# Phylogeography of some European populations of the sugar beet cyst nematode

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**Summary.** Sugar beet is an important crop of temperate climates and Serbia. The paleobotanic data suggest the sea beet was grown from ancient times, while the beets with swollen roots were cultivated in the Middle Ages in Europe. Phylogeography of the European populations of *Heterodera schachtii*, a nematode parasite on sugar beet, using Maximum Likelihood and Bayesian analyses was studied. Results based on matching the historical data with phylogenetic analyses based on the ITS rRNA region indicate the area across the Dutch-Belgian coastal region as a possible place of origin of the European *H. schachtii* populations. In addition, the dendrograms reveal a clear distinction between the two sister species (*H. schachtii* and *H. betae*) that coexist on the same host.

**Key words:** Bayesian analysis, geography, *H. betae*, *Heterodera schachtii*, ITS rRNA, ML analysis, phylogeny.

Sugar beet (*Beta vulgaris* ssp. *vulgaris* L., 1753) is an important crop of temperate climates, which provides nearly 30% of the world's annual sugar production and is a source for bioethanol and animal feed. Leafy beets have been cultivated since Roman times, but sugar beet is one of the most recently domesticated crops (Dohm *et al.*, 2014).

The sea beet *Beta vulgaris* ssp. *maritima* (L.) Arcang., 1882 is considered as the wild ancestor of all cultivated beets (Leys *et al.*, 2014). The sea beet was known from ancient times. An archeological site from Denmark, Tybrind Vig is a late Mesolithic coastal settlement, dated to the period 5600-4000 BC. The food plant remains are represented by fragments of *Quercus* sp. L., 1753 parenchyma, shell fragments of *Corylus avellana* L., 1753 and the charred fragments of parenchymatous tissue from roots of *Beta vulgaris* ssp. *maritima* (Kubiak-Martens, 1999). In the Late Neolithic site of the North Holland (around 2900 BC) the drift deposits were occupied by plants such as *B. vulgaris* ssp. *maritima* and *Suaeda maritima* (L.) Dumortier, 1827 (Kubiak-Martens *et al.*, 2015).

The sea beet is indigenous to European coastal regions, particularly the Mediterranean. In Europe *B. vulgaris* species with distinctly swollen roots were cultivated in the Middle Ages. Central European types are presumed to be descended from those used in Arabian horticulture in Spain. These plants were taken to The Netherlands, where they were cultivated beginning in 1500, later spreading throughout Germany. The crop was introduced into the USA in 1800 where it became known as a garden beet (Anonymous, 2001; *loc.cit.* Mansfeld, 1986).

Since crops are followed by their parasites during centuries (Oro *et al.*, 2014), the most economically important nematode parasite of sugar beet is the cyst nematode *Heterodera schachtii* Schmidt, 1871. At the present time, its distribution is recorded in all European countries where sugar beet is grown. The sugar beet nematode is found in Albania, Austria, Belgium, Bulgaria, Croatia, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, Moldova, The Netherlands, Poland, Mainland Portugal, Azores, Romania, Russia, Mainland Spain, Canary Islands, Sweden, Switzerland, UK and Ukraine (CABI/EPPO, 2001). Production of sugar beet in Serbia for the period 2000-2007, has more than tripled, from about 1 to about 3 million tons, which reflected new measures of agricultural policy (Božić *et al.*, 2010). *Heterodera schachtii* was recorded in 1954 for the first time in Serbia. It was found in a sugar beet field in Zvecka near Obrenovac. The field covering 20 ha of sugar beet had an average number of cysts of 4-68 $(100\ g\ soil)^{-1}$. Yield loss was more than 60% and the greatest loss was observed in a variety of sugar beet originating from The Netherlands (Grujicic, 1958).

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The latest data of Bačić (2013) indicate the presence of *H. schachtii* in sugar beet growing areas in Vojvodina, especially in the Backa and Srem districts, with the exclusion of Banat.

*Heterodera schachtii* and its sister species the yellow beet cyst nematode, *H. betae* Wouts, Rumpenhorst & Sturhan, 2001, occur together significantly more frequently than expected by chance. The analysis of the effect of habitat variables, showed that *H. schachtii* occurred preferentially in northern Europe, while *H. betae* was located in warm habitats of southern Europe, and both nematode species, found on the sea beet, exhibit a south-to-north phylogeographic pattern congruent to post-glacial recolonisation of the wild beet (Gracianne *et al.*, 2014). In contrast to what can be suspected for (crop) field populations, the study showed that wild cyst nematodes have very low dispersal capabilities and populations are strongly disconnected from each other (Gracianne *et al.*, 2016).

This study focused on phylogeography of the European populations (comprising our populations) of *H. schachtii*, using Maximum Likelihood (ML) and Bayesian analyses based on the internal transcribed spacer (ITS rRNA) region. Also, we aimed to match the current nematode molecular data and historical dispersal routes of sugar beet in order to propose a possible centre of origin of the sugar beet nematode in Europe as a consequence of the host-parasite relationship. In addition, to test the hypothesis of sequence similarity between the two sister species, the sequences of *H. betae* were also included. Molecular characterisation of *H. schachtii* populations was performed for the first time in Serbia, 66 years after the first occurrence of the sugar beet nematode.

## MATERIAL AND METHODS

The specimens of cyst nematodes were collected from sugar beet growing areas in Nova Crvenka and Kula (The Backa district) in Vojvodina, Serbia. Individual cysts were used for DNA extraction with a DNeasy Blood & Tissue Kit (Qiagen) in accordance with the manufacturer's instructions.

The PCR was done with primers for direct sequencing: TW81 (5'-GTT TCC GTA GGT GAA CCT GC-3') and AB28 (5'-ATA TGC TTA AGT TCA GCG GGT-3') (Skantar *et al.*, 2007). The ITS consensus sequences of sugar beet nematode from Nova Crvenka, Kula 1 and Kula 2 were deposited in GenBank nucleotide sequence database under the accession numbers: MF975709, MF975710 and MF975711, respectively. The sequences were aligned by using ClustalW module within MEGA 4 (Tamura *et al.*, 2007). Genetic distances among populations were calculated using pairwise distances (p-distances). Phylogenetic analyses were performed with available sequences of *H. schachtii* and *H. betae* from GenBank (Table 1) using PhyML 3.1 (Guindon & Gascuel, 2003) and MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2005) computer programs. The Maximum Likelihood (ML) tree was obtained with the General Time Reversible model (GTR), invariable sites and gamma distribution (GTR+I+G). The proportion of invariable sites and gamma distribution parameters were estimated by the program. Bayesian phylogenetic analysis was performed using GTR+I+G as nucleotide evolution model as well. The consensus dendrogram with 50% majority rule obtained by Bayesian inference (BI) was created by $3.2 \times 10^6$ generations of MCMC (Markov chain Monte Carlo), with the sample frequency of 100 and burnin function of 20%. *Heterodera avenae* (Wollenweber, 1924) Filipjev, 1934 and *H. filipjevi* (Madzidov, 1981) Mulvey & Golden, 1983 served as outgroups. The bootstrap values higher than 70% were shown next to the node.

## RESULTS AND DISCUSSION

Investigation of 730 molecular characters of selected nematode populations of *H. schachtii* and *H. betae* revealed 559 conserved sites, 185 variable sites, 128 parsimony informative and 55 singleton sites. The ITS region enclosed the partial ITS1 region spanning from the first to the 520$^{th}$ nucleotide, 5.8 S ribosomal RNA gene from the 521$^{st}$ to the 678$^{th}$ nucleotide and the partial ITS2 segment, between 679$^{th}$ and 730$^{th}$ nucleotide.

The content of nucleotides (Table 1) varied within the species level and was similar among the species. Similarly to the ITS region of potato cyst nematodes (Oro & Oro-Radovanović, 2012), the same region of sugar beet nematode also had higher percentages of guanine and thymine: 30.2 *versus* 27.4 respectively, with a distinct difference between them. The content of adenine (18%) was the lowest.

The p-distances of *H. schachtii* populations varied from 0.0% within the same population, *e.g.* among the Turkish clones nos. 4, 5, 6, 7 and 8 or between equal sequences from different localities (the Mexican one and the Belgian populations nos. 6 and 7 or between The South Korean 2 and The South African populations, to 3.0% between the clones from Turkey and the Belgian populations 2 and 12. Interestingly, the clones inside the same population from Ohain (the Belgian clones 10 and 12)

**Table 1.** List of *Heterodera* spp.: *H. schachtii* (1-55), *H. betae* (56-62), *H. avenae* (63), *H. filipjevi* (64) populations with accession numbers, locality and content of nucleotides.

| No. | Acc. no. | Country | Locality | T(U)% | C% | A% | G% |
|---|---|---|---|---|---|---|---|
| 1. | MG800690 | Algeria | unknown | 27.4 | 23.9 | 18.3 | 30.4 |
| 2. | EF611123 | Australia | Munster | 27.4 | 24.4 | 17.6 | 30.6 |
| 3. | EF611114 | Australia2 | Munster | 27.0 | 24.4 | 18.1 | 30.5 |
| 4. | EF611113 | Australia3 | Munster | 27.3 | 24.2 | 18.5 | 30.0 |
| 5. | EF611116 | Belgium | Momalle | 27.3 | 24.4 | 17.9 | 30.3 |
| 6. | EF611112 | Belgium2 | Molembaix | 27.5 | 24.0 | 18.4 | 30.1 |
| 7. | EF611111 | Belgium3 | Herme | 27.8 | 24.4 | 17.7 | 30.1 |
| 8. | EF611110 | Belgium4 | unknown | 27.1 | 24.7 | 17.9 | 30.3 |
| 9. | EF611109 | Belgium5 | Momalle | 27.0 | 24.7 | 17.9 | 30.3 |
| 10. | EF611107 | Belgium6 | Herme | 27.0 | 24.8 | 17.9 | 30.3 |
| 11. | EF611106 | Belgium7 | Momalle | 27.0 | 24.8 | 17.9 | 30.3 |
| 12. | EF611105 | Belgium8 | Molembaix | 27.4 | 24.6 | 17.9 | 30.1 |
| 13. | EF611100 | Belgium9 | Momalle | 27.1 | 24.7 | 18.2 | 30.0 |
| 14. | AY166438 | Belgium10 | Ohain | 27.3 | 24.7 | 17.9 | 30.2 |
| 15. | AY166437 | Belgium11 | Ohain | 27.3 | 24.0 | 18.2 | 30.4 |
| 16. | AY166435 | Belgium12 | Ohain | 27.6 | 23.9 | 18.4 | 30.1 |
| 17. | EU616694 | Belgium13 | Tongeren | 27.2 | 24.3 | 18.0 | 30.5 |
| 18. | EU616693 | Belgium14 | Gingelom | 27.3 | 24.5 | 18.0 | 30.2 |
| 19. | EF611103 | France | Aisne | 27.0 | 24.8 | 18.0 | 30.2 |
| 20. | EF611115 | Germany | Schladen | 27.6 | 24.4 | 17.5 | 30.5 |
| 21. | AY166439 | Germany2 | Muenster | 27.2 | 24.1 | 18.5 | 30.2 |
| 22. | KF225726 | Germany3 | Muenster | 27.4 | 24.7 | 17.8 | 30.0 |
| 23. | AF274394 | Germany4 | unknown | 27.1 | 24.4 | 18.0 | 30.5 |
| 24. | AF498389 | Iran | Fars | 27.2 | 24.3 | 18.5 | 30.0 |
| 25. | MK130992 | Mexico | Chalco | 27.0 | 24.8 | 17.9 | 30.3 |
| 26. | EF611118 | Morocco | Berkane | 27.8 | 24.3 | 17.7 | 30.1 |
| 27. | EF611108 | Morocco2 | Berkane | 27.2 | 24.6 | 17.9 | 30.3 |
| 28. | AY166436 | Morocco3 | Ouled Mbarek | 27.5 | 24.7 | 17.7 | 30.1 |
| 29. | EF611121 | The Netherlands | Rutten | 27.8 | 24.2 | 17.6 | 30.3 |
| 30. | EF611120 | The Netherlands2 | Rutten | 27.8 | 24.2 | 17.8 | 30.2 |
| 31. | EF611102 | The Netherlands3 | Borsel | 27.4 | 24.5 | 18.0 | 30.1 |
| 32. | EF611101 | The Netherlands4 | Borsel | 27.0 | 24.8 | 18.2 | 29.9 |
| 33. | LC208693 | The Netherlands5 | unknown | 27.3 | 24.7 | 18.0 | 30.0 |
| 34. | LC208692 | The Netherlands6 | unknown | 27.3 | 24.5 | 18.0 | 30.2 |
| 35. | JX024218 | The Netherlands7 | unknown | 27.8 | 24.2 | 17.6 | 30.3 |
| 36. | JX024219 | Poland | unknown | 27.3 | 24.4 | 17.9 | 30.3 |
| 37. | MN720075 | South Korea | Samcheok | 27.3 | 24.7 | 17.9 | 30.2 |
| 38. | MF043911 | South Korea2 | Jeongseon | 27.3 | 24.7 | 17.9 | 30.2 |
| 39. | MF754150 | South Africa | Tarlton | 27.3 | 24.7 | 17.9 | 30.2 |
| 40. | KT874527 | Turkey | San Liurfa | 27.7 | 24.1 | 17.9 | 30.3 |
| 41. | KT874526 | Turkey2 | San Liurfa | 27.7 | 24.2 | 17.9 | 30.2 |
| 42. | KT874525 | Turkey3 | San Liurfa | 27.5 | 24.1 | 18.2 | 30.2 |

**Table 1. (continued)** List of *Heterodera* spp.: *H. schachtii* (1-55), *H. betae* (56-62), *H. avenae* (63), *H. filipjevi* (64) populations with accession numbers, locality and content of nucleotides.

| No. | Acc. no. | Country | Locality | T(U)% | C% | A% | G% |
|---|---|---|---|---|---|---|---|
| 43. | KT874524 | Turkey4 | San Liurfa | 27.7 | 24.2 | 17.9 | 30.2 |
| 44. | KT874523 | Turkey5 | San Liurfa | 27.7 | 24.2 | 17.9 | 30.2 |
| 45. | KT874522 | Turkey6 | San Liurfa | 27.7 | 24.2 | 17.9 | 30.2 |
| 46. | KT874521 | Turkey7 | San Liurfa | 27.7 | 24.2 | 17.9 | 30.2 |
| 47. | KT874520 | Turkey8 | San Liurfa | 27.7 | 24.2 | 17.9 | 30.2 |
| 48. | KT874519 | Turkey9 | San Liurfa | 27.5 | 24.2 | 17.9 | 30.3 |
| 49. | KT874518 | Turkey10 | San Liurfa | 27.5 | 24.4 | 18.0 | 30.0 |
| 50. | KT874517 | Turkey11 | San Liurfa | 27.5 | 24.4 | 17.9 | 30.2 |
| 51. | KT874516 | Turkey12 | San Liurfa | 27.4 | 24.4 | 18.0 | 30.2 |
| 52. | AY590282 | USA | Michigan | 27.3 | 24.4 | 18.1 | 30.2 |
| 53. | MF975709 | Serbia | Nova Crvenka | 27.1 | 24.7 | 17.9 | 30.3 |
| 54. | MF975710 | Serbia | Kula1 | 27.1 | 24.7 | 17.9 | 30.3 |
| 55. | MF975711 | Serbia | Kula2 | 27.1 | 24.7 | 18.0 | 30.2 |
| 56. | LC208691 | The Netherlands | unknown | 27.7 | 24.2 | 17.8 | 30.3 |
| 57. | LC208690 | The Netherlands | unknown | 27.8 | 24.2 | 17.8 | 30.2 |
| 58. | LC208689 | The Netherlands | unknown | 27.8 | 24.2 | 17.8 | 30.2 |
| 59. | JX024221 | Germany | Elsdorf | 27.8 | 24.2 | 17.8 | 30.2 |
| 60. | JX024200 | Belgium | unknown | 27.3 | 24.6 | 17.7 | 30.4 |
| 61. | EF611122 | Germany | unknown | 27.9 | 24.3 | 17.7 | 30.0 |
| 62. | EF611122 | Germany | unknown | 27.9 | 24.2 | 17.9 | 30.0 |
| 63. | KC847094 | Serbia | Bezdan | 27.7 | 23.1 | 19.1 | 30.1 |
| 64. | JX235959 | Serbia | Gunaros | 27.8 | 22.4 | 19.3 | 30.5 |

had high level of divergence of 2.5%, similarly to the Australian clones from Münster. A similar observation was made in the study of Madani *et al.* (2007), who concluded that there was no grouping of populations according to the geographic origin.

Within *H. betae* populations, distances varied from 0.2 to 1.0%. The pairwise distances between the two species occurred between 0.0 and 3.0% indicating some species misidentification. Regarding populations found in Serbia, the distance between the Kula 1 (and Nova Crvenka) and Kula 2 clones was 0.3%. The populations of Kula 1 and Nova Crvenka had the same sequences. The greatest difference (2.5%) appeared between Kula 1 (and Nova Crvenka) and the Belgian populations 2 and 12. The closest to the population Kula 2 were the Belgian populations 9 and 11. The same sequences were shared by populations Kula 1 and Nova Crvenka and the Belgian clones 4 and 5.

The obtained phylogenetic trees (both ML and BI) depicted the same relationships among the examined species (Figs 1 & 2). The populations presented in dendrograms were assembled into three clusters (clades). The first cluster comprised the European populations as well as some non-European populations of the sugar beet nematode. There were populations from The Netherlands (nos. 3, 4, 5 and 6), Belgium (nos. 4-10), France, Germany and Serbia. A population from Algeria was in the same group with the populations from The Netherlands. The populations from South Korea and South Africa were grouped with the ones from Belgium. Kula 2 was clustered with the Dutch population 4 and the Belgian population 9. Kula 1 and Nova Crvenka were genetically close to the Belgian populations 4-5 and the Moroccan population 2. A Mexican population was clustered with the Belgian populations (nos. 6 and 7). The next clade linked to the previous one, contained the populations from Belgium (nos. 2, 3, 11 and 12) and Germany (nos. 2 and 4) together with the populations from Iran, Morocco and Australia. The placements of the populations from Belgium, Poland and USA were not resolved. The last clade encompassed some European populations of *H. schachtii*, a population from Turkey and the populations of *H. betae*.

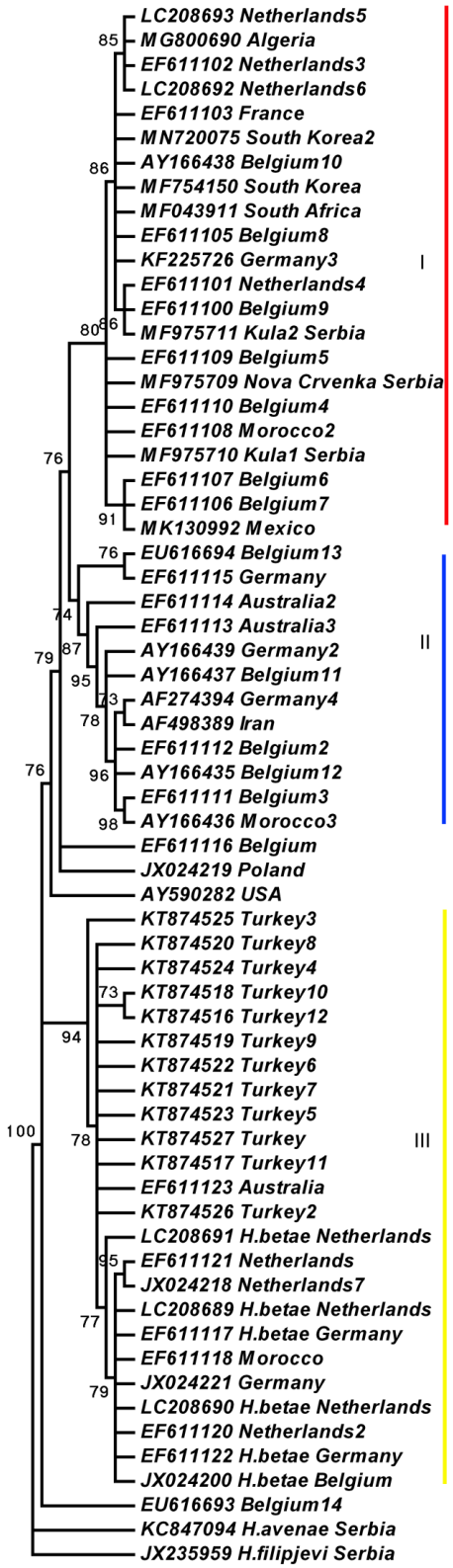


**Fig. 1.** ML phylogenetic tree of *Heterodera schachtii* and *H. betae* populations for the 64 taxon dataset, based on ITS sequence region using GTR+I+G nucleotide evolution model.

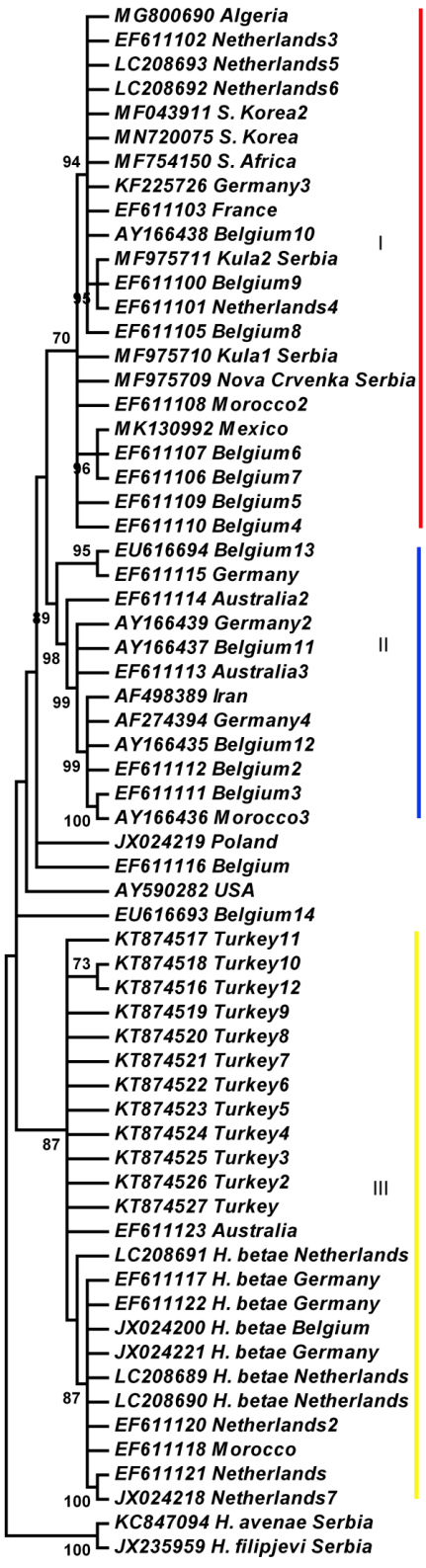


**Fig. 2.** BI phylogenetic tree of *Heterodera schachtii* and *H. betae* populations for the 64 taxon dataset derived from consensus 50% majority rule, based on ITS sequence region using GTR+I+G nucleotide evolution model.

Since the historical data consider Europe the ancestral region of sugar beet domestication, we have summarised the geographic positions of the European populations of *H. schachtii*. They are grouped towards the Dutch-Belgian direction starting from the coastal zone with the population Borsel (or probably Borssele, near the North Sea). In the 16$^{th}$ century, as mentioned, sugar beet from Spain was introduced to The Netherlands and Borssele, presumably by the medieval maritime ships. The subsequent spread followed a radial direction towards the nearby countries, such as Belgium, Germany, *etc.* (Fig. 3). The majority of populations are localised near borders, presumably because of human transport activities. The Belgian population Momalle is located close to the Dutch border, while the German population Muenster (Münster) is positioned near The Netherlands but on the opposite side. The French population Aisne is located near the Belgian border. The population from Molenbaix is situated near the French border.

Regarding the countries from other continents, their populations are always clustered with either Dutch or Belgian populations. A population from Algeria is genetically identical to the Dutch populations 3 (Borssele) and 6 (an unknown population).

The South African and South Korean nematode populations are identical to the Belgian population 10 (Ohain). The study of Escobar-Avila *et al.* (2019) has shown that the Mexican population is identical to the corresponding gene sequence of this species from Belgium, which is congruent with our dendrograms. The Moroccan population 2 is the most similar to the Belgian populations Momalle, Molenbaix, Gingelom, and the Serbian populations Kula 1 and Nova Crvenka. The remaining populations with the highest level of divergence are grouped into the *H. betae* clade.

Our phylogenetic study presents a clear distinction between populations of *H. schachtii* and *H. betae*, the two species coexisting on the same host, the fact that makes the process of species identification extremely complex and occasionally misleading. The dendrograms reveal that some populations, *i.e.* specimens identified as *H. schachtii* do not belong to that species. Bearing this in mind, we have come to an answer why some clone variants from the same locality are not arranged according to their geographic origin. They simply do not belong to the same species. The presence of congeneric species was detected in the Chinese cabbage field in South Korea (Mwamula *et al.*, 2018). Furthermore, the authors of this study frequently found *H. schachtii* cysts in mixed populations with *H. filipjevi*, when sugar beet was in rotation with wheat and maize. The presence of intercontinental populations within the clade of the European populations is presumably a result of the commodity exchange and 'import' of the phytoparasitic nematode.


**Fig. 3.** Map showing the possible spread of *Heterodera schachtii* from the ancestral localities toward countries where the sugar beet nematode was reported (the map provides approximate localities and is not drawn to scale).

The evolution of domestication is associated with the rise of many diseases attributed to an agricultural origin. The unprecedented population densities of humans, domesticated animals and plants in which efficient transmission rates were possible provided new pools for disease. This, combined with the novel juxtaposition of species as domesticates coming into contact with humans, each other and indigenous wild species in new environments, facilitated the transfer of diseases between species, often with an associated increased virulence in the adopted host. While this process has long been appreciated as an origin of many human diseases, more recently it has become apparent that the origins of many domesticated plant diseases are recent (Smith *et al.*, 2014).

Tracking the historical distribution of sugar beet seems to be an interesting alternative approach in search for the ancestral crop population of *H. schachtii* indicating that its place of origin could be the area across the Dutch-Belgian coastal region starting with the Borssele population and its further distribution to other countries. The data from countries like Spain, Portugal, *etc.* should be included in order to complete this scenario for the spread of *H. schachtii* on our continent.

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**Violeta Oro and Marijenka Tabakovic.** Филогеография некоторых европейских популяций цистообразующих нематод сахарной свеклы.

**Резюме.** Сахарная свёкла – важная культура сельхозугодий во всем поясе умеренного климата и в Сербии, в частности. Палеоботанические данные показывают, что сахарную свёклу выращивали с древности. Уже в Средние века в Европе были описаны случаи образования наростов на корнях сахарной свёклы. Филогеографию европейских популяций нематод вида *Heterodera schachtii*, паразитирующих на сахарной свёкле, оценивали с помощью метода максимального правдоподобия и с помощью Байесова анализа. Результаты, основанные на сопоставлении исторических данных с филогенетическим анализом, указывают на область в голландско-бельгийском прибрежном регионе как возможное место происхождения европейских популяций *H. schachtii*. Полученные дендрограммы позволяют также четко разграничить сестринские виды *H. schachtii* and *H. betae*, которые часто сосуществуют в одном растении-хозяине.