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► **To cite this version:**

Sylvie Bardou-Valette, Eric Grenier, Josselin Montarry. Occurrence of the tobacco cyst nematode subspecies *Globodera tabacum* subsp *virginiae* in France. *European Journal of Plant Pathology*, 2016, 144 (1), pp.199-203. 10.1007/s10658-015-0741-5 . hal-01462725

HAL Id: hal-01462725

<https://hal.science/hal-01462725v1>

Submitted on 24 Aug 2023

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**Occurrence of the tobacco cyst nematode subspecies *Globodera tabacum* subsp. *virginiae*
in France**

Sylvie Bardou-Valette, Eric Grenier and Josselin Montarry

INRA, UMR1349 IGEPP (*Institute for Genetics, Environment and Plant Protection*), F-
35653 Le Rheu, France.

Corresponding author: Josselin Montarry – josselin.montarry@rennes.inra.fr

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Abstract Tobacco cyst nematode is a sexually diploid species composed of three subspecies, *Globodera tabacum* subsp. *tabacum* (Gtt), *G. tabacum* subsp. *virginiae* (Gtv) and *G. tabacum* subsp. *solanacearum* (Gts). In Europe, only Gtt have been reported to date. Here, we report from a plant in a French tobacco field, the identification of a mixture between cysts of the subspecies Gtt and cysts genetically different. The objective of the present study was to determine the subspecies status of those particular cysts. To this aim, we performed CAPS analyses on two genes and a principal component analysis on microsatellite data. The results obtained with both CAPS and microsatellite markers demonstrate for the first time the occurrence of *G. tabacum* subsp. *virginiae* in France.

Keywords cleaved amplified polymorphic sequence; microsatellite; *Nicotiana tabacum*, plant-parasitic nematode; principal component analysis

1 Plant-parasitic nematodes are major parasites of most crops and cause considerable economic
2 losses in agriculture (Nicol et al. 2011). Cysts nematodes are biotrophic diploid sedentary
3 endoparasites. Their life cycle involves different stages: first to fourth-stage juveniles and the
4 adult stages, separated by molts. Root exudates stimulate the hatching of the infective second-
5 stage juveniles (J2) that actively disperse in the soil to locate and penetrate the host roots.
6 Juveniles become sedentary by establishing a permanent feeding site close to the vascular
7 tissues (Jones and Northcote 1972). Sex is determined epigenetically, with the frequency of
8 males increasing under crowding or poor nutrition conditions. After egg production is
9 complete, the female dies and its body wall forms a cyst enclosing hundreds of eggs which
10 constitute a survival stage that can stay viable for several years in soils.
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25 Tobacco cyst nematode (TCN) is a sexually diploid species which produces from three
26 to five generations per year (Adams et al. 1982; Johnson 1998). TCN is a species complex
27 composed of three subspecies (Stone 1983): *Globodera tabacum* subsp. *tabacum* (Lownsbery
28 and Lownsbery, 1954), *G. tabacum* subsp. *virginiae* (Miller and Gray, 1968) and *G. tabacum*
29 subsp. *solanacearum* (Miller and Gray, 1972), named here after Gtt, Gtv and Gts,
30 respectively. These subspecies show very analogous morpho-biometric features which make
31 their identification difficult. Studies attempting to identify morphological criteria for
32 characterization of these subspecies (Mota and Eisenback 1993a, 1993b, 1993c) found only a
33 few characters of value in females and cysts. TCN are worldwide distributed but the
34 distribution of the subspecies remains mainly unknown except in France and USA. In the
35 United States, Gtt is limited to Connecticut and Massachusetts, whereas Gtv and Gts occur in
36 Virginia and North Carolina, as well as in one county in Maryland (Johnson 1998; Miller
37 1986). Gtt and Gts are more economically important parasites of tobacco in the US (Johnson
38 1998; Komm et al. 1983). Conversely, Gtv does not reproduce well on *Nicotiana* species
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(Miller 1978). In France, Alenda et al. (2014) sampled 18 infested tobacco (*Nicotiana tabacum* L.) fields in Aquitaine and Midi-Pyrénées regions and were able to identify only the Gtt subspecies.

With the initial objective to investigate the possible causes of nematode heterozygote deficits at the plant scale (Montarry et al. 2015), a stronger sampling effort was conducted in some of the fields investigated in Alenda et al. (2014). When cysts collected during this study were genotyped using microsatellite markers, we observed that in one population from Guinarthe Parenties (Pyrénées-Atlantique) some juveniles (coming from 16 cysts among the 47 cysts genotyped for this population) were genetically different compared to all the other TCN juveniles genotyped during that project. In the present study, we have investigated their subspecies status by i) cleaved amplified polymorphic sequence (CAPS) analyses on two genes as defined by Alenda et al. (2013) and ii) a principal component analysis (PCA) on microsatellite data, using also representatives of each TCN subspecies (Gui04 from France, Gv2 from USA and 75140 from Mexico representative of Gtt, Gtv and Gts, respectively).

In order to detect all the alleles existing in the offspring of the 16 cysts identified as divergent, total genomic DNA was extracted from whole individual cyst using a sodium hydroxide method as described in Boucher et al. (2013). The genotypes were acquired with two multiplex panels for a total of nine microsatellite loci (Alenda et al. 2014). The PCR for the genotyping reaction was performed in a 10 μ L volume containing 2 μ L of nematode DNA, 1X Type-it® Microsatellite PCR kit (Qiagen), and 0.4 μ M of each forward and reverse primer. After dilution (1:800), 3 μ L of PCR products were mixed with 0.05 μ L of GeneScan 500 LIZ Size Standard® (Applied Biosystems) and 5 μ L of formamide (Applied Biosystems). Analyses of PCR products were conducted on ABI Prism® 3130xl sequencer (Applied Biosystems). Allele size determinations were achieved using the GeneMapper® software v4.1

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(Applied Biosystems) by manual identification of the peaks and validation of each chromatogram.

Because each cyst contained hundreds of juveniles, each allele was coded as ‘1’ when present, or ‘0’ when absent in a binary matrix. That matrix was used to define multilocus genotypes (MLGs) and among the 16 cysts genotyped only five distinct MLGs were found. Three MLGs (MLGs a, b and e) were represented by a single cyst and the two other were represented by six (MLG d) and seven (MLG c) cysts sharing the same alleles at all loci. Five samples, representing the five distinct MLGs, were then kept for the CAPS and PCA analysis and compared to the three reference TCN populations.

The subspecies status was first assessed by using a CAPS analysis on a set of two different genes coding for CLE peptides (*Cle5* and *Cle1*) as developed by Alenda et al. (2013). The amplification of fragments to be submitted to CAPS yielded a single fragment of approximately 200 pb for *Cle5* and 500 pb for *Cle1* for all the tested samples. Figure 1 described the profiles obtained after enzyme restriction. The *MboI* endonuclease applied to the *Cle5* fragment differentiated Gtt from Gtv and Gts and showed that none of our five samples belong to Gtt (Fig. 1A). The *BstEII* endonuclease applied to the *Cle1* fragment separated Gts from Gtt and Gtv and therefore shows that none of our five samples belong to Gts (Fig. 1B). It appears therefore from the combination of these analyses that our five samples belong to Gtv. Indeed, all the patterns displayed by our five samples were congruent with the *G. tabacum* subsp. *virginiae* pattern observed for the reference sample.

A matrix of genotype distances based on the number of different alleles was calculated between our five MLGs and the three reference populations using the Microsoft EXCEL add-in GENALEX version 6.501 (Peakall and Smouse 2006). The principal component analysis (PCA) was performed using the distance matrix between each sample using GENALEX. Axes 1 and 2 of the PCA accounted respectively for 46.60% and 26.03% of the total genetic

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variability. PCA revealed three genetic clusters (Fig. 2): the first contained the Gts, the second referred to Gtt and the last cluster grouped together our five samples with the Gtv reference population.

The fact that two repeated MLGs (sharing the same alleles at all loci) were found, whereas *G. tabacum* is a sexually reproducing nematode species, revealed the low allelic richness of the microsatellite markers that were used. The nine microsatellites loci used in this study were developed from the species *Globodera rostochiensis* and *Globodera pallida*, explaining the few polymorphisms observed as cross-amplifying markers between species could lead to a loss of information due to mutations within the microsatellite arrays (FitzSimmons et al. 1995; Fredholm and Wintero 1995; Selkoe and Toonen 2006). Despite this, these markers were useful and very efficient to detect the presence of a different subspecies.

G. virginiae was a species initially described on horsenettle (*Solanum carolinense* L.) in Virginia U.S.A. (Miller et al. 1962). The results obtained in this study with both CAPS and microsatellite markers demonstrate clearly the occurrence of *G. tabacum* subsp. *virginiae* in Europe. To our knowledge this is the first report of this TCN subspecies outside the US. The second-stage juveniles from that Gtv French population (Fig. 3) were highly similar with those previously described from the three subspecies, Gtt, Gtv and Gts, both in terms of morphology (Mota and Eisenback 1993a) and morphometrics, *i.e.* stylet and tail length (Mota and Eisenback 1993c). The detection of the Gtv subspecies in France raises questions about the origin of those cysts and will need further investigations.

In a previous study by Alenda et al. (2014) using 18 *G. tabacum* populations sampled from fields in two agricultural cooperative societies of the southwestern France, no Gtv was detected, all the 425 genotyped juveniles belonging to the Gtt subspecies. The Gtv cysts were found here only parasitizing one tobacco plant (among five sampled plants in that field) and

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were in sympatry with other cysts belonging to the Gtt subspecies, the frequency of Gtv being 34% (16 cysts among the 47 genotyped cysts). Altogether those elements showed that the prevalence of the subspecies Gtv remain actually very low in France.

Host resistance represents an attractive means of control in infested fields because of the current phasing out of the most effective nematicides. However, nematode populations could adapt to the pressure imposed by the resistance and then breakdown the resistance (e.g. Fournet et al. 2013). Consequently, the resistance is often specific of a species or even a group of populations in this species. Therefore, the presence of an additional *G. tabacum* subspecies could have consequences on the definition of control strategies and on tobacco resistant cultivars deployment. Indeed, Gtt and Gtv could display contrasted (a)virulence spectrum on tobacco cultivars. Moreover, Gtt and Gtv being able to produce fertile hybrids under artificial conditions (Stone 1983), the occurrence of both subspecies in sympatry parasitizing the same tobacco plant could favor hybridization and raises questions about the fitness of hybrids and their evolutionary potential. Although previous results showed limited gene flow between French *G. tabacum* populations (Alenda et al. 2014), the prevalence of Gtv in France has now to be followed to ensure that this subspecies remains as low as it is now. In case it becomes more important, Gtv will also have to be taken into account for the selection and deployment of tobacco cultivars in France.

Acknowledgements Financial support from INRA SPE is gratefully acknowledged. We acknowledge B. Cailleateau (Imperial Tobacco, France) for his help in soil sampling, and Dr. Sylvain Fournet who took the pictures used in Fig. 3.

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Figure legends

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5 **Fig. 1** Cleaved amplified polymorphic sequences (CAPS) patterns of eight cysts, *i.e.* the five
6 multilocus genotypes (MLG) labeled a, b, c, d, and e and the three reference populations
7 labeled REF Gtt (*Globodera tabacum tabacum*), REF Gtv (*G. tabacum virginiae*) and REF
8 Gts (*G. tabacum solanacearum*), obtained from [A] *Mbo*I restriction on *Cle5* fragment, and
9 [B] *Bst*EII restriction on *Cle1* fragment.
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19 **Fig. 2** Principal component analysis (PCA) performed on the eight cysts, *i.e.* the five
20 multilocus genotypes (MLG) labeled a, b, c, d, and e and the three reference populations
21 labeled REF Gtt (*Globodera tabacum tabacum*), REF Gtv (*G. tabacum virginiae*) and REF
22 Gts (*G. tabacum solanacearum*), based on nine microsatellite markers.
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31 **Fig. 3** Anterior region [A] and tail [B] of a second-stage juvenile from the French *Globodera*
32 *tabacum virginiae* population. Stylet length = 22.0 μm and tail length = 47.1 μm .
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Figure 1

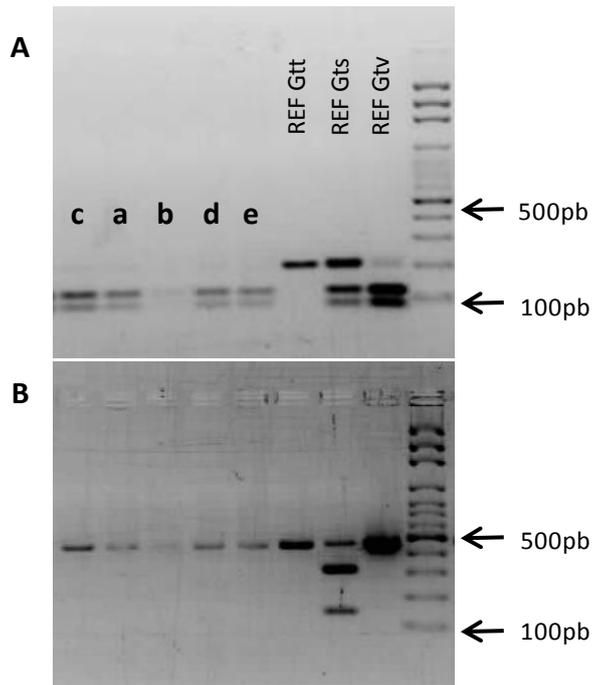
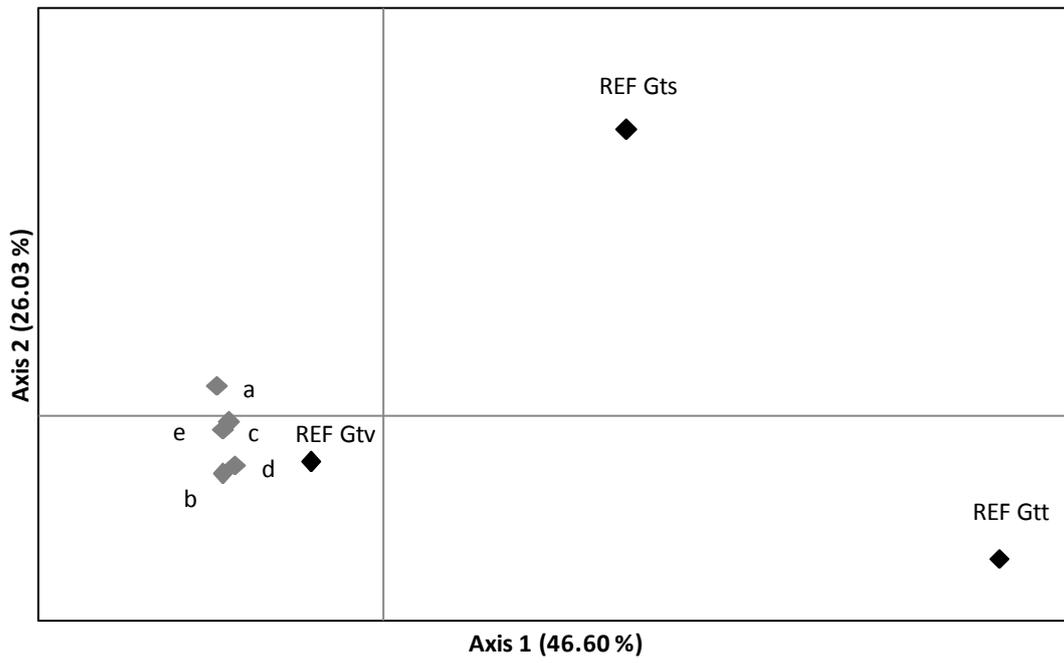
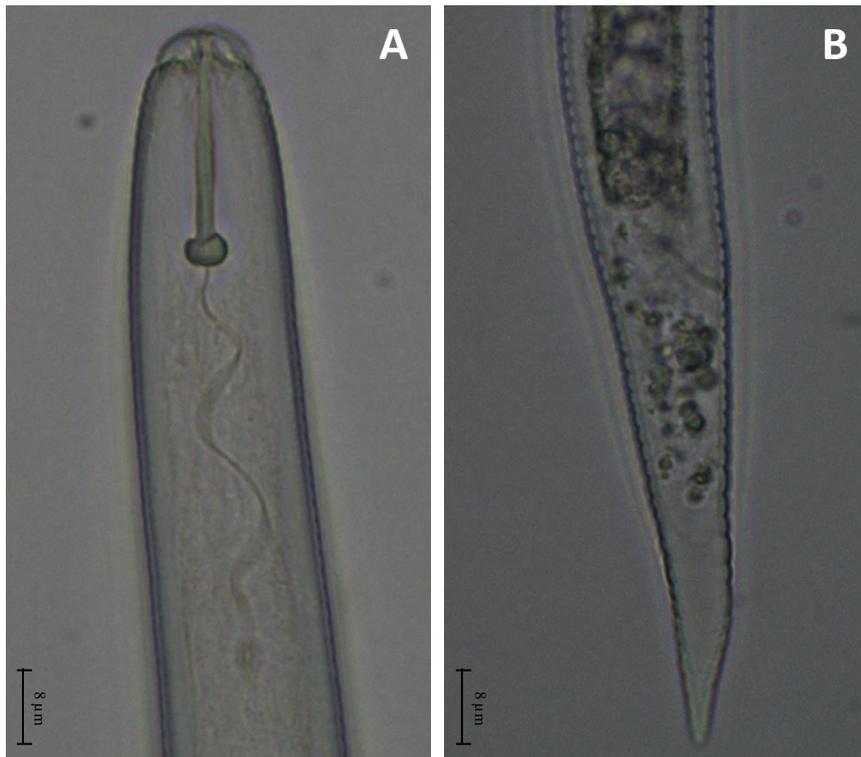


Figure 2



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Figure 3



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