+Business Media BV, 21-43.

447 Osborne P, 1971. First record of *Heterodera catotae* in Scotland. *Plant Pathology* **20**, 148.

448 Overall ADJ, Nichols RA, 2001. A method for distinguishing consanguinity and population  
449 substructure using multilocus genotype data. *Molecular Biology and Evolution* **18**, 2048-  
450 56.

451 Picard D, Plantard O, Scurrah M, Mugniery D, 2004. Inbreeding and population structure of  
452 the potato cyst nematode (*Globodera pallida*) in its native area (Peru). *Molecular Ecology*  
453 **13**, 2899-908.

454 Plantard O, Picard D, Valette S, Scurrah M, Grenier E, Mugniéry D, 2008. Origin and genetic  
455 diversity of Western European populations of the potato cyst nematode (*Globodera*  
456 *pallida*) inferred from mitochondrial sequences and microsatellite loci. *Molecular*  
457 *Ecology* **17**, 2208-18.

458 Plantard O, Porte C, 2004. Population genetic structure of the sugar beet cyst nematode  
459 *Heterodera schachtii*: a gonochoristic and amphimictic species with highly inbred but  
460 weakly differentiated populations. *Molecular Ecology* **13**, 33-41.

461 Pritchard JK, Stephens M, Donnelly P, 2000. Inference of population structure using multilocus  
462 genotype data. *Genetics* **155**, 945-59.

463 Raymond M, Rousset F, 1995. Genepop Version1.2: population genetics software for exact  
464 tests and ecumenicism. *Journal of Heredity* **86**, 248-9.

465 Rousset F, 1997. Genetic differentiation and estimation of gene flow from F-statistics under  
466 isolation by distance. *Genetics* **145**, 1219-28.

467 Subbotin SA, Mundo-Ocampo M, Baldwin JG, 2010. *Systematics of Cyst Nematodes*  
468 (*Nematodes: Heteroderinae*), Volume 8, Part B. Leiden, The Netherlands: Brill.

469 Vovlas A, Santoro S, Radicci V, Leonetti P, Castillo P, Palomares-Rius JE, 2017. Host-  
470 suitability of black medick (*Medicago lupulina* L.) and additional molecular markers for  
471 identification of the pea cyst nematode *Heterodera goettingiana*. *European Journal of*  
472 *Plant Pathology* **149**, 193-9.

473 Wang HM, Zhao HH, Chu D, 2015. Genetic structure analysis of populations of the soybean  
474 cyst nematode, *Heterodera glycines*, from north China. *Nematology* **17**, 591-600.

475 Wang J, 2017. The computer program structure for assigning individuals to populations: easy  
476 to use but easier to misuse. *Molecular Ecology Resources* **17**, 981-90.

477 Wang X, Ma J, Liu H, Liu R, Li H, 2018. Development and characterization of EST-derived  
478 SSR markers in the cereal cyst nematode *Heterodera avenae*. *European Journal of Plant*  
479 *Pathology* **150**, 105-13.

480 Weir BS, Cockerham CC, 1984. Estimating F-statistics for the analysis of population structure.  
481 *Evolution* **38**, 1358-70.

482 Yu Q, Ponomareva E, Van Dyk D *et al.*, 2017. First report of the carrot cyst nematode  
483 (*Heterodera carotae*) from carrot fields in Ontario, Canada. *Plant Disease* **101**, 1056.  
484

485 **Data Availability**

486 Two files containing the genotypic data (Genepop format) for the different *Heterodera carotae*  
487 populations sampled at the plant scale (H\_carotae\_plants.txt) and at the field scale  
488 (H\_carotae\_fields.txt) are available at data.inra.fr (<https://doi.org/10.15454/MLMDVE>).

489



490 **Supporting Information legends**

491

492 **Fig. S1** Factorial correspondence analysis (FCA) performed on the 822 individuals collected at  
493 the field scale (circles). The 114 individuals which formed a distinct group (black circles) from  
494 all the other individuals (grey circles), were clearly grouped with the 64 *H. cruciferae*  
495 individuals coming from two reference populations (white triangles). All the other individuals  
496 were clearly grouped with the 36 individuals from the two reference *H. carotae* populations  
497 (white squares). The two first axes explained 22% and 3.5% of the genetic variability,  
498 respectively.

499

500 **Fig. S2** Likelihood surfaces showing estimated  $\theta$  and  $C$  values with 95 and 99% confidence  
501 envelopes for the 12 *H. carotae* populations sampled at the spatial scale of the plant showing  
502 significant heterozygote deficits (eight populations from the field Cre4 and four populations  
503 from the field Den3).

504

505

506 **Figure legends**

507

508 **Fig. 1** Bayesian clustering analysis (STRUCTURE) of the 595 *H. carotae* individuals (i.e. the  
509 dataset free of any missing data) coming from the 19 populations sampled at the field scale.  
510 Assignment probabilities of individuals are presented for  $K = 3$ . On the graph at the bottom,  
511 each vertical line represents an individual for which the genetic assignment is partitioned into  
512 three clusters, represented by three colors, and vertical white dotted lines separate each of the  
513 19 populations. The map at the top shows the geographical location of the 19 *H. carotae*  
514 populations and their membership proportion of clusters. In the production area of Créances,  
515 the dot line represents the main south-north road.

516

517 **Fig. 2** Matrix of pairwise  $F_{ST}$  between the 19 *H. carotae* populations sampled at the field scale.  
518 ns indicates non-significant differentiation between two populations. Populations were  
519 horizontally separated according to the three carrot production areas: Créances, Val-de-Saire  
520 and Mont St-Michel.

521

522 **Fig. 3** Isolation by distance pattern between genetic differentiation, measured as  $F_{ST} / (1 - F_{ST})$ ,  
523 and geographic distance (natural logarithm of the distance in m) for pairwise *H. carotae*  
524 populations.

525

526

527 **Table 1** *Heterodera carotae* populations collected at the field scale in three carrot production  
528 areas in Lower Normandy. For each of the 19 populations, the table shows the production area,  
529 the district, the GPS coordinates of each field, the number of successfully genotyped individuals  
530 (N), the genetic diversity indices ( $H_{nb}$  and Ar) and the departure from Hardy-Weinberg  
531 equilibrium ( $F_{IS}$ ).  $F_{IS}$  values significantly different to zero are indicated by a star.

532

Population	Production area	District	GPS coordinates	N	$H_{nb}$	Ar (n=16)	$F_{IS}$
Bre1	Créances	Bretteville / Ay	49°15'01.90"N 1°39'02.83"O	33	0,447	2,56	0,185*
Bre2c	Créances	Bretteville / Ay	49°15'46.95"N 1°38'34.74"O	36	0,372	2,52	0,198*
Bre2p	Créances	Bretteville / Ay	49°15'46.95"N 1°38'34.74"O	36	0,389	2,43	0,139*
Cre1	Créances	Créances	49°12'10.52"N 1°34'56.90"O	37	0,332	2,42	0,037
Cre2	Créances	Créances	49°12'13.78"N 1°34'57.51"O	40	0,380	2,52	0,103*
Cre3	Créances	Créances	49°12'27.59"N 1°34'46.36"O	39	0,366	2,45	0,154*
Cre4	Créances	Créances	49°12'44.06"N 1°34'37.87"O	40	0,295	2,30	0,090*
Cre5	Créances	Créances	49°12'36.14"N 1°34'46.58"O	40	0,343	2,32	0,088*
Cre6	Créances	Créances	49°12'09.51"N 1°35'03.02"O	39	0,373	2,35	0,142*
Cre7	Créances	Créances	49°12'42.03"N 1°35'42.04"O	39	0,356	2,47	-0,041
Cre8	Créances	Créances	49°12'31.48"N 1°34'31.51"O	38	0,336	2,33	0,046
StG1	Créances	Saint Germain / Ay	49°14'33.88"N 1°37'35.42"O	39	0,336	2,36	0,166*
StG2	Créances	Saint Germain / Ay	49°14'23.13"N 1°37'27.58"O	34	0,386	2,54	0,135*
Den2	Créances	Denneville	49°18'34.75"N 1°40'51.46"O	34	0,381	2,28	0,062
Den3	Créances	Denneville	49°18'46.96"N 1°40'51.86"O	40	0,333	2,43	0,133*
SIL5	Val-de-Saire	Gatteville-le-Phare	49°40'9.90"N 1°16'37.57"O	36	0,427	2,52	0,094*
VDS4	Val-de-Saire	Montfarville	49°39'26.73"N 1°14'53.35"O	22	0,437	2,75	0,118*
StM3	Mont S <sup>t</sup> -Michel	Le Mont Saint-Michel	48°36'56.20"N 1°31'47.51"O	40	0,346	2,35	0,195*
StM4	Mont S <sup>t</sup> -Michel	Beauvoir	48°36'19.85"N 1°31'51.02"O	40	0,438	2,77	0,119*

533

534 **Table 2** *Heterodera carotae* populations collected at the plant scale in two carrot fields (Cre4  
535 and Den3). For each of the 19 populations, the table shows the number of genotyped individuals  
536 (N), the genetic diversity index ( $H_{nb}$ ), the  $F_{IS}$  values and their significance (values significantly  
537 different to zero are indicated by a star) and  $\theta$  and C values, corresponding to the maximum-  
538 likelihood, for each population showing a significant heterozygote deficit.

539

Population	N	$H_{nb}$	$F_{IS}$	$\theta$	C
Cre4-1	40	0.30	0.132*	0.00	0.57
Cre4-2	40	0.32	0.164*	0.67	0.48
Cre4-3	39	0.29	0.093*	0.71	0.00
Cre4-4	39	0.28	0.121*	0.13	0.00
Cre4-5	37	0.28	0.088*	0.68	0.51
Cre4-6	38	0.30	0.037	.	.
Cre4-7	39	0.30	0.205*	0.00	0.76
Cre4-8	38	0.26	0.142*	0.72	0.48
Cre4-9	40	0.28	0.114*	0.07	0.17
Den3-1	39	0.31	0.055	.	.
Den3-2	39	0.35	0.145*	0.60	0.75
Den3-3	36	0.33	0.112*	0.11	0.00
Den3-4	36	0.31	0.077	.	.
Den3-5	37	0.32	-0.033	.	.
Den3-6	40	0.34	0.056	.	.
Den3-7	39	0.36	0.158*	0.64	0.38
Den3-8	40	0.34	0.161*	0.05	0.43

---

Den3-9	40	0.35	0.066	.	.
Den3-10	39	0.39	0.000	.	.

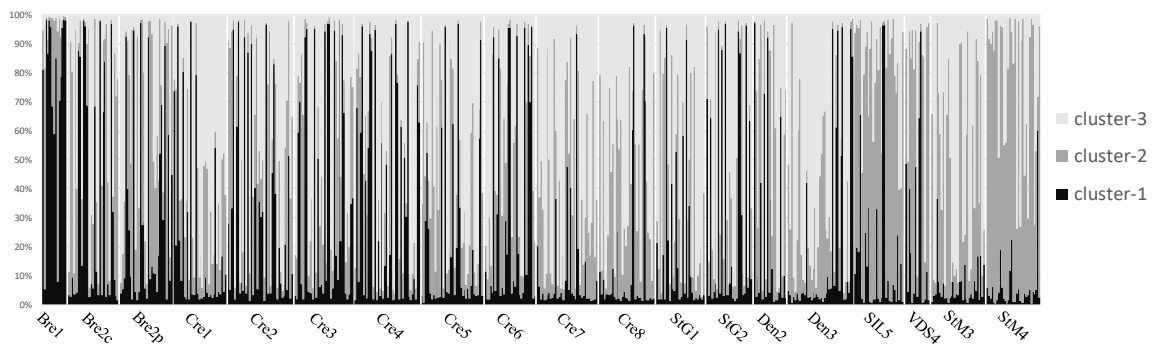
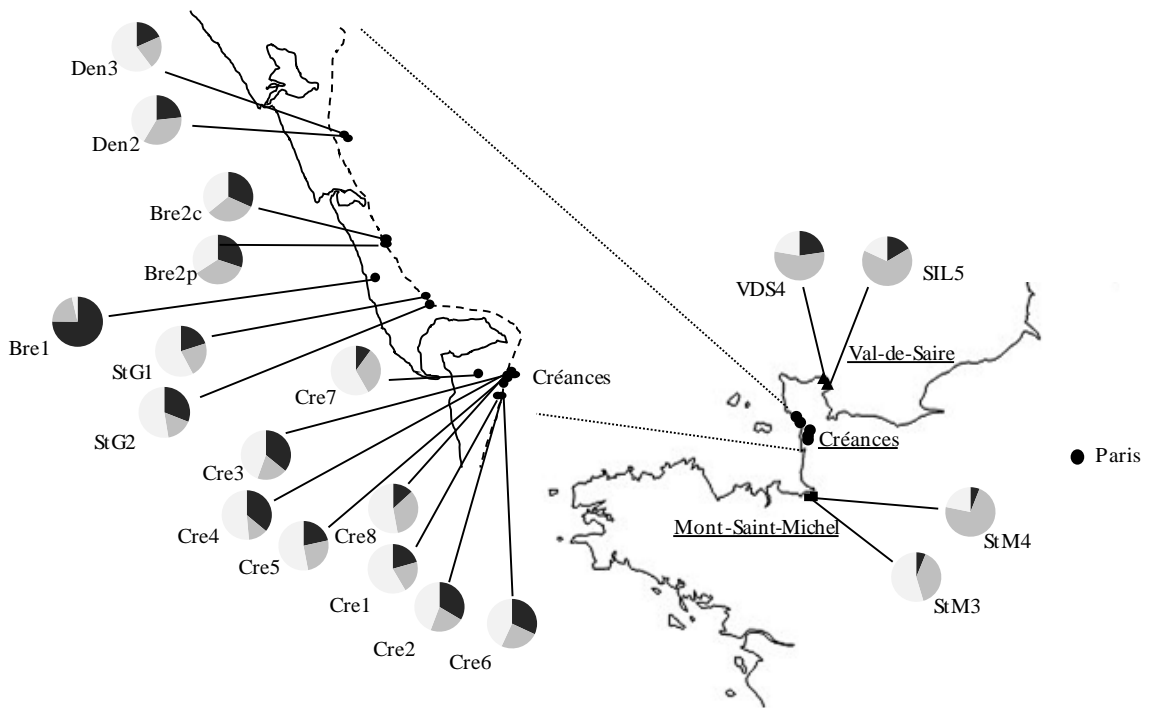
---

540

541

542 **Figure 1 – Esquibet *et al.***

543



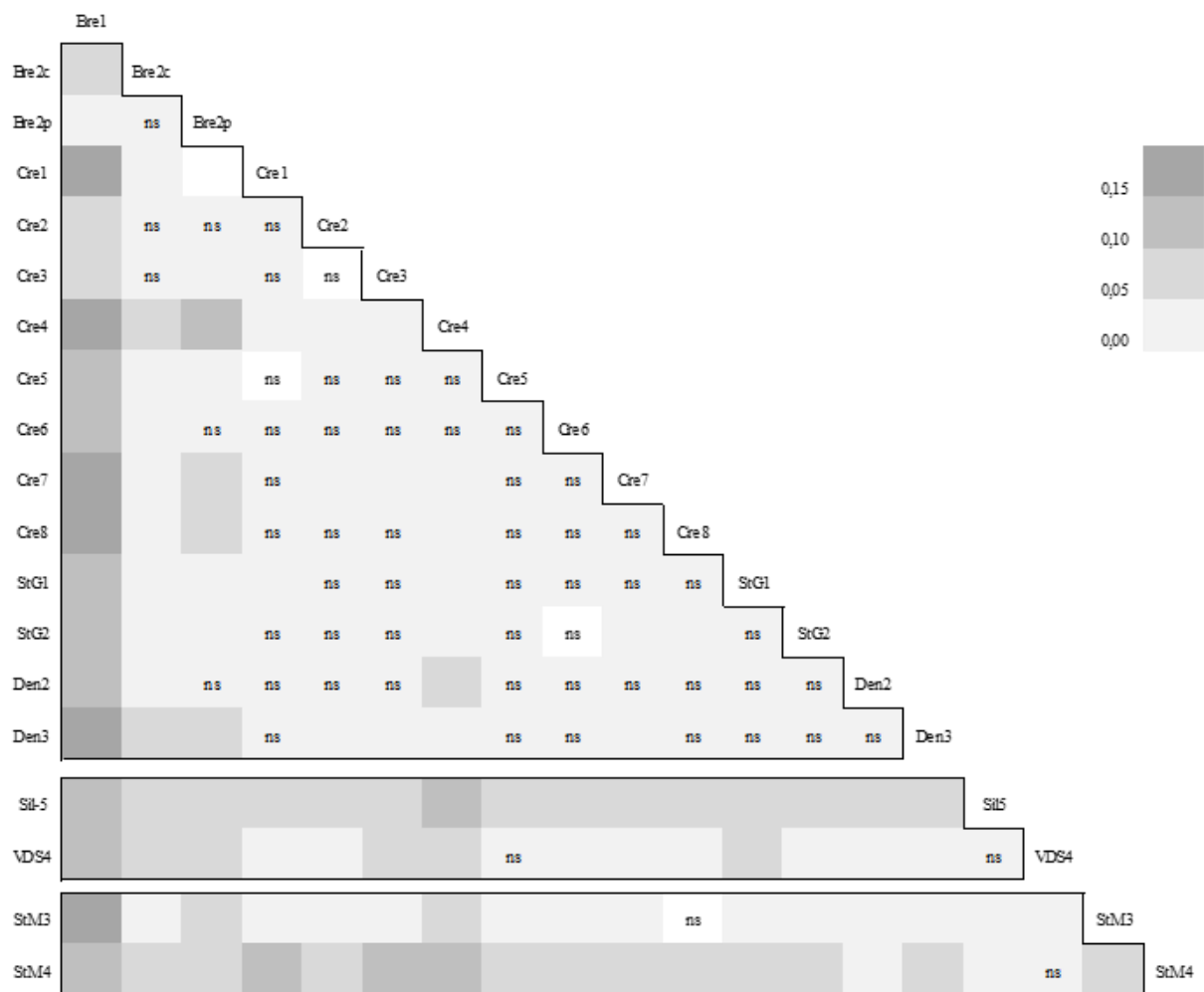
544

545

546

547 **Figure 2 – Esquibet *et al.***

548

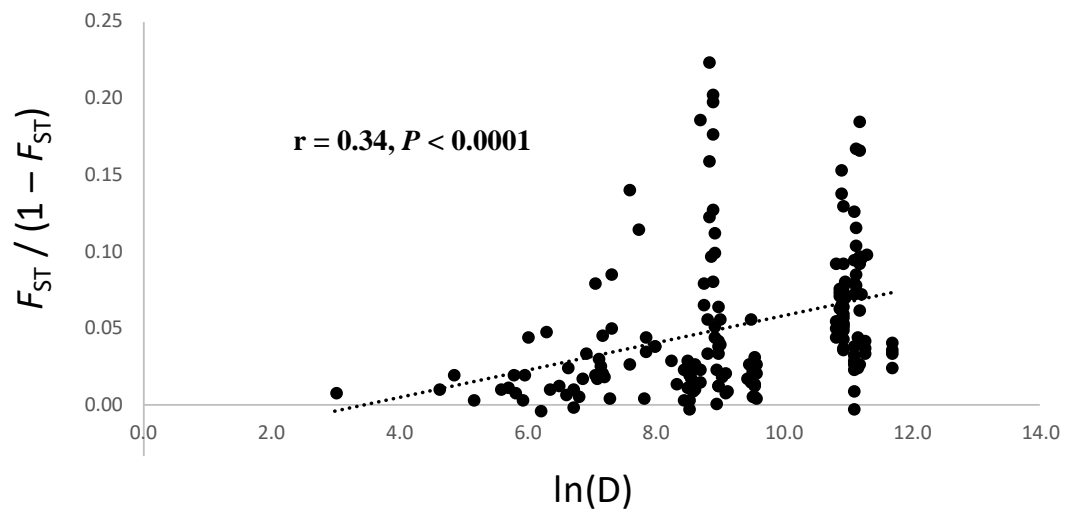


549

550

551 **Figure 3 – Esquibet *et al.***

552



553

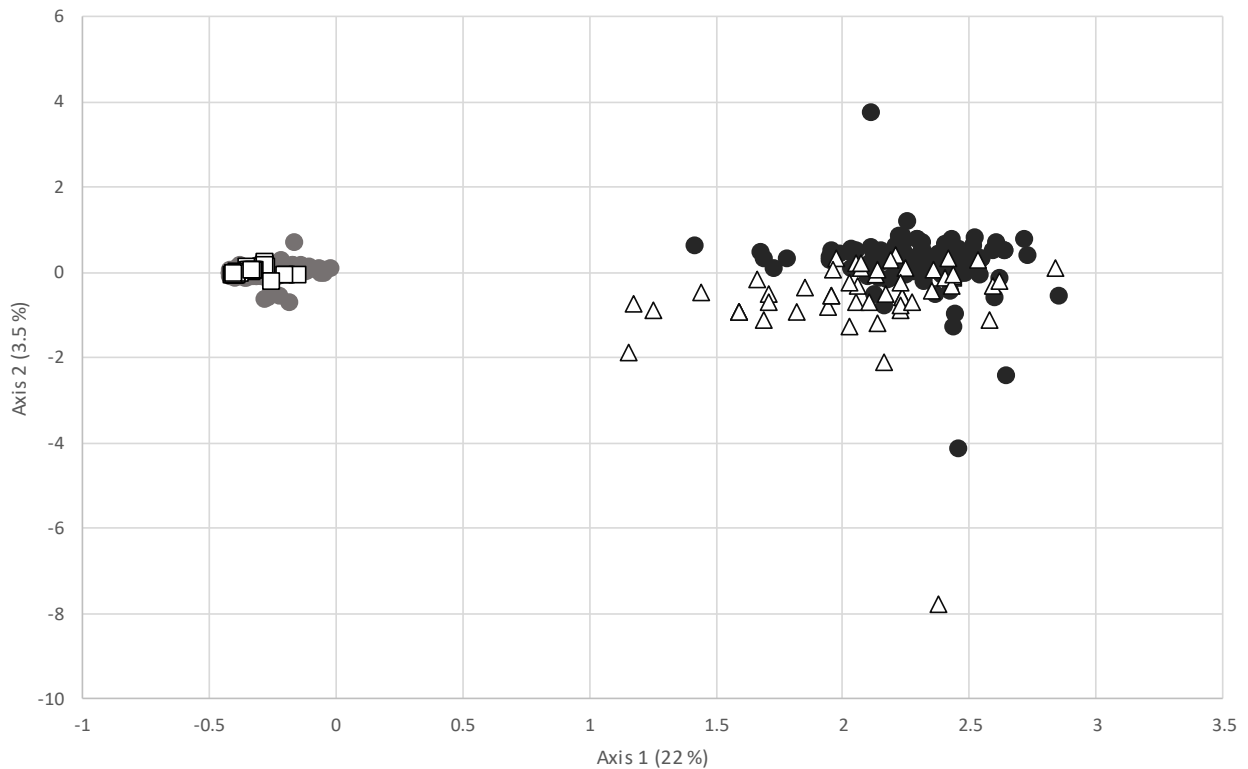
554



555 **Figure S1 – Esquibet *et al.***

556 Factorial correspondence analysis (FCA) performed on the 822 individuals collected at the field  
557 scale (circles). The 114 individuals which formed a distinct group (black circles) from all the  
558 other individuals (grey circles), were clearly grouped with the 64 *H. cruciferae* individuals  
559 coming from two reference populations (white triangles). All the other individuals were clearly  
560 grouped with the 36 individuals from the two reference *H. carotae* populations (white squares).  
561 The two first axes explained 22% and 3.5% of the genetic variability, respectively.

562



563

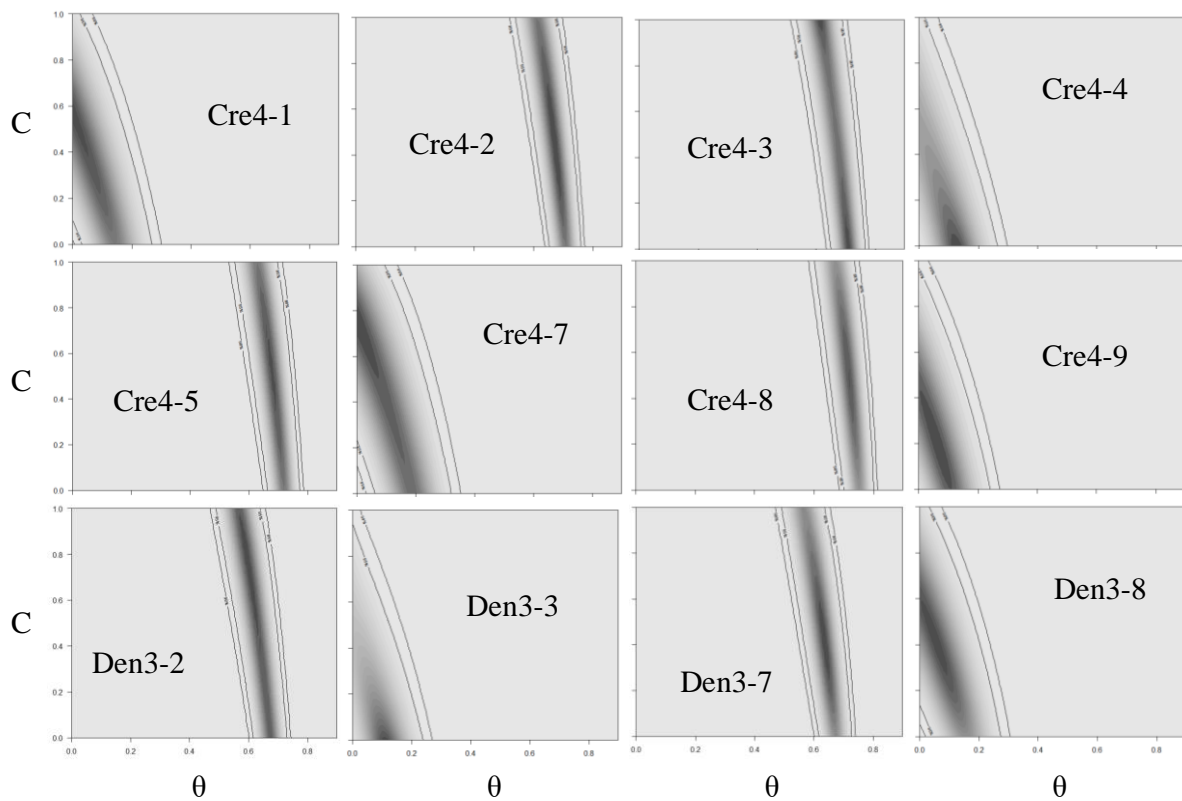
564

565

566 **Figure S2 – Esquibet *et al.***

567 Likelihood surfaces showing estimated  $\theta$  and C values with 95 and 99% confidence envelopes  
568 for the 12 *H. carotae* populations sampled at the spatial scale of the plant showing significant  
569 heterozygote deficits (eight populations from the field Cre4 and four populations from the field  
570 Den3).

571



572