Final Report

Project title (Acronym)
Global warming and distribution of root-knot nematode species of the tropical group (MeloTrop)

Project Duration:

Start date:	2017-04-01
End date:	2020-03-31

Contents

1. Research Consortium Partners

Applicant / Coordinator – Partner 1			
Organisation	Agricultural Institute of Slovenia (KIS)		
Name of contact (incl. Title)	Dr. Saša Širca	**Gender**:	M
Postal address	Hacquetova ulica 17, Si-1000 Ljubljana, Slovenia		
E-mail	sasa.sirca@kis.si		
Phone	+386 (0)1 280-5262		
Research participants	Saša Širca, Barbara Gerič Stare, Polona Strajnar, Matej Knapič, Uroš Žibrat		

Applicant – Partner 2			
Organisation	Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail (ANSES)		
Name of contact (incl. Title)	Dr Laurent Folcher	**Gender**:	M
Postal address	LSV Domaine de la Motte au Vicomte,BP 35327, 35653 Le Rheu Cedex, France		
E-mail	laurent.folcher@anses.fr		
Phone	33 2 99 30 48 28		
Research participants	Laurent Folcher, Fabrice Ollivier, Alain Buisson, Anne-Marie Chappe		

Applicant – Partner 3			
Organisation	National Institute for Agricultural and Veterinarian Research (INIAV)		
Name of contact (incl. Title)	Dr. Maria Lurdes Inácio	**Gender**:	F
Postal address	Quinta do Marquês, 2780-159 Oeiras, Portugal		
E-mail	lurdes.inacio@iniav.pt		
Phone	+351 916626641; +351 214463727		
Research participants	Maria Lurdes Inácio, Leidy Rusinque, Filomena Nóbrega, Eugénia Andrade		

Applicant – Partner 4			
Organisation	Netherlands Food and Consumer Product Safety Authority (NVWA)		
Name of contact (incl. Title)	Prof. Dr. Gerrit Karssen	**Gender**:	M
Postal address	Geertjesweg 15, 6706 EA Wageningen, the Netherlands		
E-mail	g.karssen@nvwa.nl		
Phone	+31 6 15891726		
Research participants	Evelyn van Hesse, Gerrit Karssen, Tim Warbroek		

Applicant – Partner 5			
Organisation	Institut Tamiš, Agricultural Extension Service Institute Tamiš (AES)		
Name of contact (incl. Title)	Dr. Jasmina Bačić	**Gender**:	F
Postal address	Novoseljanski put 33, 26000 Pančevo, Serbia		
E-mail	jasmina.bacic@jasminabacic.rs		
Phone	+381 13 313 092		
Research participants	Jasmina Bačić		

Applicant – Partner 6			
Organisation	University of Coimbra (UC)		
Name of contact (incl. Title)	Prof. Isabel L.P.M. Conceição	**Gender**:	F
Postal address	Calçada Martim de Freitas, 3000-456 Coimbra, Portugal		
E-mail	isabelluci@gmail.com		
Phone	+351 239 240 759		
Research participants	Isabel Abrantes, Isabel L.P.M. Conceição, Carla Maleita		

2. Short project report

2.1. Executive Summary

Root-knot nematodes (RKN) (*Meloidogyne* spp.) represent a relatively small but economically important group of obligate plant parasites. Several RKN species belong to the 'tropical' RKN group which can cause significant economic losses in agriculture, especially in vegetable crops. The species of this group reproduce by mitotic parthenogenesis and are pests of important food crops, vegetables, fruits and ornamentals grown in tropical, subtropical and temperate climates. The damage and yield losses caused by this group are greater in tropical regions than in temperate regions because of more favourable environmental conditions for pest colonization, development, reproduction and dispersal. However, climate change will influence the spread of these pests and their dispersal across temperate regions.

The project aimed to organise surveys in several partnering countries to map the occurrence and distribution of tropical RKN species, to validate biochemical and/ or molecular diagnostic tests and to study the survival ability of RKN species at the open field conditions in the continental climate and Mediterranean/ Atlantic conditions.

Data on the distribution of tropical RKN species in partner countries were obtained focussing on sites of field cultivation and ornamental production and by summing national record data on the occurrence of tropical RKN. In total, 107 locations from France, Portugal, Serbia and Slovenia were covered. The most frequently identified species was *Meloidogyne incognita*, recorded at 47 locations. The second most common species was *M. arenaria* (21 locations), followed by *M.* javanica (11 locations), *M. hispanica* (7 locations), *M. luci* (6 locations) and of *M. enterolobii* (2 locations). Mixed species were also found at 13 sites. Forty-three different host species were recorded. Tropical RKN species in the open areas was predominantly distributed in areas with Mediterranean climatic conditions, but a small proportion of sites were recorded in areas with semi-continental climates and mild winter conditions.

Five laboratories from partner institutions participated in the test performance study to identify RKN species using biochemical and molecular tests. For isozyme phenotyping, the average agreement of results was 85.1%, while successful identification among laboratories ranged from 58.3 to 100%. Not all species were identified with the same success. The species that were correctly identified 100% of the time were *M. arenaria* and *M. enterolobii.* For *M. hispanica, M. javanica* and *M. luci,* 90% agreement was achieved, while 80% for *M. incognita,* 70% for *M. ethiopica* and 60% for *M. inornata.* A modified DNA barcoding method, in which four mtDNA coding regions were sequenced, did not prove to be a successful method for identifying all eight selected species. Only 36% of the samples were correctly identified by all laboratories involved in the test performance study. Sequencing of additional mtDNA regions as well as having valid reference data for more species in public nucleotide sequences databases (e.g. Genbank, ENA, Q-bank) could achieve better resolution and correct species identification, but the success rate of identification among laboratories remains to be tested.

The survival ability of *M. incognita* and *M. arenaria* in the field under continental climatic conditions was studied. Both species survived and maintained their infectivity in a micro-plot (semi-field conditions) filled with soil during three consecutive winters at the Ljubljana site, defined as continental conditions. The potential suitability of European territory for survival

and development of selected tropical RKN species was assessed with CLIMEX software and simulation of the influences of climate change scenario were performed.

2.2. Project aims

Root-knot nematodes (RKN, *Meloidogyne* spp.) are considered to be the most harmful plant-parasitic nematodes. Among plant parasites, only RKN lead to crop losses of around 15% in the sub-tropical countries. Moreover, yield losses of 50-80% caused by these nematodes in vegetable crops are common. The 'tropical' RKN group (*M. incognita, M. arenaria, M. javanica, M. enterolobii, M. ethiopica, M. hispanica, M. paranaensis* etc.) may cause substantial economic losses in agriculture particularly in vegetable production. The damage and yield losses caused by this group are greater in tropical than in temperate regions due to more favourable environmental conditions for pest colonization, development, reproduction and dispersal. However, climate change is likely to influence the future distribution of the pests and it is expected that *Meloidogyne* species previously found in tropical and subtropical regions will become important pests in temperate regions as well. Tropical RKN species can move northwards and both temperate and tropical RKN can have more generations per year.
In addition, an intensive global trade, trends such as environment friendly plant production methods and lack of adequate management strategies pose a serious risk of these pests for the agricultural production in Europe. Two species, *M. incognita* and *M. javanica*, from the tropical RKN group were recognised as the most rapidly spreading plant pests globally, as measured by country saturation rate of 424 plant pests and diseases. Several species of the tropical group are able to survive open field winter conditions particularly in the Mediterranean countries. Open field occurrence represents additional risk, especially considering climate change and the fact that infestations at larger acreages are much more difficult to manage.
To cope with such threats, detection and identification methods/schemes for tropical RKN species are needed. Due to the hybrid origin of parthenogenetic RKN, the use of nuclear markers, such as ribosomal DNA (rDNA) and methods that focus on a single molecular marker are inappropriate for species identification within this group. The most promising results were obtained by analysing multiple marker genes of the mitochondrial DNA (mtDNA), but the diagnostic tests based on these markers are not developed yet. Currently, one of the most reliable tests for the group diagnostics is the isozyme phenotyping, which shows poor reproducibility.
The project aimed to obtain the distribution map of tropical RKN species, assess the survival ability of two tropical RKN species in continental European climate conditions and develop models for the open field spreading of tropical RKN, using different climate change scenarios in Europe. Additionally, the project included the validation of biochemical and molecular methods for the identification of tropical RKN species.

2.3. Descriptions of the main activities

The following activities were performed:

a) The distribution of the tropical RKN species was determined for the participating countries. Sampling was performed in open field crops and ornamental plants. Some samples were collected also in the protected areas of vegetables production.

Additionally, the national record data of the tropical RKN occurrence was added in the project tropical RKN database from KIS, ANSES, INIAV, UC and Institut Tamiš.

b) The study of biochemical and molecular tests for the identification of tropical RKN species included isozyme phenotyping and nucleic acids based tests. The aim of this activity was to obtain validation data for the isozyme electrophoresis esterase (Est, EC 3.1.1.1) and malate dehydrogenase (Mdh, EC 1.1.1.37), using specimens of tropical RKN from partners' collections. Isozyme migration rates were calculated and compared among the laboratories. Validation data were also obtained for selected DNA barcoding approach. A modified sequencing approach, previously described by Janssen *et al*. (2016, Sci Rep 6: 22591 DOI: 10.1038/srep22591), that analyses four mtDNA coding regions (Nad2, Nad5, Cox2 and Cox3) was tested in the participating laboratories.

c) Testing the ability of survival of two tropical RKN, *M. incognita* and *M. arenaria*, in continental and Mediterranean climate conditions. As both species were detected in crops in the fields at Bilje (Slovenia, Mediterranean climate), the test was performed only in Ljubljana (continental climate). The test was set up in a micro-plot (semi-field) of 1 m^2, filled with soil.

d) The effect of climate change on the tropical RKN species potential spreading in the open field agricultural production areas in Europe was also analysed.

2.4. Main results

2.4.1. Distribution of the tropical RKN species

The data on the tropical RKN distribution in partnering countries were obtained by sampling in open field crop production and ornamental plants. The samples were analysed for the presence of RKN. The positive samples were further analysed for RKN species identification, using morphological, biochemical or molecular methods. Additionally, the national record data of the tropical RKN occurrence was added to the project tropical RKN database. In total, the Melotrop database contained 107 locations from France, Portugal, Serbia and Slovenia. The most frequent species was *M. incognita*, recorded at 47 locations. Second most common species was *M. arenaria* (21 locations), followed by *M. javanica* (11 locations), *M. hispanica* (7 locations), *M. luci* (6 locations) and *M. enterolobii* (2 locations). Besides, mixed populations were detected in several locations: *M. hispanica* + *M. incognita* (4 locations); *M. enterolobii* and *M. arenaria* (3 locations); *M. arenaria* and *M. incognita* (2 locations); and *M. luci* and *M. incognita*, *M. hapla* and *M. incognita*, *M. arenaria* and *M. hispanica* and *M. javanica*, *M. javanica* and *M. arenaria* and *M. hapla* were detected in 4 locations. Forty-three host species were recorded. At 11 locations, the host species were not identified as the samples were collected at bare fields. The distribution of tropical RKN species at the open fields was predominant in areas with the Mediterranean climatic conditions; however, a small proportion of locations were recorded in areas of semi-continental climate with milder winter conditions. Distribution maps of tropical RKN were created. More information is available from the Appendices.

2.4.2. Validation of biochemical and molecular diagnostic methods for tropical RKN species

Five laboratories from partnering institutions participated in the test performance study of isozyme phenotyping. The laboratories were encoded as Lab 1 to Lab 5. Lab 1, Lab 2 and Lab 3 used PhastSystem, while Lab 4 and Lab 5 used the PAGE method. For seven species of tropical RKN (*M. arenaria, M. enterolobii, M. ethiopica, M. hispanica, M. inornata, M. javanica* and *M. luci*) two populations were tested and for *M. incognita* only one. Among 15 analysed populations, one population was found to be either miss-identified or the population consisted of a mixed population and was, therefore, excluded from the result analyses. Besides, the quality of two samples in Lab 2 and one sample in Lab 4 were not adequate and were also excluded from the result analyses.

In total, 67 isozyme phenotyping analyses were performed. For 57 analysed samples, the results were in conformance with sample identity, while the results of 10 analysed samples were not. The average conformity of results was 85.1%, while the successful identification between laboratories ranged from 58.3 to 100%. Not all species were identified with the same success. The species that were 100% correctly identified in both samples were *M. arenaria* and *M. enterolobii.* For *M. hispanica, M. javanica,* and *M. luci* 90% conformity was achieved, whereas 80% was achieved for *M. incognita,* 70% for *M. ethiopica* and 60% for *M. inornata.*

Results of the relative migration (Rm) test were variable when comparing values of the same sample among laboratories regardless the method used. Therefore, it was concluded that Rm is not a stable character when analysing isozyme EST and MDH electrophoretic phenotypes and would be challenging to use it for method validation purposes. Regardless of the obtained variability in Rm value, the isozyme phenotype pattern seems to be a stable characteristic of particular species included in the test performance study. The isozyme phenotype patterns enabled species identification of provided samples reaching 85.1% average conformity. Therefore, it can be concluded that isozyme phenotyping remains the most efficient method for the identification of species within tropical RKN group.

A modified DNA barcoding method in which four mtDNA coding regions (Nad2, Nad5, Cox2 and Cox3) were sequenced did not prove successful in identifying all eight selected species of tropical RKN. One hundred percent accurate identification was achieved for *M. incognita* and *M. javanica*, while *M. enterolobii* and *M. arenaria* were correctly identified in most (but not all) of the cases. *M. ethiopica, M. incognita, M. inornata* and *M. hispanica* species could not be identified with the same success rate. The modified DNA barcoding method did not show high inter-laboratory reproducibility. Only five out of 14 samples (36%) were correctly identified by all laboratories (see Table 5 in the Appendix).

Sequencing of additional mtDNA regions (e.g. 16S, Cox1, CytB, Nad1, Nad3) could achieve better resolution and correct species identification, but identification success rate between different laboratories remains to be tested.

Further, quick development of the High-Throughput Sequencing (HTS) approach, where almost complete nuclear genome and complete mtDNA sequences can be determined, bares a promise that other informative regions differentiating between species will be identified and new identification tests could be developed in the near future. More information is available from the Appendices.

2.4.3. Survival ability of *M. incognita* and *M. arenaria* at the open field in continental climate conditions

Both species, *M. incognita* and *M. arenaria*, survived and maintain infection ability in a micro-plot (semi-field) filed with soil for three successive winters from 2017 – 2020 at Ljubljana location (continental conditions).
The results suggest that both species can survive winter period in the open field, especially in southern Europe or in similar climate conditions. More information is available from the Appendices.

2.4.4. Assessment of the effect of climate change scenarios on the tropical RKN species potential spreading in the open field agricultural production areas in Europe

The basic modelling principle was to look for a realistic worst-case scenario of survival of selected tropical RKN species based on nematode species findings in Bilje (location in southwest of Slovenia near Italian border), Montemor-o-Velho in Portugal and from reports in the literature. For each nematode species, 2 maps of Ecoclimatic index (EI) were developed, one based on dataset from 1975 till 2000 and one considering simple climate change scenario with the temperature rise of 2°C.
From modelling results it can be concluded that *M. arenaria* and *M. incognita* threaten the larger area of European territory which appears to be much more endangered under the climate change scenarios considered in this study. The modelling results are presented through Ecoclimatic index (EI) which reflects the suitability of environmental conditions for development and reproduction of certain organism. From all European countries, Cyprus seems to be the most endangered country for tropical RKN species, followed by some regions in Portugal.
To assess the suitability of climate conditions where some of the tropical RKN can survive, project partners compared the climate data of locations where nematodes were already detected at the open field (Bilje as worst-case scenario) to the climate data of European region.
The weights were put only on temperature data. However, such modelling approach should be used only as first indication where similar climate conditions in Europe exist compared to the climate of Bilje. More information is available from the Appendices.

2.5. Conclusions and recommendations to policy makers

Data on distribution of tropical RKN species in partner countries were obtained by sampling of field cultivation and ornamental production and by summing national record data on the occurrence of tropical RKN. In total, Melotrop database contained 107 locations from France, Portugal, Serbia and Slovenia. The most common species was *M. incognita*, recorded at 47 locations. The second most common species was *M. arenaria* at 21 locations, followed by 11 locations of *M. javanica*, 7 locations of *M. hispanica*, 6 locations of *M. luci* and 2 locations of *M. enterolobii*. Mixed populations were also recorded at 13 locations. Fourty-three different host species were recorded. The distribution of tropical RKN species in the open fields was predominantly in areas with Mediterranean climatic conditions. Nevertheless, a small proportion of locations were recorded in areas with semi-continental climatic conditions with

milder winter conditions. Such areas in Europe should be considered as potential spreading areas for these pests and appropriate monitoring programs should be established in the future.

Isozyme phenotyping showed an average agreement of results of 85.1%, while successful identification among laboratories ranged from 58.3 to 100%. Not all species were identified with the same success. The species that were correctly identified 100% of the time in both samples provided were *M. arenaria* and *M. enterolobii.* For *M. hispanica, M. javanica,* and *M. luci,* 90% agreement was achieved, while 80% for *M. incognita,* 70% for *M. ethiopica* and 60% for *M. inornata.* A modified DNA barcoding method, in which four mtDNA coding regions were sequenced, did not prove to be a successful method for identifying all eight selected species. Only five of 14 samples (36%) were correctly identified by all the laboratories involved in the assay. Sequencing of additional mtDNA regions could achieve better resolution and accurate species identification, but the success rate of identification among different laboratories remains to be tested. Regardless of variability achieved in Rm value, the isozyme phenotype pattern appears to be a stable feature of the individual species included in the test performance study. The isozyme phenotype patterns allowed species identification of the provided samples and reached an average agreement of 85.1%. Therefore, it can be concluded that isozyme phenotyping remains the most efficient method for species identification within the tropical RKN group.

The survivability of *M. incognita* and *M. arenaria* in the field under continental climatic conditions was assessed in Slovenia. Both species, *M. incognita* and *M. arenaria*, survived and maintained their infectivity in a soil-filled microplot (semi-field conditions) three consecutive winters at the Ljubljana location, defined as continental conditions. The results suggest that both species pose a serious threat to agricultural production in the field, especially in southern Europe or similar climatic conditions.

CLIMEX software was used to evaluate the potential suitability of the European territory for the survival and development of selected tropical RKN species and to simulate the impact of a possible climate change scenario. From the modelling results, it can be concluded that *M. arenaria* and *M. incognita* threaten the larger area of the European territory, which is much more at risk under the climate change scenario. The modelling results are represented by the ecoclimate index, which reflects the suitability of environmental conditions for the development and reproduction of a given organism. Among all European countries, Cyprus seems to be the most vulnerable country for tropical RKN species, followed by some regions in Portugal and the rest of the Mediterranean countries. Furthermore, modelling results suggest that tropical RKN species could benefit from climate change in the future and move into areas that were unsuitable for their establishment and development in the past. The research consortium recommends the following:

i. Appropriate monitoring programs for tropical RKN should be established in areas of Europe with Mediterranean climatic conditions and semi-continental climatic conditions with milder winter conditions.
ii. Available diagnostic methods for species identification of tropical RKN are unsatisfactory as they do not allow definite and unambiguous species identification with high reliability. Development of new diagnostic approaches for the group of tropical RKN is urgently needed and should be supported.

iii. Training for national reference laboratories (NRL) on existing and new diagnostic methods for tropical RKN identification and the implementation of ring tests could improve NRL efficiency in tropical RKN identification.

2.6. Benefits from trans-national cooperation

The trans-national cooperation enabled a valuable exchange of biological material as the majority of the national reference laboratories do not have available all tropical RKN species in their national collections.

To our knowledge, a test performance study for tropical RKN species identification was conducted for the first time. Two identification methods were tested: isozyme phenotyping based on malate dehydrogenase and esterase phenotypes and barcoding based on four mtDNA genes. Collecting data on the distribution of tropical RKN in different European countries resulted in more robust and accurate input data for modelling potential distribution maps for individual tropical RKN species.

3. Publications

3.1. Article(s) for publication in the EPPO Bulletin

None.

3.2. Article for publication in the EPPO Reporting Service

None.

3.3. Article(s) for publication in other journals

Gerič Stare, Barbara, Strajnar, Polona, Širca, Saša, et al. (2018). **Challenges of the Euphresco project MELOTROP: global warming and distribution of root-knot nematode species of the tropical group.** V: 70th International Symposium on Crop Protection, May 22, 2018, Ghent, Belgium: abstracts, str. 173. http://www.ugent.be/bw/crop-protection/iscp/en/programme. [COBISS.SI-ID 5542504]

3.4. Other planned dissemination activities

- Saša Širca, Barbara Gerič Stare, Anne-Marie Chappe, Fabrice Ollivier, Laurent Folcher, Maria L. Inácio, Filomena Nóbrega, Leidy Rusinque, Eugénia Andrade, Carla Maleita, Isabel Luci Conceição, Isabel Abrantes, Evelyn Y.J. van Heese, Gerrit Karssen, Jasmina Bačič**.** Detection and diagnostics of tropical *Meloidogyne spp.* within the Euphresco project *MeloTrop*. Accepted presentation at the 7th ICN 2020, planned to be held 1-6 May 2022 at the Palais des Congrès in Antibes Juan-Les-Pins (France).
- The Final Report on Melotrop: Global warming and distribution of root-knot nematode species of the tropical group is planned to be published as an extended publication containing all generated data.
- The final results of Melotrop project will be presented in future events such as ESN and ICN Congress.

4. Open Euphresco data

None.

Appendix 1 – Full scientific report

WP 1: Distribution of the tropical RKN species

Participants: KIS, ANSES, INIAV, UC, Institut Tamiš

The objective of this WP was to obtain the data on the tropical RKN distribution for the participating countries. Sampling was performed in the open field crops and ornamental plants. Small proportion of samples was collected also in the protected areas of vegetables production. Additionally, the national record data of the tropical RKN occurrence was added to the project tropical RKN database from KIS, ANSES, INIAV, UC and Institut Tamiš.

In Slovenia, 150 samples (128 soil + 22 plant material) were collected and analysed by KIS. The survey was carried out in the open field crop (115 samples) and protected area greenhouse (35 samples). Nineteen samples tested positive for RKN tropical group. At the open field, *M. incognita* was detected in 11 samples, *M. arenaria* in 6 samples and a mixture of *M. incognita* and *M. arenaria* in one sample. Both species were well established at open field agricultural production in the area close to the Adriatic Sea with the Mediterranean climatic conditions.

In France, 91 samples were collected and analysed by ANSES. The tropical group of RKN was detected in 20 samples, of which 8 were sampled at the open fields. In positive samples, from open fields, *M. arenaria* was detected in 6 samples from areas around the Mediterranean with the influence of the Mediterranean climate and areas with milder winters influenced by an oceanic climate. *Meloidohyne incognita* was detected in two samples collected from areas with milder winters influenced by an oceanic climate.

In Portugal, 546 samples were collected and analysed in the frame of Melotrop and other RKN national surveys and projects. RKN were detected in 115 samples and 48 RKN species from the tropical group were identified at the open field samples. In addition, the national data on tropical RKN species occurrence was added to the Melotrop database. Thus, the open field occurrence database from Portugal contained 79 samples/locations of tropical RKN species.

In Serbia, three samples tested positive on tropical RKN. Two samples were obtained from the open fields, and in both samples *M. arenaria* was detected.

In all partnering countries involved in WP1, *M. hapla* was frequently found in samples from open fields. Several samples tested positive for *Meloidogyne* spp., but the species identification was not done or not possible to perform. These samples were not included into Melotrop open field occurrence database.

The distribution maps of tropical RKN were created (Appendix 2).

WP 2: Validation of biochemical and molecular diagnostic methods for the tropical RKN species

T2.1: Exchange of biological material - collections of live populations of the tropical RKN; Participants: KIS, ANSES, INIAV, NVWA, UC

T2.2: Validation of a biochemical diagnostic method for the tropical RKN; Participants: KIS, ANSES, INIAV, NVWA, UC

T2.3: Validation of molecular diagnostic method for the tropical RKN species; Participants: KIS, ANSES, INIAV, NVWA, UC

T2.1: Exchange of biological material - collections of live populations of the tropical RKN; Participants: KIS, ANSES, INIAV, NVWA, UC

A list of RKN species kept as live populations in the collections of project partners was prepared. RKN species (live populations) from other collaborators were added and provided with authorisation for their use in the frame of the project objectives. The list included 52 RKN isolates/populations of the tropical group species. Eight tropical RKN species were selected (*M. arenaria, M. enterolobii, M. ethiopica, M. hispanica, M. incognita, M. inornata, M. javanica* and *M. luci*), each represented by two different populations. In total 16 RKN populations were selected for the WP2, to be used for testing biochemical (isoenzymes) and molecular diagnostic tests. Exchange of biological material was initiated when project partners sent live material to KIS. The material was re-cultured and multiplied at KIS. All populations were randomly coded as RKN 1 to RKN 16. Thirteen populations reproduced successfully. Two populations (one *M. inornata* and one *M. enterolobii*) needed to be re-cultured for an additional life cycle. One culture of *M. incognita* did not reproduce successfully and was therefore excluded. The material was sent to the partners in September 2018. If the received material was not in good condition, additional samples of certain populations were re-sent on request. Nevertheless, some samples were of poor quality and the analysis was difficult to carry out.

In total, 15 samples of tropical RKN populations, coded as RKN 1 to RKN 15, and a population of *M. javanica* to be used as a reference in isozyme analysis were provided to all partners involved in T2.2 and T2.3.

Table 1: List of tropical root-knot nematode (RKN) populations coded as RKN 1 to RKN 15 used in T2.2 'Validation of a biochemical diagnostic method for the tropical RKN and T2.3 'Validation of molecular diagnostic method for the tropical RKN species.

CODE	***Melodogyne* species**	**Geographical origin**	**Institution collection**	**Identification methods**
RKN 1	*M. arenaria*	Italy	NVWA	Morphology, isozyme profile, sequence data
RKN 2	*M. incognita*	France	ANSES	Morphology, specific PCR
RKN 3	*M. enterolobii*	Senegal	ANSES	MorphologY, specific PCR
RKN 4	*M. ethiopica*	Chile	KIS	Isozyme profile
RKN 5	*M. hispanica*	Portugal	UC	Isozyme profile
RKN 6	*M. ethiopica*	Brasil	UC	Morphology, isozyme, profile, sequence data
RKN 7	*M. arenaria*	Sri Lanka	NVWA	Morphology, isozyme profile, sequence data
RKN 8	*M. inornata*	France	ANSES	Morphology
RKN 9	*M. javanica*	Israel	ANSES	Morphology, isozyme profile, specific PCR
RKN 10	*M. luci*	Slovenia	KIS	Isozyme profile, sequence analysis of mtDNA
RKN 11	*M. inornata*	Chile	KIS	Morphology, isozyme profile, sequence data
RKN 12	*M. luci*	Iran	NVWA	Morphology, isozyme profile, sequence data
RKN 13	*M. javanica*	Portugal	UC	Isozyme profile

RKN 14	*M. enterolobii*	China	NVWA	Morphology, isozyme profile, sequence data
RKN 15	*M. hispanica*	Portugal	UC	Isozyme profile

T2.2: Validation of a biochemical diagnostic method for the tropical RKN;
Participants: KIS, ANSES, INIAV, NVWA, UC
A test performance study was organised to assess the method for isozyme phenotyping of Esterase (EST) and Malate Dehydrogenase (MDH). Laboratories at KIS, ANSES and NVWA performed isozyme phenotyping according to EPPO PM 7/41 standard using PhastSystem (GE Healthcare/Amersham Pharmacia) equipment (Karssen et al., 1995). Laboratories at INIAV and UC performed isozyme phenotyping using an '*in house'* prepared polyacrylamide gel followed by protein electrophoresis. Native polyacrylamide gel electrophoresis (PAGE) was carried out on vertical polyacrylamide gels, 1 mm thick, in a Mini-Protean II (Bio-Rad Laboratories, Hercules, California, USA). The separating gel was 7% polyacrylamide, pH 8.8, while the stacking gel was 3% polyacrylamide, pH 6.8, and Tris-glycine solution pH 8.3 was used as running buffer. The electrophoresis was carried out at 6 mA/gel during the first 10 minutes and then at 20 mA/gel for about 50 minutes or until the bromophenol blue had migrated to the end of the gel.
Enzymatic reaction and staining solutions for EST (EC 3.1.1.1) activity and for MDH (EC 1.1.1.37) was conducted according to EPPO PM 7/41 standard at all participating laboratories. Detailed instructions for isozyme analysis were prepared by KIS and sent to other participating partners.

T2.2.1 Test performance study of isozyme phenotyping
Five laboratories from partnering institutions participated in the test performance study of isozyme phenotyping. The laboratories were encoded as Lab 1 to Lab 5. Lab 1, Lab 2 and Lab 3 were using PhastSystem, while Lab 4 and Lab 5 used PAGE method. RKN samples coded as RKN 1 to RKN 15 were analysed (Table 1) according to the instructions provided. For the tropical RKN *M. arenaria, M. enterolobii, M. ethiopica, M. hispanica, M. inornata, M. javanica* and *M. luci* two populations were tested, while for *M. incognita* only one was tested.

All participating laboratories provided analytical results. The provided material of RKN 7 and RKN 15 was not suitable for analyses in Lab 2, and similarly RKN 14 in Lab 4. Therefore, these results were excluded from the laboratory result analyses. Obtained results from different laboratories and further analysis showed that sample coded as RKN 8 (listed as *M. inornata*) was either identified as *M. arenaria* or *M. inornata* or the population consisted of a mix population of *M. arenaria* and *M. inornata*. Therefore, sample RKN 8 was excluded from the result analysis. In total, 67 isozyme analyses were performed. The results of 57 (85.1%) analyses were in conformance with population identity, while the results of 10 (14.9%) analyses miss-identified provided samples. Success of correct identification for different laboratories was as follows: 100.0% for Lab 1, 58.3% for Lab 2, 85.7% for Lab 3, 92.3% for Lab 4 and 85.7% for Lab 5. The average percentage of results conformability was 85.1% (Table 2). The species that were 100% correctly identified by all participating laboratories and in both provided populations were *M. arenaria* and *M. enterolobii.* For *M. javanica, M. hispanica* and *M. luci* 90% conformity was achieved among laboratories, while 80% conformity was achieved for *M. incognita* samples, 70% for *M. ethiopica* samples and 60% for *M. inornata* samples (Table 3).

Table 2: Results of test performance using isozyme phenotyping analysis of the five laboratories participating in the Melotrop project. Green colour – identity of the provided root-knot nematode population; red colour – incorrect identification; ND – not determined.

Code	*Meloidogyne* species	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Analyzed	Conform	Not-conform	% conform
RKN 1	*M. arenaria*	*M. arenaria*	*M. arenaria*	*M. arenaria*	*M. arenaria*	*M. arenaria*	5	5	0	100.0
RKN 2	*M. incognita*	*M. incognita*	*M. hispanica*	*M. incognita*	*M. incognita*	*M. incognita*	5	4	1	80.0
RKN 3	*M. enterolobii*	*M. enterolobii*	*M. enterolobii*	*M. enterolobii*	*M. enterolobii*	*M. enterolobii*	5	5	0	100.0
RKN 4	*M. ethiopica*	*M. ethiopica*	*M. enterolobii*	*M. inornata*	*M. ethiopica*	*M. inornata*	5	2	3	40.0
RKN 5	*M. hispanica*	*M. hispanica*	*M. enterolobii*	*M. hispanica*	*M. hispanica*	*M. hispanica*	5	4	1	80.0
RKN 6	*M. ethiopica*	*M. ethiopica*	*M. ethiopica*	*M. inornata*	*M. ethiopica*	*M. ethiopica*	5	4	1	80.0
RKN 7	*M. arenaria*	*M. arenaria*	ND	*M. arenaria*	*M. arenaria*	*M. arenaria*	4	4	0	100.0
RKN 9	*M. javanica*	*M. javanica*	*M. luci*	*M. javanica*	*M. javanica*	*M. javanica*	5	4	1	80.0
RKN 10	*M. luci*	*M. luci*	*M. luci*	*M. luci*	*M. luci*	*M. luci*	5	5	0	100.0
RKN 11	*M. inornata*	*M. inornata*	*M. inornata*	*M. inornata*	*M. ethiopica*	*M. ethiopica*	5	3	2	60.0
RKN 12	*M. luci*	*M. luci*	*M. ethiopica*	*M. luci*	*M. luci*	*M. luci*	5	4	1	80.0
RKN 13	*M. javanica*	*M. javanica*	*M. javanica*	*M. javanica*	*M. javanica*	*M. javanica*	5	5	0	100.0
RKN 14	*M. enterolobii*	*M. enterolobii*	*M. enterolobii*	*M. enterolobii*	ND	*M. enterolobii*	4	4	0	100.0
RKN 15	*M. hispanica*	*M. hispanica*	ND	*M. hispanica*	*M. hispanica*	*M. hispanica*	4	4	0	100.0
Analyzed		14	12	14	13	14	67	57	10	**85.1** (average)
Conform		14	7	12	12	12				
Not-conform		0	5	2	1	2				
% conform		100.0	58.3	85.7	92.3	85.7				

Table 3: Concordance of species identification of provided samples. Percentages are calculated as average of two tested populations per laboratory and five participating laboratories, except for *Meloidogyne incognita* and *M. inornata* where the result of only one population/species was available – designated with *.

Meloidogyne species	Results concordance (%)
M. arenaria	100
M. enterolobii	100
M. hispanica	90
M. javanica	90
M. luci	90
*M. incognita**	80
M. ethiopica	70
*M. inornata**	60

The relative migration rate (Rm) was calculated as measured length from the start point (sample loading) to the middle of the isozyme band, as described in the instructions provided to participating laboratories. All laboratories provided the results for all analysed samples (Table 4). Rm analysis revealed variability of Rm values for the same species between different laboratories. Variability was also observed when Rm values were compared between laboratories using the same method (PhastSystem or PAGE) and for both analysed isozymes (EST and MDH) (Table 4, Figures 1, 2). Furthermore, the variability of Rm was also detected in the reference population, *M. javanica,* when comparing results of different analyses (runs) in the same laboratory/method.

Table 4: Relative migration (Rm) rates of esterase (EST) and malate dehydrogenase (MDH) phenotypes of all analysed *Meloidogyne* populations of the test performance study from the five partner laboratories (Lab 1 – Lab 5). *M. javanica* was included in each gel as reference population. ND – not determined.

	Lab 1		Lab 2		Lab 3		Lab 4		Lab 5	
	RKN 1	*M. arenaria*								
	RKN 1	*M. javanica*	RKN 1	*M. javanica*	RKN 1	*M. javanica*	RKN 1	*M. javanica*	RKN 1	*M. javanica*
EST	1.06	1.00	1.12	1.00	1.07	1.00	1.18	1.00	1.16	1.00
	1.10	1.10	1.18	1.09	1.10	1.14	1.27	1.22	1.22	1.16
	1.14	1.15		1.16	1.18	1.20		1.31		1.29
MDH	0.51	0.50	0.50	0.50	0.52	0.50	0.50	0.50	0.50	0.50
									0.56	
									0.66	
	RKN 2	*M. incognita*								
	RKN 2	*M. javanica*	RKN 2	*M. javanica*	RKN 2	*M. javanica*	RKN 2	*M. javanica*	RKN 2	*M. javanica*
EST	1.02	1.16	1.03	1.00	1.01	1.00	1.00	1.00	1.04	1.00
		1.11	1.09	1.09		1.14	1.08	1.18	1.11	1.20
		1.00		1.16		1.20		1.29		1.33
MDH	0.51	0.50	0.51	0.50	0.49	0.50	0.50	0.50	0.50	0.50
									0.65	
	RKN 3	*M. enterolobii*								
	RKN 3	*M. javanica*	RKN 3	*M. javanica*	RKN 3	*M. javanica*	RKN 3	*M. javanica*	RKN 3	*M. javanica*
EST	1.00	1.00	0.88	1.00	0.98	1.00	0.73	1.00	0.75	1.00
	0.88	1.11	1.02	1.12	1.13	1.13	0.93	1.17	0.92	1.14

		1.16		1.18		1.20		1.27		1.25
MDH	0.64	0.50	ND	0.50	0.72	0.50	0.70	0.50	0.69	0.50
	RKN 4	*M. ethiopica*								
	RKN 4	*M. javanica*	RKN 4	*M. javanica*	RKN 4	*M. javanica*	RKN 4	*M. javanica*	RKN 4	*M. javanica*
EST	0.99	1.00	0.81	1.00	0.93	1.00	0.93	1.00	0.88	1.00
	1.07	1.09	0.96	1.12	1.07	1.13	1.12	1.20	1.07	1.14
	1.14	1.14		1.18	1.15	1.20	1.22	1.29	1.18	1.25
MDH	0.49	0.50	ND	0.50	0.51	0.50	0.50	0.50	0.50	0.50
	RKN 5	*M. hispanica*								
	RKN 5	*M. javanica*	RKN 5	*M. javanica*	RKN 5	*M. javanica*	RKN 5	*M. javanica*	RKN 5	*M. javanica*
EST	0.84	1.00	0.85	1.00	0.82	1.00	0.87	1.00	0.85	1.00
	0.92	1.10	0.98	1.09	0.91	1.14	0.96	1.21	0.92	1.14
	0.96	1.15		1.16	0.97	1.21	1.04	1.32	1.00	1.25
MDH	0.51	0.50	0.47	0.50	0.57	0.50	0.50	0.50	0.50	0.50
	RKN 6	*M. ethiopica*								
	RKN 6	*M. javanica*	RKN 6	*M. javanica*	RKN 6	*M. javanica*	RKN 6	*M. javanica*	RKN 6	*M. javanica*
EST	0.99	1.00	1.05	1.00	1.01	1.00	0.92	1.00	0.96	1.00
	1.08	1.08	1.14	1.09	1.13	1.14	1.08	1.16	1.13	1.16
	1.14	1.13	1.21	1.16	1.22	1.21	1.20	1.27	1.23	1.26
MDH	0.52	0.50	0.52	0.50	0.52	0.50	0.50	0.50	0.50	0.50
	RKN 7	*M. arenaria*								
	RKN 7	*M. javanica*	RKN 7	*M. javanica*	RKN 7	*M. javanica*	RKN 7	*M. javanica*	RKN 7	*M. javanica*
EST	1.17	1.00	1.07	1.00	1.21	1.00	1.14	1.00	1.17	1.00
	1.12	1.09	1.12	1.08	1.30	1.16	1.22	1.16	1.24	1.17
		1.17		1.14		1.24	1.29	1.27		1.27
MDH	0.52	0.50	ND	0.50	0.56	0.50	0.50	0.50	0.50	0.50
	0.59				0.68		0.59		0.60	
	0.65				0.79		0.71		0.69	
	RKN 9	*M. javanica*								
	RKN 9	*M. javanica*	RKN 9	*M. javanica*	RKN 9	*M. javanica*	RKN 9	*M. javanica*	RKN 9	*M. javanica*
EST	1.01	1.00	0.88	1.00	0.99	1.00	1.00	1.00	1.00	1.00
	1.12	1.11	1.11	1.10	1.12	1.13	1.21	1.21	1.17	1.17
	1.18	1.16	1.18	1.18	1.19	1.20	1.32	1.32	1.29	1.29
			1.24							
MDH	0.51	0.50	0.51	0.50	0.53	0.50	0.50	0.50	0.50	0.50
	RKN 10	*M. luci*								
	RKN 10	*M. javanica*	RKN 10	*M. javanica*	RKN 10	*M. javanica*	RKN 10	*M. javanica*	RKN 10	*M. javanica*
EST	1.00	1.00	0.84	1.00	1.00	1.00	1.000	1.00	1.00	1.00
	1.06	1.10	1.02	1.10	1.07	1.13	1.10	1.19	1.10	1.16
	1.12	1.15	1.05	1.18	1.16	1.20	1.23	1.29	1.23	1.26
			1.14							
MDH	0.51	0.50	0.53	0.50	0.53	0.50	0.50	0.50	0.50	0.50
	RKN 11	*M. inornata*								
	RKN 11	*M. javanica*	RKN 11	*M. javanica*	RKN 11	*M. javanica*	RKN 11	*M. javanica*	RKN 11	*M. javanica*
EST	0.96	1.00	1.00	1.00	1.03	1.00	0.94	1.00	0.96	1.00
	1.05	1.08	1.09	1.09	1.17	1.16	1.13	1.17	1.10	1.16
	1.11	1.13	1.17	1.16	1.28	1.24	1.23	1.30	1.20	1.26

MDH	0.51	0.50	0.43	0.50	0.59	0.50	0.50	0.50	0.50	0.50
	RKN 12	*M. luci*								
	RKN 12	*M. javanica*	RKN 12	*M. javanica*	RKN 12	*M. javanica*	RKN 12	*M. javanica*	RKN 12	*M. javanica*
EST	1.01	1.00	1.00	1.00	0.98	1.00	1.00	1.00	1.00	1.00
	1.06	1.08	1.08	1.08	1.07	1.16	1.13	1.19	1.10	1.16
	1.10	1.14	1.14	1.15	1.18	1.24	1.26	1.30	1.20	1.26
MDH	0.51	0.50	ND	0.50	0.53	0.50	0.50	0.50	0.50	0.50
	RKN 13	*M. javanica*								
	RKN 13	*M. javanica*	RKN 13	*M. javanica*	RKN 13	*M. javanica*	RKN 13	*M. javanica*	RKN 13	*M. javanica*
EST	1.02	1.00	1.02	1.00	1.06	1.00	1.00	1.00	1.00	1.00
	1.10	1.08	1.09	1.08	1.19	1.13	1.19	1.19	1.19	1.19
	1.14	1.14	1.16	1.15	1.25	1.20	1.28	1.28	1.30	1.30
MDH	0.51	0.50	0.51	0.50	0.57	0.50	0.50	0.50	0.50	0.50
	RKN 14	*M. enterolobii*								
	RKN 14	*M. javanica*	RKN 14	*M. javanica*	RKN 14	*M. javanica*	RKN 14	*M. javanica*	RKN 14	*M. javanica*
EST	0.93	1.00	0.87	1.00	0.86	1.00	0.95	1.00	0.76	1.00
	1.00	1.09	0.92	1.10	1.00	1.13	1.02	1.18	0.96	1.16
		1.14	0.96	1.14		1.20		1.27		1.26
			1.03							
MDH	0.58	0.50	0.61	0.50	0.76	0.50	0.68	0.50	0.69	0.50
	RKN 15	*M. hispanica*								
	RKN 15	*M. javanica*	RKN 15	*M. javanica*	RKN 15	*M. javanica*	RKN 15	*M. javanica*	RKN 15	*M. javanica*
EST	0.81	1.00	1.04	1.00	0.84	1.00	0.86	1.00	0.86	1.00
	0.90	1.12		1.10	0.91	1.14	0.93	1.16	0.93	1.16
	0.94	1.17		1.14	0.98	1.22	1.02	1.26	1.03	1.26
MDH	0.51	0.50	ND	0.50	0.49	0.50	0.50	0.50	0.53	0.50

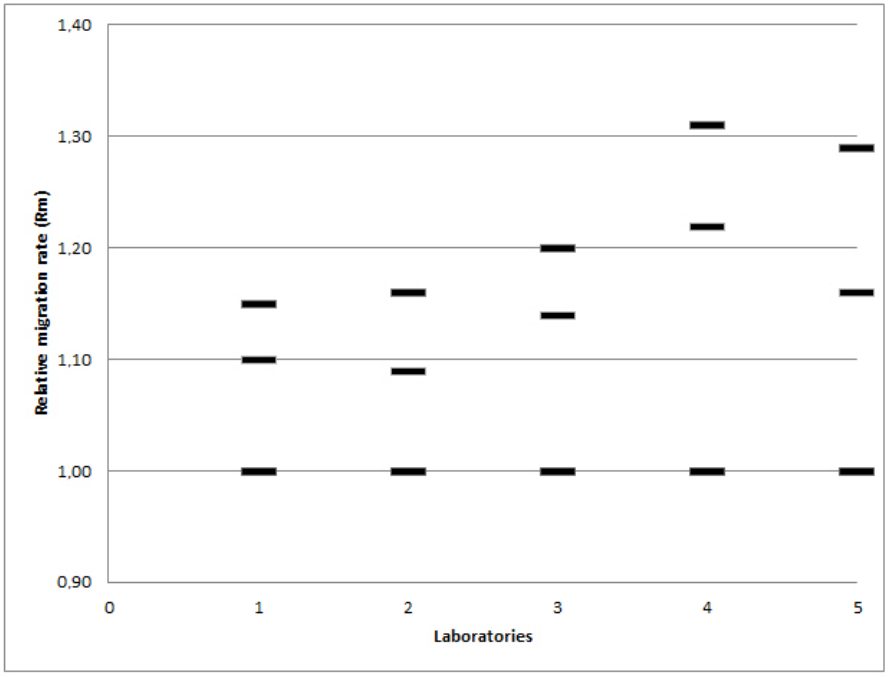

Figure 1: Comparison of relative migration (Rm) rates of the esterase (EST) phenotype for the reference species *Meloidogyne javanica,* from the five laboratories (1 – 5). Band 1 (lower) represents migration rate=1, migrations of band 2 (middle) and band 3 (upper) are calculated as relative migration compared to band 1.

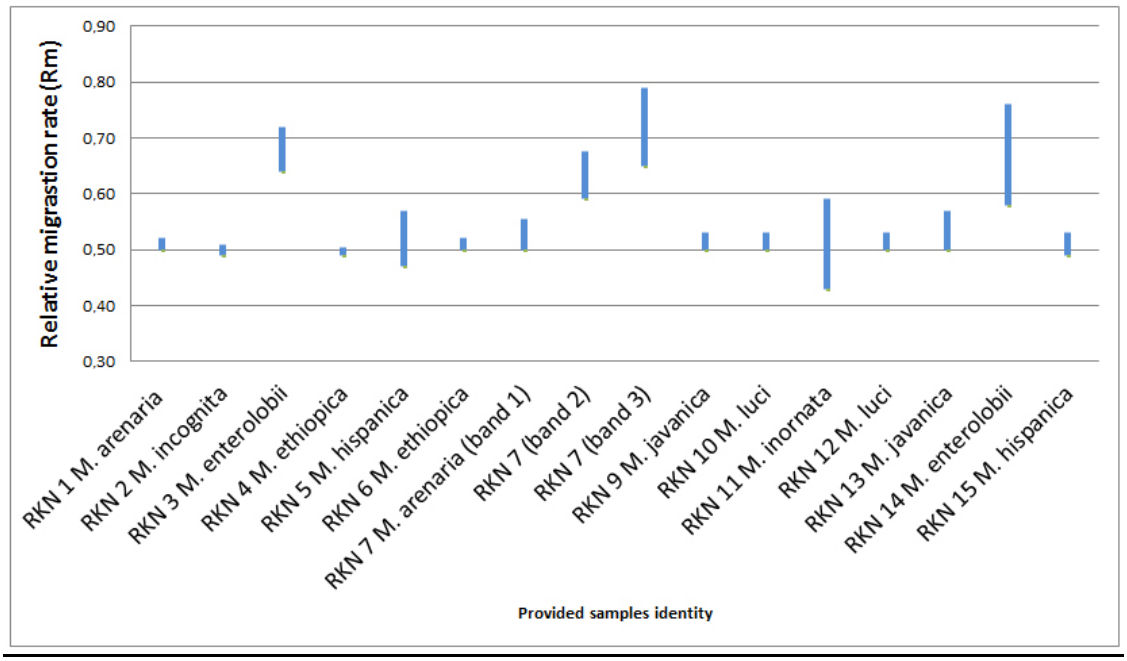

Figure 2: Variation in relative migration (Rm) rates of malate dehydrogenase (MDH) phenotypes for all *Meloidogyne* populations included in the study (see Table 1). Bars represent the variation in Rm of the same sample obtained from different laboratories.

2.3: Validation of molecular diagnostic method for the tropical RKN species;

Participants: KIS, ANSES, INIAV, NVWA, UC

T2.3.1 Literature review of available methods, laboratories' experience with accuracy of these methods and selection of a method to be tested in four participating laboratories.

Literature was reviewed for available molecular methods for tropical RKN species (= clade I species in genus *Meloidogyne*). Thirty-two articles describing molecular methods for tropical RKN species were found from the period from 1993 till 2017. Experience of participating laboratories with tested methods was exchanged.

Many species-specific primers have been developed for tropical RKN and some of them seem to be more reliable and robust than others. While species-specific primers are developed for the most common RKN species, they are not available or are not working reliably for many other 'minor' species. Similar is true for PCR-RFLP approach; while it seems to work for identification of some species, the approach does not enable identification of all or at least the majority of the tropical RKN species. Further, sequencing of different regions has also been tested. Sequences of ITS, SSU and LSU of the rDNA regions are not appropriate markers for studying relationships among the tropical group of RKN as genetic variability is greater within the species than between species.

The approach from Janssen et al. (2016, Sci Rep 6: 22591 DOI: 10.1038/srep22591), based on sequencing of several mtDNA coding regions seemed to be the most promising for differentiation of tropical RKN species.

A general approach enabling identification of any tropical RKN species would be a big step forward from using species-specific primers allowing only for identification or elimination of single species with one PCR reaction. On the other hand, DNA barcoding does not provide a yes/no answer but does help to identify unforeseen plant threats or unknown lineages/ species.

The project consortium selected the Janssen et al. (2016, Sci Rep 6: 22591 DOI: 10.1038/srep22591) DNA barcoding for testing in participating laboratories and modified it in a way to test if sequencing of four mtDNA coding regions (Nad2, Nad5, Cox2 and Cox3) instead of six would be sufficient for tropical RKN species identification. Selected DNA barcoding method based on the referred regions was tested to see whether correct identification is possible and whether the results can be reproducible among laboratories.

T2.3.2 Testing of selected modified DNA barcoding method for molecular identification of tropical RKN

Instructions for molecular identification with modified DNA barcoding method were prepared by Barbara Gerič Stare (KIS) and Evelyn van Heese (NVWA) and distributed to all participants. All participants of T.2.3 have received biological material of the 15 selected live tropical RKN populations to be tested from KIS via express mail (same material as for T2.2; Table 3). Selected RKN populations belonged to the following eight species: *M. arenaria, M. enterolobii, M. ethiopica, M. hispanica, M. incognita, M. inornata, M. javanica* and *M. luci.*

Partners were instructed to use at least eight populations (RKN 1, RKN 2, RKN 3, RKN 4, RKN 5, RKN 9, RKN 11, RKN 12; Table 1) in the testing of the barcoding method for identification of tropical RKN species. Laboratories with sufficient resources were urged to perform the molecular testing on the 15 populations (RKN 1 – RKN 15). Two laboratories tested all populations and three laboratories tested eight populations, where Lab 4 and Lab 5 tested the eight populations specified in the instructions, while the Lab 3 tested the first eight populations (RKN1-RKN8) (Table 5). As explained in T2.2.1, the sample encoded RKN 8 proved to be unsuitable for test performance study and it was excluded from the result analyses.

Partners used different methods of DNA extraction (lysis buffer and proteinase K; Wizard Genomics DNA Purification Kit by Promega; DNA extraction kit for individual nematodes by ClearDetection; Dneasy® Blood & Tissue Kit by Qiagen (the latter by two laboratories), different Taq enzyme in the

PCR reactions GoTaq G2 Flexi polymerase by Promega; Phusion® High-Fidelity DNA Polymerase by New England Biolabs (by two laboratories); Taq DNA polymerase by Bioline; Supreme NZT Taq II 2x green master mix by Nzytech supreme), different Sanger sequencing reaction kit (Genewiz; BigDye® Terminator v1.1 Cycle Sequencing Kit by Life Technologies; BigDye™ Terminator v3.1 Cycle Sequencing Kit and BigDye XTerminator™ Purification Kit, both by Applied Biosystems or, commercial service) and different computer software for sequence analysis (Geneious R11; Geneious v.11.1.2; Geneious R10; BioEdit; CLC Sequence View).

Overall, correct species identification was achieved for 25 out of 51 tests (49%) with the modified DNA barcoding method. Success of correct identification for individual laboratories ranged from 36, over 43, 50 (two participants) to 75% among the five participants. All laboratories accurately identified *M. incognita* and *M. javanica*, while *M. enterolobii* and *M. arenaria* were correctly identified in the majority of cases. Closely related species *M. ethiopica, M. luci* and *M. inornata* also referred to as the *M. ethiopica* group (MEG) could not be correctly identified to the species level, but were designated to the MEG group in 11 out of 18 tests, which represents a 61% success in designating the correct subgroup within the group of tropical RKN. *M. hispanica* was correctly identified only by Lab 3 (Table 5).

Table 5: Tropical root-knot nematodes species (*Meloidogyne* spp.) identification by molecular method based on DNA barcoding in five partner laboratories. ID – identification; Y – yes; N – no.

Sample Code	True ID	Lab1		Lab 2		Lab 3		Lab 4		Lab 5	
		ID based on DNA barcoding	Correct ID	ID based on DNA barcoding	Correct ID	ID based on DNA barcoding	Correct ID	ID based on DNA barcoding	Correct ID	ID based on DNA barcoding	Correct ID
RKN 1	*M. arenaria*	*M.arenaria/incognita/javanica*	N	*M. arenaria*	Y	*M. arenaria*	Y	*M. arenaria*	Y	*M. arenaria*	Y
RKN 2	*M. incognita*	*M. incognita*	Y	*M. incognita*	Y	*M. incognia*	Y	*M. incognita*	Y	*M. incognita*	Y
RKN 3	*M. enterolobii*	*M. enterolobii*	Y	Not possible	N	*M. enterolobii*	Y	*M. enterolobii*	Y	*M. enterolobii*	Y
RKN 4	*M. ethiopica*	*M. luci/inornata*	N	*M. luci*	N	Not possible	N	*M. luci/ethiopica/inornata*	N	Inconclusive	N
RKN 5	*M. hispanica*	*M.arenaria/incognita/javanica*	N	*M. arenaria*	N	*M. hispanica*	Y	*Meloidogyne* sp.	N	Inconclusive	N
RKN 6	*M. ethiopica*	*M. luci/inornata*	N	*M. luci/inornata/ethiopica*	N	Not possible	N				
RKN 7	*M. arenaria*	*M. arenaria*	Y	*M. javanica???*	N	*M. arenaria*	Y				
RKN 9	*M. javanica*	*M. javanica*	Y	*M. javanica*	Y	*M. javanica*	Y	*M. javanica*	Y	*M. javanica*	Y
RKN 10	*M. luci*	*M. incognita*	N	*M. inornata/luci/ethiopica*	N	Not possible					
RKN 11	*M. inornata*	*M. luci/inornata*	N	*M. inornata/luci/ethiopica*	N	Not possible		*M. luci/ethiopica/inornata*	N	Inconclusive	N
RKN 12	*M. luci*	*M. luci/inornata*	N	*M. luci/inornata?*	N	Not possible		*M. luci/ethiopica/inornata*	N	Inconclusive	N
RKN 13	*M. javanica*	*M. javanica*	Y	*M. javanica*	Y	Not possible					
RKN 14	*M. enterolobii*	*M. enterolobii*	Y	*M. enterolobii*	Y	Not possible					
RKN 15	*M. hispanica*	*M.arenaria/incognita/javanica*	N	*M. arenaria*	N	Not possible					

WP 3: Survival ability of *M. incognita* and *M. arenaria* at the open field in continental climate conditions

Participant: KIS

Objective of this WP is to assess the ability of survival of two tropical RKN, *M. incognita* and *M. arenaria,* in continental and Mediterranean climate conditions. Because both species were found infesting crops in the fields at Bilje (Mediterranean climate), the assay was performed only in Ljubljana (continental climate) and was set up in a micro-plot (semi-field) of 1 m^2, filed with soil at Ljubljana location. In May 2017, four tomato plants were planted separately into two microplots and inoculated with eggs of *M. incognita* and *M. arenaria*. In 2018 and 2019, tomato seedlings were re-planted. Every year, in autumn, the tomato stems were cut, while the infected roots were left in the soil. The survival of species was examined in the autumn 2018, 2019 and 2020, by checking the infection on tomato roots (Figure 3). Re-identification of the species was made at the end in 2020 on freshly isolated females with isozyme phenotyping using PhastSystem gel electrophoresis.

Both species survived and maintained infection ability in Ljubljana climate conditions. Temperature conditions at location Ljubljana Bežigrad in winter month from 2017 – 2020 are presented in Table 6. The results suggest that both species can survive winter period and, therefore, represent a serious risk for agriculture production in the open field, especially in southern Europe or similar climate conditions.

Figure 3: Tomato roots from micro-plot infested with *Meloidogyne incognita*, examined in autumn 2020.

Table 6: Air temperature conditions at location Ljubljana Bežigrad in winter month from 2017 – 2020 measured at 2 m. Temperatures are presented as means of average daily temperatures and minimum average daily temperatures in a decade.

Year	Month	Decade	Mean Decade Temp (°C)	Minimum Daily Temp (°C)
2017	Nov	I	9,1	5,9
		II	4,6	2,6
		III	4,9	1,3
2017	Dec	I	0,7	-2,4
		II	2,8	0,3
		III	2,0	-1,0
2018	Jan	I	5,9	3,8
		II	3,4	0,8
		III	5,1	0,7
2018	Feb	I	1,8	0,6
		II	0,6	-2,2
		III	-3,4	-5,1
2018	Mar	I	1,7	-1,4
		II	5,3	3,0
		III	6,5	2,6
2018	Nov	I	12,9	11,1
		II	8,0	6,4
		III	4,0	2,5
2018	Dec	I	4,7	1,8
		II	-0,1	-2,5
		III	2,0	-1,0
2019	Jan	I	0,2	-2,9
		II	2,7	-0,8
		III	-0,6	-2,7
2019	Feb	I	3,5	-0,1
		II	4,9	-1,3
		III	6,8	0,0
2019	Mar	I	10,4	4,7
		II	7,2	2,5
		III	9,4	2,2
2019	Nov	I	9,8	7,5
		II	8,5	6,8
		III	8,0	6,2
2019	Dec	I	2,5	-0,3
		II	5,4	2,5
		III	3,0	-0,3
2020	Jan	I	-0,3	-4,1
		II	2,2	-1,5
		III	3,7	0,0
2020	Feb	I	6,6	2,7
		II	7,2	2,9
		III	6,7	1,6
2020	Mar	I	6,5	2,8
		II	10,1	3,3
		III	5,2	1,3

WP 4: Assessment of the effect of climate change scenarios on the tropical RKN species potential spreading in the open field agricultural production areas in Europe

Participants: KIS, INIAV, ANSES, UC, Institut Tamiš

T4.1 – Geographical maps of possible open field spreading for tropical RKN species
T4.2 – Simulations of different climate change scenarios

CLIMEX software was used for assess of the potential suitability of European territory for survival and development of selected tropical RKN. In modeling process we included *M. arenaria*, *M. hispanica*, *M. incognita*, *M. javanica* and *M. luci*. Inside Climex we used module "Compare locations (1 species)". Although some data of stress conditions were not available, like determination of wet and dry stress, from the literature tried to obtain useable modelling results. We used relevant data from each nematode reproductive curve and climate data from locations Bilje (Slovenia) and Montemor-o-Velho (Portugal), where some of the RKN species were found in the nature or were survived in the trials on the open field. We used Climond climate dataset at 10' resolution. Climate data serves as a realistic worst case scenario for minimum temperature requirements for particular nematode species survival. To follow worst case scenario we used two consecutive average weekly minimum temperature in winter period of those years when nematodes were survived outside at Bilje (Table 7) or 75th percentile of long term monthly minimum winter temperature average at Montemor-o-Velho (Table 7). Two different rates were used for cold stress accumulation (THCS). For *M. arenaria*, *M. incognita* and *M. luci*, which were found on open field or survived in pot experiment in Bilje, we set THCS value to -0.001. First simulation with THCS value from Climex template for *M. javanica* and *M. hispanica* in Mediterranean region seems to be too strict so we decreased THCS to -0.002. Actually, we adjust also other parameters related to cold stress – TTCS, DHCS, DVCS to be able to produce a reasonable output.

Having in mind that data of reproductive curve were obtained in growth chambers where reasonable estimation could be made that temperature in the substrate and air temperature in growth chamber are equal. We have looked for simple regression equation to be able to transform the air temperature to the soil temperature at certain depth and obtain more realistic modelling results. Best regressions of air to soil temperature transformation are rarely linear and depend from soil type and from other climate parameters. However, it was decided to took the linear regression equation with good correlation (r=0.93) for the upper 5 cm which serves well to estimate differences in nematodes growth using soil temperature.

Basic parameters used for Climex modelling are listed in Table 1 and the influence of possible climate change scenario was also simulated. We modified +3 degrees scenario that is one of default scenarios in Climex model in a way to change the constant rise of temperature on plus 2°C in winter and in summer period. Nevertheless, due to the lack of relevant data in the modelling process we didn't include several parameters like wet and dry stress, and their combinations with other stress parameters.

Table 7: Basic parameters* used for modelling of each nematode species (DV0 to DV3 is soil temperature on 5 cm depth)

Nematode species	DV0	DV1	DV2	DV3	TTCS	THCS	PDD
M arenaria	14.6	18.2	30.8	33	-0.5	-0.001	287
M. incognita	12.9	17.3	30.8	33.5	-0.5	-0.001	336
M. hispanica	13.2	18.2	33.5	35.3	3.5	-0.002	475
M. javanica	15.6	18.2	35.5	38	3.5	-0.002	322
M. luci	12	17.3	33.5	35.3	-0.5	-0.001	560

*

DV0= No population growth takes place at or below this average weekly temperature.
DV1= The lower limit of the range of ideal temperatures for population growth.
DV2= The upper limit of the range of ideal temperatures for population growth.
DV3= No population growth takes place when the average weekly maximum temperature equals or exceeds this value.
TTCS= The parameter represents the average weekly minimum temperature below which Cold Stress accumulates.
THCS= The rate at which Cold Stress accumulates once temperatures drop below the threshold value of TTCS.
PDD= Minimum degree-days above DV0 necessary to complete a generation.

http://meteo.arso.gov.si/met/sl/archive/
https://weatherspark.com/m/32306/2/Average-Weather-in-February-in-Montemor-o-Velho-Portugal#Sections-Temperature

Modelling results
The basic modelling principle is to look for a realistic worst case scenario of survival of selected tropical RKN species based on nematodes species findings in Bilje (location in southwest of Slovenia near Italian border), Montemor-o-Velho (Portugal) and from reports in the literature. For each nematode species, 2 maps of Ecoclimatic index (EI) were prepare, one based on dataset from 1975 till 2000 and one considering simple climate change scenario with the temperature rise of 2°C.
From modelling results we can conclude that *M. arenaria* and *M. incognita* threaten the larger area of European territory, which was much more endangered under the climate change scenario. The modelling results are presented through EI, which reflects suitability of environmental conditions for development and reproduction of certain organism. From all European countries, Cyprus seems to be the most endangered country for tropical RKN species, followed by some regions in Portugal and rest of the Mediterranean countries.

One of the possible illustrations, where some of the tropical RKN could survive, is to compare the climate data of location where nematodes were found (Bilje as worst case scenario) with the climate data of the European region. We put weights only on temperature data. However, such modelling approach should be used only as first indication where similar climate conditions in Europe exist compared to the climate of Bilje (Figure 14).

CLIMEX model to assess the potential suitability of European territory for survival and development of selected tropical RKN species of *Meloidogyne* genus. The images represent both the potential suitability for survival and spread of a particular RKN species expressed as calculated EI. Areas colored in blue mark suitable environment for particular species to survive at the open field conditions. Areas colored in red mark suitable environment where particular species is expected to survive and generate several reproduction cycles at the open field conditions.

Meloidogyne arenaria

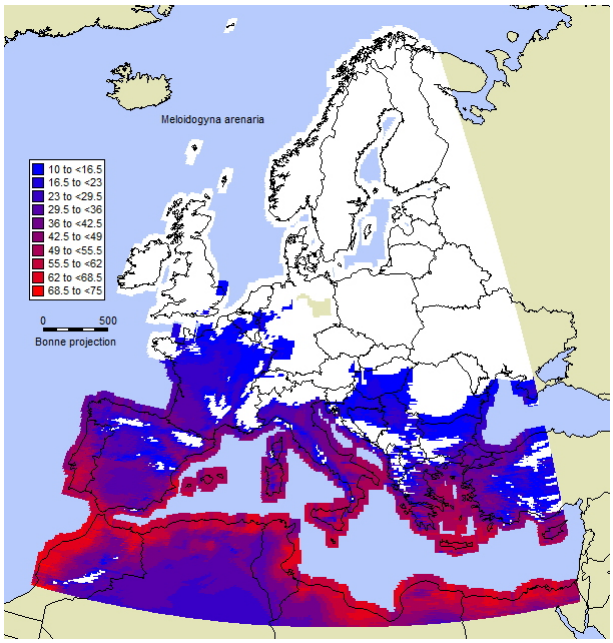

Figure 4: Potential suitability for survival and development of *Meloidogyne arenaria* expressed with EI (ecoclimatic index) (generations range = 1 to 5 for European region).

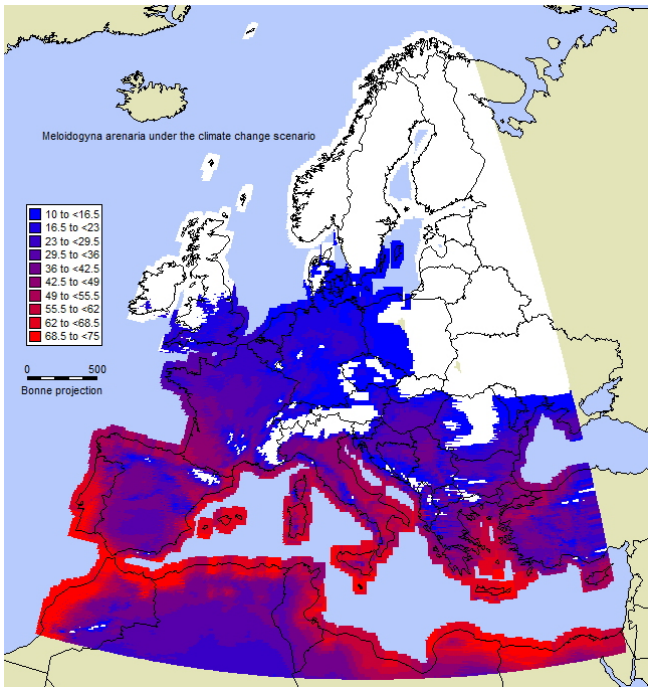

Figure 5: Potential suitability for survival and development of *Meloidogyne arenaria* expressed with EI (ecoclimatic index) under the climate change scenario (+2°C, generations range = 1 to 8 for European region).

Meloidogyne incognita

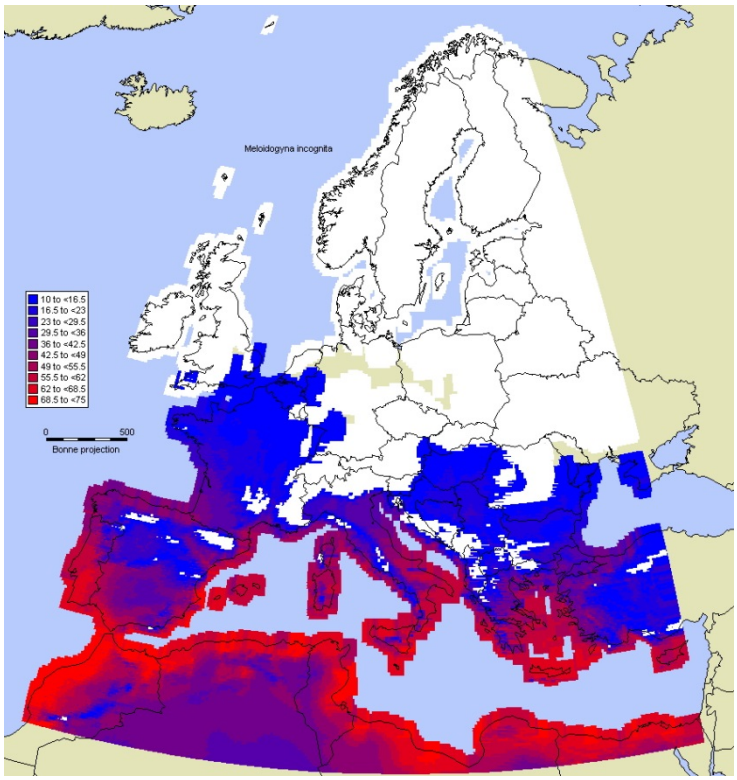

Figure 6: Potential suitability for survival and development of *Meloidogyne incognita* expressed with EI (ecoclimatic index) (generations range = 1 to 7 for European region).

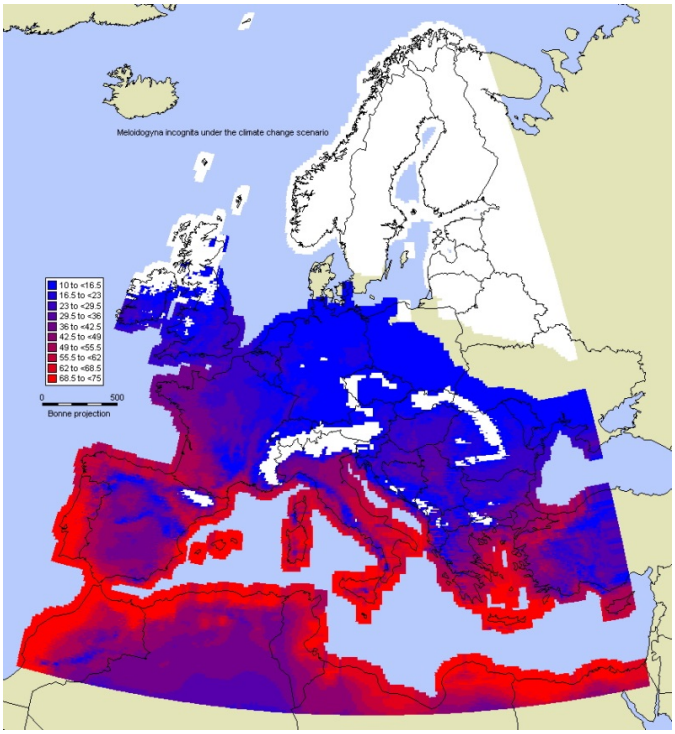

Figure 7: Potential suitability for survival and development of *Meloidogyne incognita* expressed with EI (ecoclimatic index) under the climate change scenario (generations range = 1 to 8 for European region).

Meloidogyne hispanica

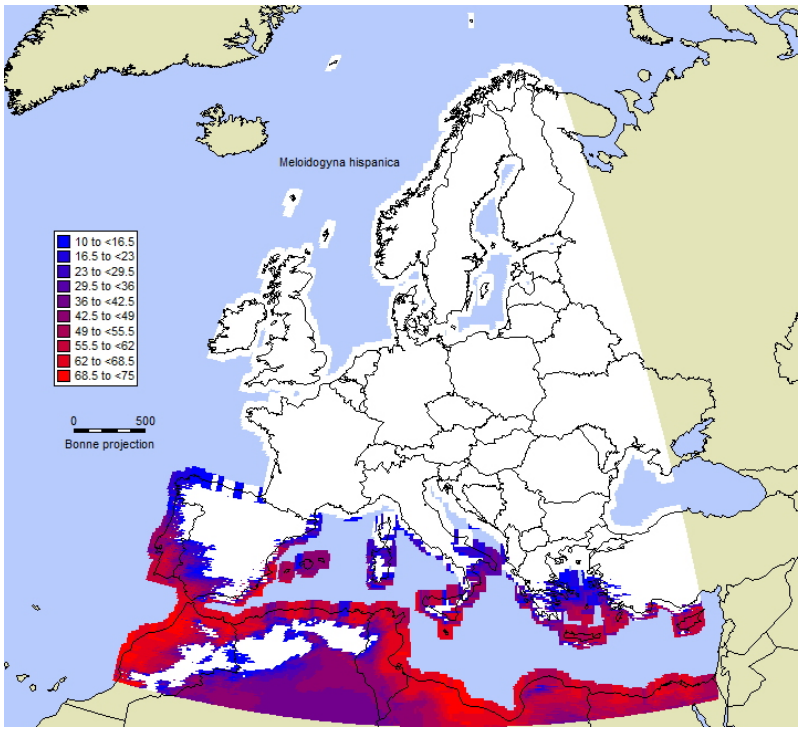

Figure 8: Potential suitability for survival and development of *Meloidogyne hispanica* expressed with EI (ecoclimatic index) (generations range = 1 to 5 for European region).

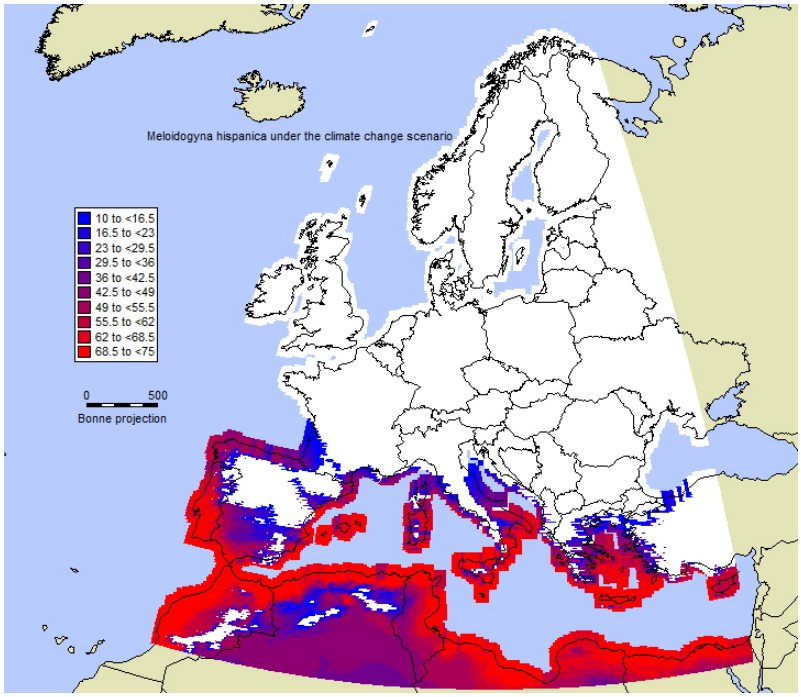

Figure 9: Potential suitability for survival and development of *Meloidogyne hispanica* expressed with EI (ecoclimatic index) under climate change scenario (generations range = 1 to 6 for European region).

Meloidogyne javanica

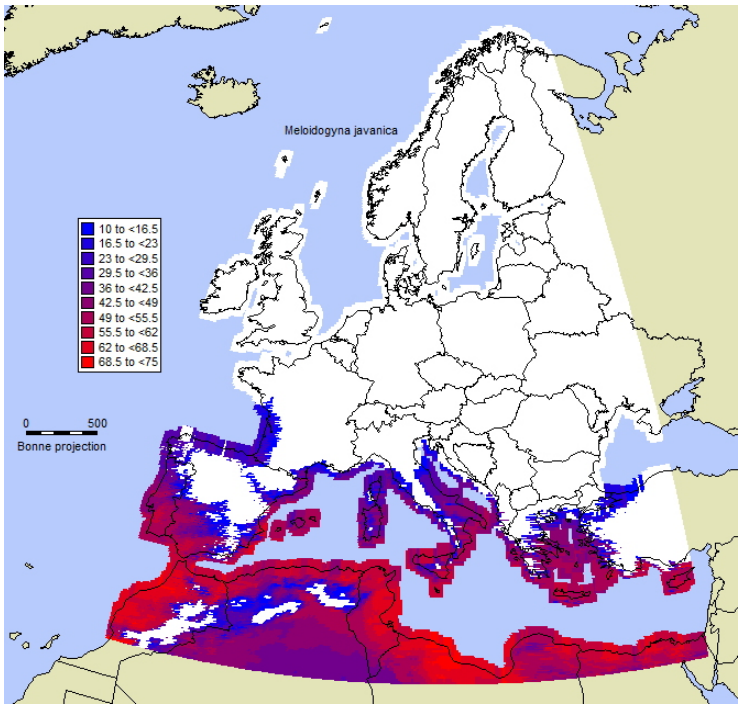

Figure 10: Potential suitability for survival and development of *Meloidogyne javanica* expressed with EI (ecoclimatic index) (generations range = 1 to 5 for European region).

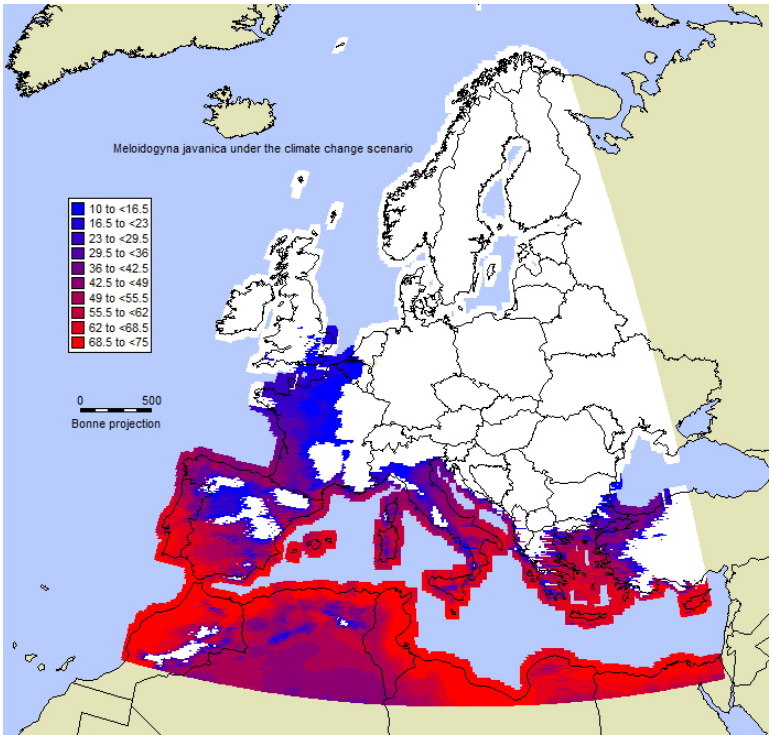

Figure 11: Potential suitability for survival and development of *Meloidogyne javanica* expressed with EI (ecoclimatic index) under the climate change scenario (generations range = 1 to 6 for European region).

Meloidogyne luci

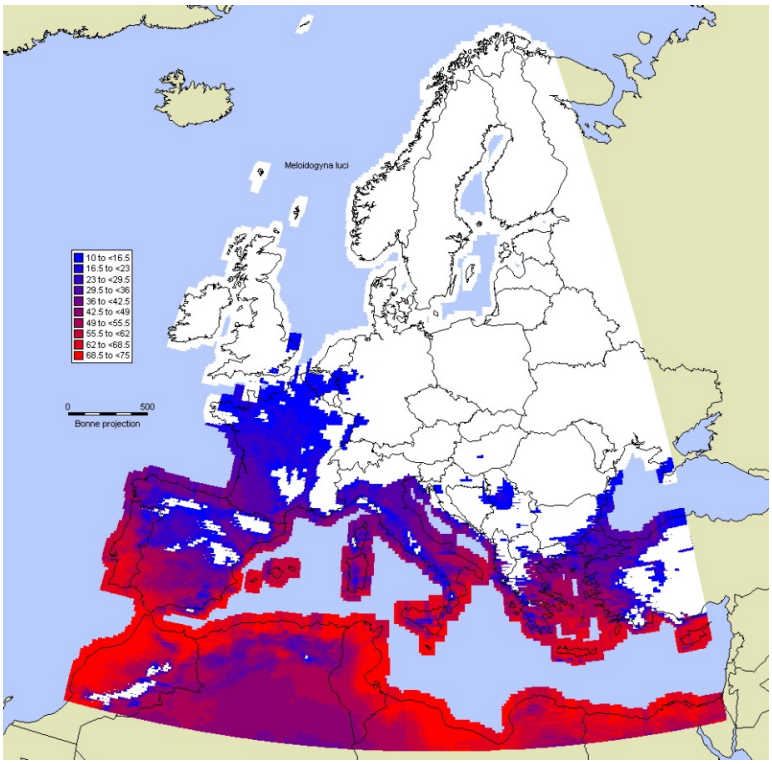

Figure 12: Potential suitability for survival and development of *Meloidogyne luci* expressed with EI (ecoclimatic index) (generations range = 1 to 4 for European region).

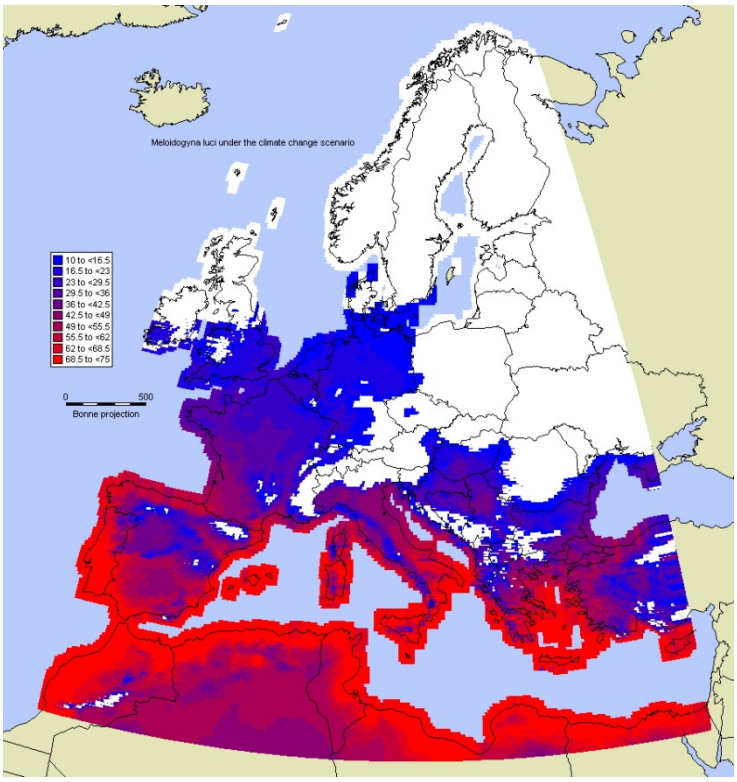

Figure 13: Potential suitability for survival and development of *Meloidogyne luci* expressed with EI (ecoclimatic index) under climate change scenario (generations range = 1 to 5 for European region).

Match climate modelling

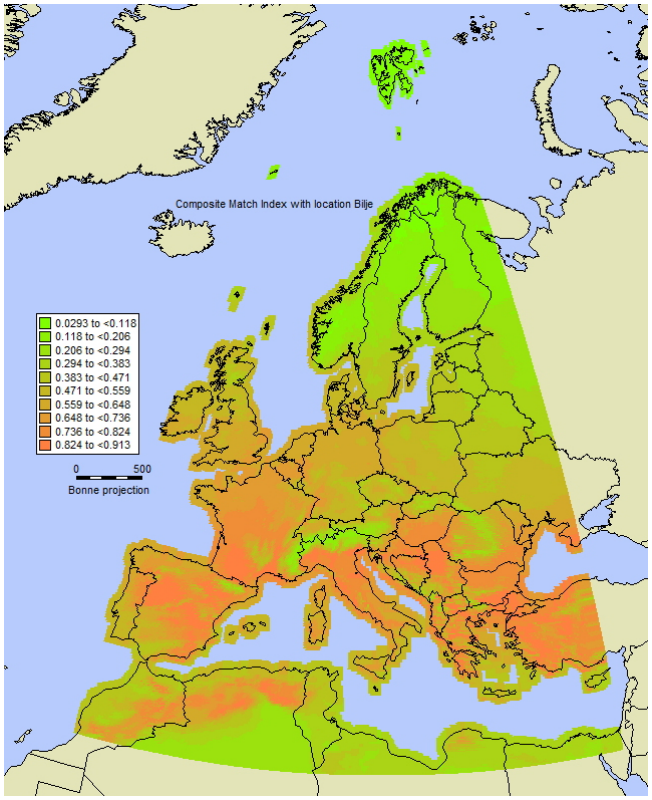

Figure 14: Results of match climate modelling of Bilje location (green= unlikely, dark orange=likely).

Main conclusions

The data on the tropical root-knot nematode (RKN) species distribution in partnering countries were obtained by sampling in the open field crop production and production of ornamental plants and addition of the national record data of the tropical RKN occurrence. In total, Melotrop database contained 107 locations from France, Portugal, Serbia and Slovenia. The most frequent species was *M. incognita*, recorded at 47 locations. The second most common species was *M. arenaria* (21 locations), followed by *M. javanica* (11 locations), *M. hispanica* (7 locations), *M. luci* (6 locations) and *M. enterolobii* (2 locations). Besides, mixed populations were detected at 13 locations. Forty-three host species were recorded. The distribution of tropical RKN species at the open fields was predominant in areas with the Mediterranean climatic conditions; however, a small proportion of locations was recorded in areas of semi-continental climate with milder winter conditions.

Isozyme phenotyping had average conformity of results of 85.1%, while the successful identification among laboratories ranged from 58.3 to 100%. Not all species were identified with the same success. The species that were 100% correctly identified in both provided samples were *M. arenaria* and *M. enterolobii.* For *M. hispanica, M. javanica,* and *M. luci* 90% conformity was achieved, while 80% was achieved for *M. incognita,* 70% for *M. ethiopica* and 60% for *M. inornata.* Results of Rm were variable when comparing values of the same sample between laboratories and when comparing values of the same sample of different laboratories with the same method. Regardless of the obtained variability in Rm value, the isozyme phenotype pattern seems to be a stable characteristic of particular species included in the test performance study. The isozyme phenotype patterns enabled species identification of provided samples reaching 85.1% average conformity. Therefore, it can be concluded that isozyme phenotyping remains the most efficient method for the identification of species within tropical RKN group.

A modified DNA barcoding method, where four mtDNA coding regions were sequenced (nad2, nad5, cox2 and cox3), resulted in a lower conformity than isozyme analysis. Only five out of 14 samples (36%) were correctly identified by all laboratories involved in the test. This relative new method requires more routine skills form the participating labs. Sequencing of additional mtDNA regions and more reliable sequence data of reference material of tropical RKN species in public databases could achieve better resolution and correct species identification.

The survival ability of *M. incognita* and *M. arenaria* at the open field in continental climate conditions was tested. Both species, *M. incognita* and *M. arenaria*, survived and maintained infection ability in a micro-plot (semi-field) filed with soil for three successive winters at Ljubljana location, defined as having continental conditions. The results suggest that both species represent a serious risk for agriculture production in the open field, especially in southern Europe or similar climate conditions.

CLIMEX software was selected for the assessment of potential suitability of European territory for survival and development of selected tropical RKN species. Simulated influence of possible climate change scenario was also performed. From the modelling results, it can be concluded that *M. arenaria* and *M. incognita* threaten the larger area of European territory, which was much more endangered under the climate change scenario.

The modelling results are presented through the Ecoclimatic index, which reflects the suitability of environmental conditions for development and reproduction of certain organism. From all European countries, Cyprus seems to be the most endangered country for tropical RKN species, followed by some regions in Portugal and the other Mediterranean countries.

Appendix 2 - Tropical RKN distribution maps

The distribution maps present the occurrence of tropical RKN species at the open field crop and ornamental plants production. Presented locations are illustrative, positioned in the area where the positive samples were detected. The maps of countries in this report are for illustrative purposes only.

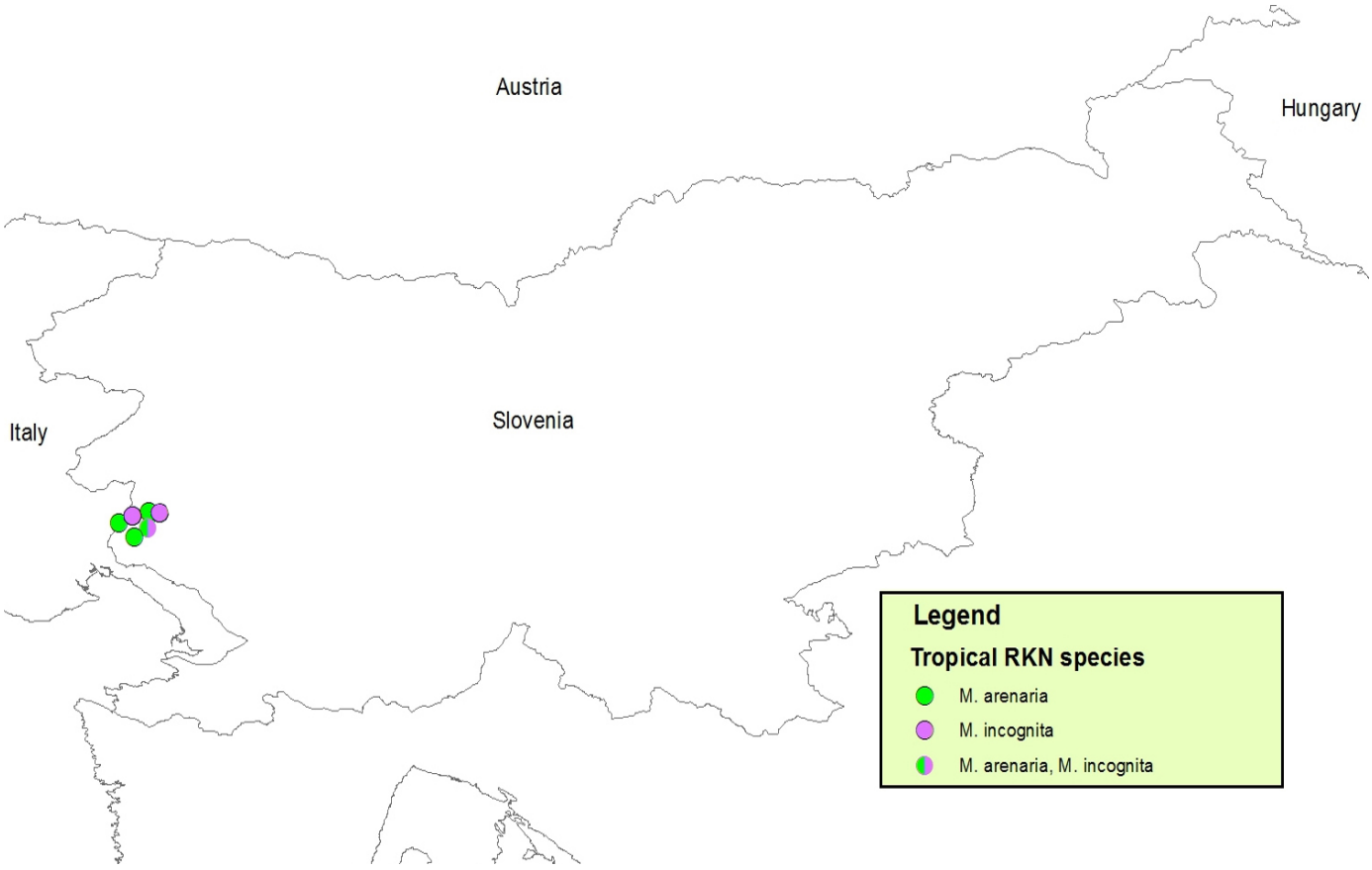

Figure 1: Distribution map of tropical root-knot nematode species in Slovenia.

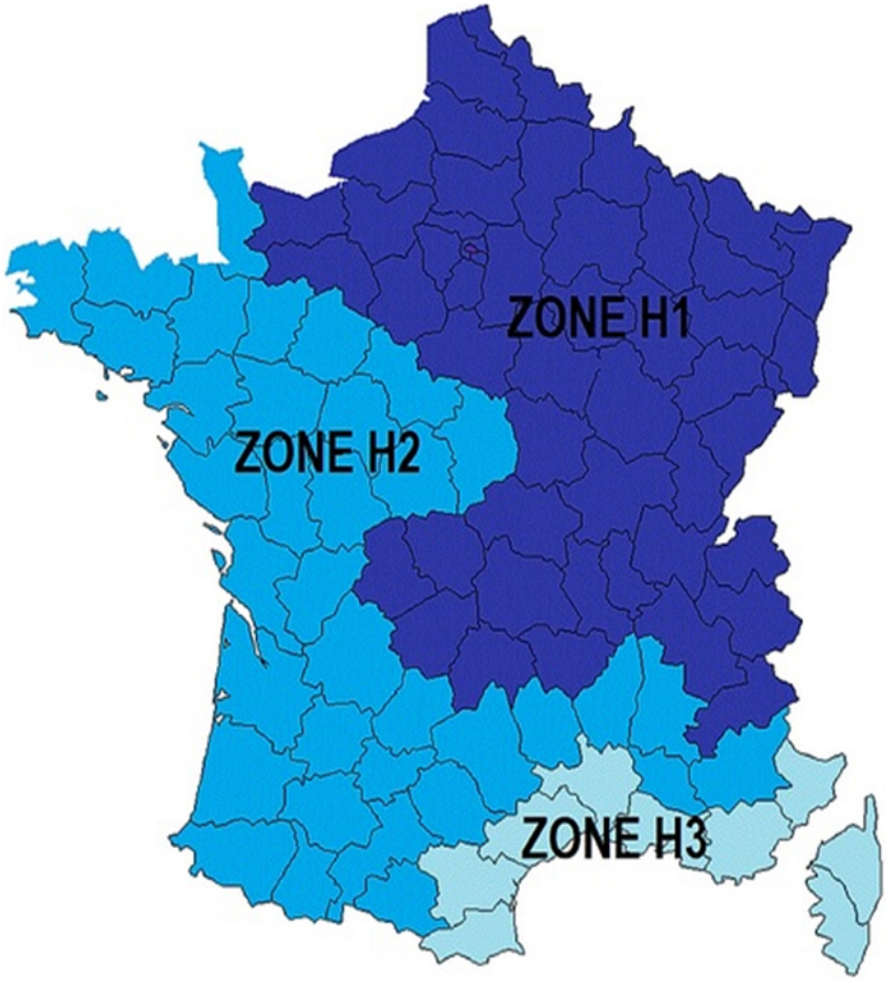

Figure 2: Climate zones in France. ZONE H1: area where winter temperatures are the coldest, under the influence of a semi-continental climate. ZONE H2: area with milder winters influenced by an oceanic climate. ZONE H3: around the Mediterranean, under the influence of the Mediterranean climate. *M. arenaria* was detected in 6 samples from areas in ZONE H2 and ZONE H3. *M. incognita* was detected in two samples from areas in ZONE H2.

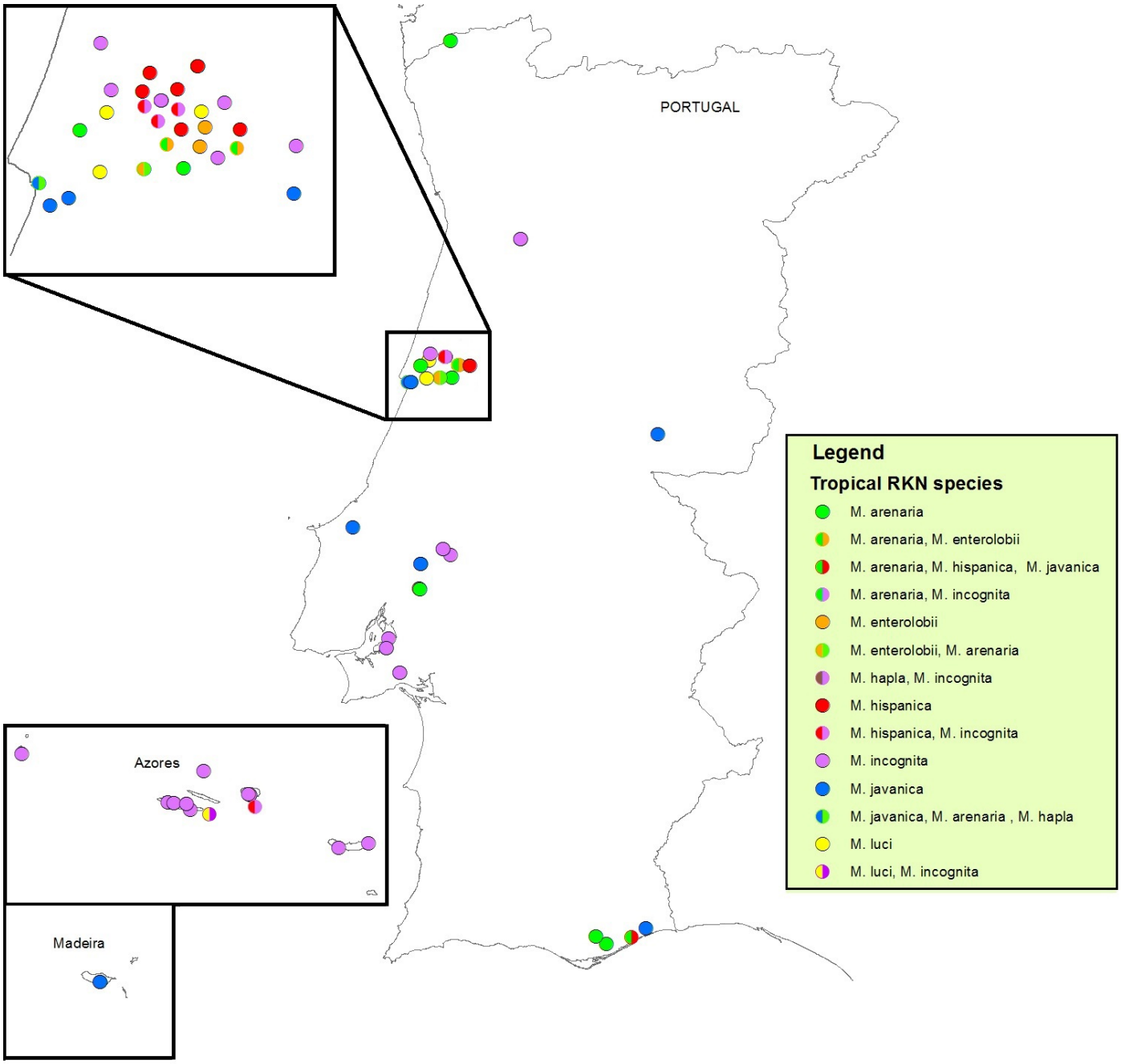

Figure 3: Distribution map of tropical root-knot nematode species in Portugal.

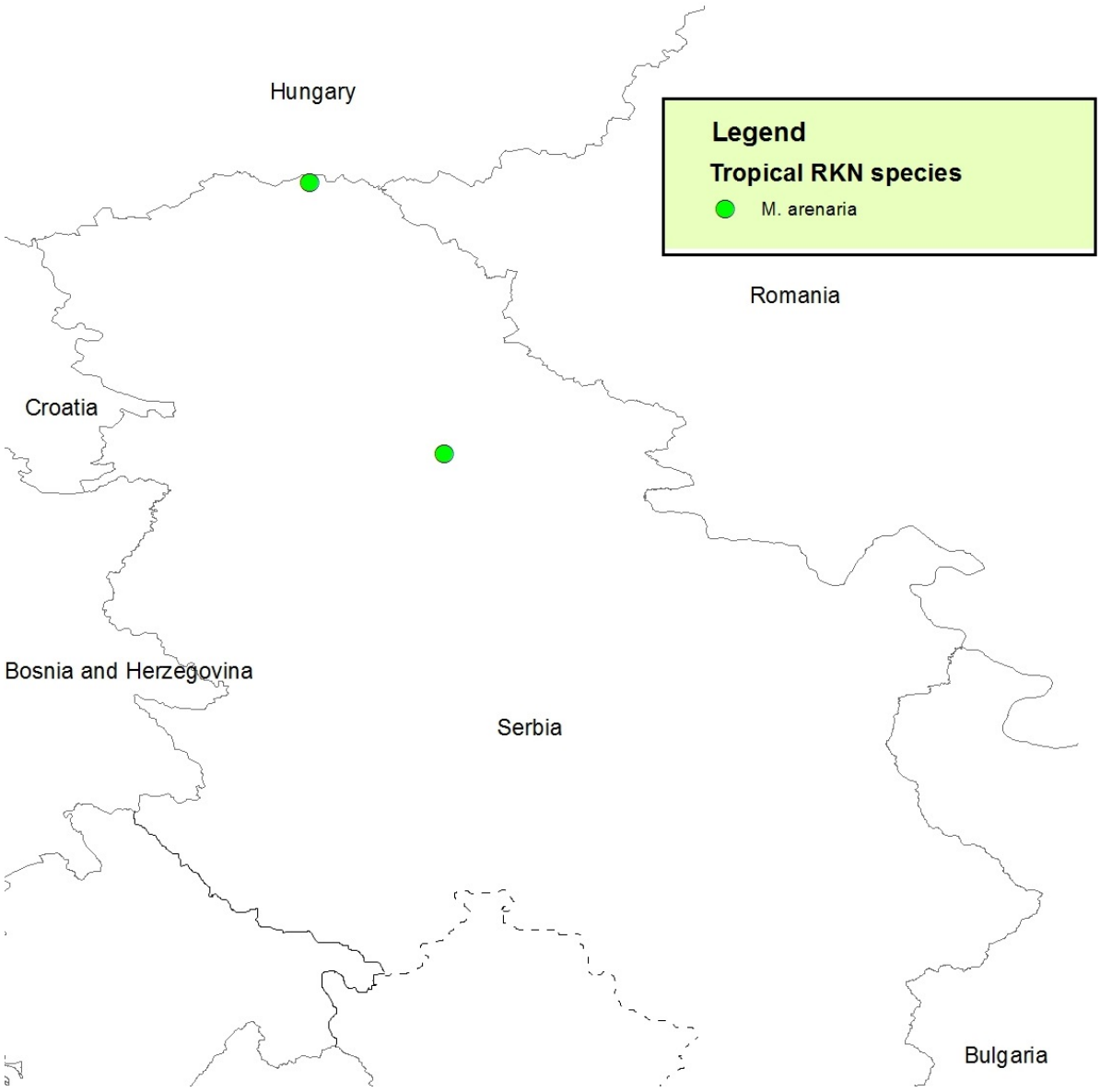

Figure 4: Distribution map of tropical root-knot nematode species in Serbia.