*e-Xtra

New distribution and phylogeography of the reniform nematode *Rotylenchulus macrosoma* Dasgupta, Raski and Sher, 1968 (Nematoda: Rotylenchulinae) in Europe

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ABSTRACT

Reniform nematodes of the genus *Rotylenchulus* are semi-endoparasites of numerous herbaceous and woody plant roots and occur largely in regions with temperate, subtropical and tropical climates. In this study, we provide new records of this nematode in eight European countries (Czech Republic, France, Germany, Hungary, Italy, Romania, Serbia and Portugal), additionally to the already reported six Mediterranean countries (Greece, Israel, Jordan, Spain, Syria, and Turkey). Four new host-plants such as corn, pea, wheat and almond-peach hybrid rootstock we added to the already studied host-plants (*viz.* bean, chickpea, hazelnut, peanut, soybean, wild and cultivated olive). Molecular analyses, based upon *coxI* and D2-D3 segments of 28S RNA markers, showed a great diversity and a strong genetic structure in *R. macrosoma* populations. However, the complexity of phylogeographic patterns in plant-parasitic nematodes may be related to the intrinsic heterogeneity in distributions of soil organisms, rare occurrence of species or the potential human impact associated with agricultural practices.

Keywords: reniform nematodes, 28S rDNA D2-D3, *coxI*, phylogeny, phylogeography, taxonomy.

Reniform nematodes of the genus *Rotylenchulus* Linford and Oliveira 1940 are semi-endoparasites of numerous wild and cultivated plants. This genus comprises 11 valid species (Van den Berg et al. 2016) which have been reported in 54 countries of Africa, Asia, Australia, Europe, North and South America, but being mainly distributed in tropical and warm temperate climate conditions (Fig. 1). These nematodes have a life cycle beginning from a first molt within the egg and the second-stage juvenile (J2) hatch from the eggs. Male and juvenile specimens (from J2 to pre-adult fourth-stage or J4) are found in the soil. Vermiform immature females penetrate the root tissues and induce different type of feeding sites (that is, single uninucleate giant cell or syncytium, depending of the species-plant host combination) and then become sedentary, keeping the anterior portion of the body embedded into the root whilst the enlarged posterior region protrudes from the root surface (Palomares-Rius et al. 2017; van den Berg et al. 2012). Mature females lay around 50-60 eggs in a gelatinous matrix secreted by the female forming an egg mass externally on the root. *Rotylenchulus* species are characterized by sexual dimorphism, mature females being swollen to kidney shape and males vermiform with less developed stylet and pharynx as males and do not feed (Dasgupta et al. 1968; van den Berg et al. 2012) (Fig. 2). *Rotylenchulus reniformis* Linford and Oliveira 1940 is the most economically important and widest distributed species, and a severe soil pathogen in cotton and other crops in 30 countries from North and South America, Africa, Asia, Europe (Robinson et al. 1997), while *R. parvus* (Williams 1960) Sher 1961, *R. macrosoma* Dasgupta, Raski and Sher 1968 among other species have a limited distribution and are of less economic importance (Gaur and Perry 1991; Robinson et al. 1997). In particular, *R. macrosoma* has been reported in the Mediterranean region in Greece, Israel, Jordan, Spain, Syria, and Turkey (Robinson et al. 1997; Palomares-Rius et al. 2018), but no data from outside this area are known. This nematode was reported in several host-plants, including wild and cultivated olive, bean, chickpea, hazelnut, peanut, soybean, (Dasgupta et al. 1968; Cohn and Mordechai 1988; Robinson et al. 1997, Sikora et al. 2018). Overall, it is noteworthy that the phytopathological perspective of this group of nematodes is currently changing given the expansion of *R. reniformis* to cotton and subtropical producing areas in Spain (Palomares-Rius et al. 2018; Castillo and Gómez-Barcina 1993; Artero et al. 1977) and the consideration as an emerging problem in USA cotton production (Robinson 2007).

The biology of *R. reniformis* and other reniform nematodes have several competitive advantages over other damaging nematodes such as *Meloidogyne* spp. including: (1) their ability to survive in dry soil in its vermiform stages as an anhydrobiotic form; (2) shorter life cycles; (3) lesser damage to root tissue, and the establishment of feeding sites along primary,

secondary, and tertiary roots with survival at greater depths in the soil; (4) effective protection against biological agents with the retention of body cuticles (J2 to J4 are not parasitic and retain the cuticles of the previous stages after molting); and (5) wide ecological adaptation to different soil types (Gaur and Perry 1991; Robinson 2007). However, other factors such as the low population density in soil, no apparent harvest losses in some crops or the difficulties for an accurate identification for some plant-parasitic nematode (PPN) species could restrict the precise geographical distribution. For these reasons, *Rotylenchulus* spp. could be "neglected" as potentially dangerous pathogens because their ecological conditions could change in the future under projected global climate change scenarios (IPCC 2019). Consequently, it is important to know their current distribution and adaptation to different crops and environmental conditions in order to design precise management practices.

The genus *Rotylenchulus* has an interesting genome and ribosomal set of genes. Ribosomal RNA (rRNA) genes have showed the presence of high levels of intraspecific and intra-individual variation (Van Den Berg et al. 2016; Palomares-Rius et al. 2018; Qing et al. 2019). The majority types of the D2 region of 28S rRNA (type A and B) for *R. reniformis* have been characterized as functional by reconstruction of secondary structure models and mutation mapping (Van den Berg et al. 2016). These different sequences are paralogs that are located at different rRNA clusters or chromosomes and the number of tandem arrays may still be expanding (Qing et al. 2019). Additionally, the size of the *R. reniformis* genome based on flow cytometry is estimated to be 190 Mb, being two and four times larger than that of *Caenorhabditis elegans* and the root-knot nematode (*Meloidogyne incognita*) genome, respectively (Ganji et al. 2013). Several genomes of this species have been recently published (Nyaku et al. 2014; Showmaker et al. 2019) in which the last one showed a size of 314 Mb that could be the result of unresolved haplotypes derived from the heterogeneity within the *R. reniformis* population used for DNA extraction (Showmaker et al. 2019). The complete mtDNA sequence has been deposited in GenBank (accession CM003310), which shows a length of 24,572 bp, but without annotations of putative regions and genes. However, these species showed a high intraspecific variability of some diagnostic features in immature females (usually the development stage used for species identification) (Van Den Berg et al. 2016) and for this reason, it is necessary to use molecular markers for species identification. In this regard, the use of rRNA markers is difficult, as mentioned before, by the presence of several copies not well-homogenized in the genome and several sizes and sequences could be found in the specific amplification of rRNA genes (Van Den Berg et al. 2016; Palomares-Rius et al. 2018; Qing et al. 2019). A high level of genetic diversity between populations from

several states of the USA and Japan has been found in *R. reniformis* using microsatellites (Arias et al. 2009; Leach et al. 2012). However, for other species of this genus the information is scarce and only based in rRNA markers. Microsatellites of *R. reniformis* obtained by Leach et al. (2012) did not amplify bands in *R. macrosoma* samples (Palomares-Rius, *unpublished*). For this reason, we studied the use of mitochondrial cytochrome c oxidase subunit 1 (*coxI*) in combination with rRNA data for population genetic analyses of *R. macrosoma* populations, as this mitochondrial marker has been useful for other nematodes (Derycke et al. 2008; Gutiérrez-Gutiérrez et al. 2011; Subbotin et al. 2018; Xu et al. 2020).

As mentioned, the current data about the occurrence of *R. macrosoma* shows a limited distribution only to the area of the Mediterranean Basin (Palomares-Rius et al. 2018). However, we suspect an unknown spatial expansion of this species revealing a wider distribution and infecting new host plants as shown in other species of this genus (Palomares-Rius et al. 2018; Castillo and Gómez-Barcina 1993; Artero et al. 1977). Likewise, we hypothesized that the current phylogenetic pattern of *R. macrosoma* might also reflect ancient geographic and climate changes (Hewitt 2001; Gomez and Lunt 2006). To test these assumptions, we: (1) described the new areas of distribution of *R. macrosoma* in Europe and their association with new important crops such as corn or wheat; (2) studied the putative cryptic species diversity using different sequence-based species delimitation methods to objectively interpret species boundaries in the sampled populations; and (3) addressed the issues of how genetically distinct and cohesive the different populations were, how they are related to one another, and how the geological and climate history changes affected their phylogeography in Europe based on mitochondrial and nuclear markers.

MATERIAL AND METHODS

Nematode population sampling, extraction and morphological identification. Samples with specimens of R. macrosoma populations were obtained from different ways: ii) Samples already studied in Palomares-Rius et al. (2018); ii) samples collected in different crops through Europe by different companies in our Nematological identification service; and iii) spontaneous samples obtained by authors in different personal travels (Table 1). The samples were not obtained from a designed sampling strategy. Nematode populations of corn and wheat crops were extracted from soil samples provided by farmers to the IAS-CSIC nematology laboratory (Spain) for its nematological diagnostic service. To avoid possible bias, a standardized method was used to collect soil samples from all populations. Soil samples

were collected with a mattock and a sampler from 10 up to 40 cm depth depending on soil condition from the rhizosphere of three to five plants randomly chosen in each field. Nematode specimens from 500 cm^3 of soil samples were extracted using the centrifugal flotation method (Coolen 1979). Specimens were examined using a Leica DM6 compound microscope with differential interference contrast at magnifications up to 1,000x, and a Leica DFC7000 T digital camera. Nematodes were identified at the species level using an integrative approach combining molecular and morphological techniques to obtain efficient and accurate identification (Palomares-Rius et al. 2018). Nematode population density in soil was also assessed as the total number of adults and juvenile stages for each sample and calculated as the average of the soil counts.

DNA extraction, PCR and sequencing of *coxI* and *D2-D3* sequences. Juveniles or adults were used for molecular identification and molecular characterization. Nematode DNA was extracted from single individuals as described by Subbotin et al. (2000). PCR and sequencing were completed in the laboratory of IAS-CSIC, Spain. *coxI* gene was amplified using the primers JB3 (5′-TTTTTTGGGCATCCTGAGGTTTAT-3′) and JB5 (5′-AGCACCTAAACTTAAAACATAATGAAAATG-3′) (Bowles et al. 1992). From those individuals with different haplotype or from different populations, the 28S rRNA fragment was also sequenced. The D2-D3 expansion segments of 28S rRNA was amplified using the D2A (5'-ACAAGTACCGTGAGGGAAAGTTG-3') and D3B (5'-TCGGAAGGAACCAGCTACTA-3') primers (De Ley et al. 1999).

PCR cycle conditions for the 28S rRNA marker were: one cycle of 94 °C for 2 min, followed by 35 cycles, of 94 °C for 30 s, an annealing temperature of 55 °C for 45 s, 72 °C for 1 min, and finally one cycle of 72 °C for 10 min. The cycle for *coxI* was as follows: 95 °C for 15 min, 39 cycles at 94 °C for 30 s, 53 °C for 30 s, and 68 °C for 1 min, followed by an extension at 72 °C for 7 min. PCR products were purified after amplification using ExoSAP-IT (Affimetrix, USB products), and used for direct sequencing in both directions using the mentioned primers to. The resulting products were purified and run on a DNA multicapillary sequencer (Model 3130XL genetic analyzer; Applied Biosystems, Foster City, CA, USA), using the BigDye Terminator Sequencing Kit v.3.1 (Applied Biosystems, Foster City, CA, USA), at the Stab Vida sequencing facilities (Caparica, Portugal). The sequence chromatograms from the two markers, *coxI* and D2-D3 expansion segments of 28S rRNA, were investigated with DNASTAR LASERGENE SeqMan v. 7.1.0. The newly obtained

bidirectional sequences were submitted to the GenBank database under accession numbers indicated on Table 1.

Data analyses and population genetic structure. Sequences from the two markers were separately aligned by ClustalW. Number of sequences and sampling points are listed in Table 1. *coxI* sequences were translated to amino acid sequences before the alignment in order to check the presence of stop codons using the Alternative Flatworm Mitochondrial Code (transl_table=14) genetic code in NCBI web (https://www.ncbi.nlm.nih.gov/Taxonomy/Utils/wprintgc.cgi?mode=c#SG14). However, some haplotypes presented stop codons in this region (TAG), but their amino-acid sequence corresponds to COXI protein. Nucleotide substitutions map for *coxI* region was calculated with the package adegenet (Jombart, 2008) in R v. 3.5.1 freeware (R Core Team 2019). Genetic diversity within sampling sites was investigated by calculating nucleotide diversity (π) and haplotype diversity (h) using DnaSP v. 6 (Rozas et al. 2017) according to Nei (1987). To investigate whether sequence evolution followed a neutral model, Tajima's D and Fu's Fs neutrality tests were performed. These tests were performed to check if the populations experienced an expansion (Rogers and Harpending 1992). This is important because of reniform nematodes are assumed to be distributed in warmer temperate and tropical climates rather than colder climates in Central or Northern Europe. To investigate whether *R. macrosoma* distribution is either natural or the result of human distribution, several analysis using hierarchical AMOVA were performed (Excoffier et al., 1992) using Kimura 2P distance method: i) all population in one group; ii) populations per country; and iii) two geographical groups (Eastern *vs* Western populations) considering the major area of diversity (Hungary, Romania, Serbia and Crete, Greece) *vs* lower diversity areas (Spain, France, Germany, Italy and Czech Republic). All population genetic analyses were performed using the Arlequin 3.5.2.2 software (Excoffier and Lischer, 2010) and only sampling sites with more than three sequenced individuals for *coxI* were included in the analysis (resulting a total of 22 sampling points in nine countries). To investigate evolutionary relationships and mutational differences between haplotypes, as well as the geographical distribution of haplotypes, a haplotype network was built based on TCS network (Clement et al. 2002) implemented in Popart v. 1.7 (http:/popart.otago.ac.nz). A rarefraction approach was studied using the package spider 1.5.0. (Brown et al. 2012) in R v. 3.5.1. Isolation by distance (IBD) was assessed through Mantel testing in adegenet package (Jombart 2008) in R based on *coxI* marker between genetic distances as Edwards' distance (Euclidean) *vs* geographical distances using 10,000

randomizations. Distances between populations points were calculated using the great-circle distance between populations in the gdistance package (van Etten 2017). Plots were drawn using the MASS package (Venables and Ripley 2002) in R v. 3.5.1.

For comparing specific hypotheses for the demographic history, we utilized the Approximate Bayesian Computation (ABC) approach implemented in diyabc 2.0.3 (Comuet et al. 2014). This software tests different scenarios by calculating summary statistics rather than exact likelihoods (Csilléry et al. 2010). Two alternative scenarios were examined that represented either gradual diversification from putative refuge areas: i) Crete, Greece->Iberian Peninsula->rest of Europe, and ii) rest of Europe->Iberian Peninsula->Crete, Greece (Fig. S1). Priors were set to follow uniform distributions ranging from 10,000 to 20,000 for effective population sizes (Ne) and from 1,000 to 50,000 and from 80,000 to 100,000 for diversification times (t1 and t2, in generations and considering 4 generations/year, respectively for t1 and t2, with t2 the approximately representing the Last Glacial Maximum 22,000 years ago). For each scenario 1,000,000 simulations were run (total of 2,000,000 simulations), after which summary statistics were drawn for: number of haplotypes and FST (selected using prior and real data evaluation in the program). All other settings were left as suggested in the diyabc 2.0 handbook. Posterior probabilities for scenarios were calculated by logistic regression considering the 20,000 simulated datasets that were closest to the observed values.

Phylogenetic analyses. Sequences obtained in this study were used for phylogenetic analyses. Outgroup taxa for each dataset were chosen following previously published studies (Van Den Berg et al. 2016; Palomares-Rius et al. 2018). Multiple sequence alignments of the different genes were made using the FFT-NS-2 algorithm of MAFFT V.7.450 (Katoh et al. 2019). Sequence alignments were visualized using BioEdit (Hall 1999) and edited by Gblocks ver. 0.91b (Castresana 2000) in Castresana Laboratory server (http://molevol.cmima.csic.es/castresana/Gblocks_server.html) using options for a less stringent selection (minimum number of sequences for a conserved or a flanking position: 50% of the number of sequences + 1; maximum number of contiguous non-conserved positions: 8; minimum length of a block: 5; allowed gap positions: with half). Phylogenetic analyses of the sequence datasets were based on Bayesian inference (BI) using MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003) and Maximum likelihood (ML) using PAUP * 4b10 (Swofford 2003). The best-fit model of DNA evolution was obtained using JModelTest V.2.1.7 (Darriba et al. 2012) with the Akaike Information Criterion (AIC). The best-fit model, the base frequency, the proportion of invariable sites, and the gamma distribution shape

parameters and substitution rates in the AIC were then given to MrBayes for the phylogenetic analyses. We used an unlinked general time-reversible model with invariable sites and a gamma-shaped distribution (GTR + I + G) for the D2-D3 expansion segments of 28S rRNA and the partial *coxI*. These BI analyses were run separately per dataset using four chains for 2 × 10^6 generations for each molecular marker. The Markov Chains were sampled at intervals of 100 generations. Two runs were conducted for each analysis. After discarding burn-in samples and evaluating convergence, the remaining samples were retained for further analyses. The topologies were used to generate a 50% majority-rule consensus tree. Posterior probabilities (PP) are given on appropriate clades. In the ML analysis, the estimation of the support for each node was obtained by bootstrap analysis with 200 fast-step replicates. Trees from all analyses were visualized using FigTree software V.1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/) (Page 1996).

Species delimitation. To test whether sequence datasets constituted single or multiple species, the General Mixed Yule Coalescent (GMYC) (Fujisawa and Barraclough, 2013; Pons et al., 2006) and Automatic Barcode Gap Discovery (ABGD) (Puillandre et al., 2012) methods were applied. The ABGD analyses were conducted on the online server (https://bioinfo.mnhn.fr/abi/public/abgd/) with the default program settings. Distances were calculated utilizing the Jukes-Cantor (JC69) model and Kimura (K80) model of nucleotide substitution.

The GMYC algorithm compares two alternative models: 1) a single coalescence model that assumes a single species, and 2) a model that combines a coalescent model of intraspecific branching with a Yule model for interspecific branching, thus assuming multiple species. The location of the switch (threshold T) from speciation to coalescence nodes is then fitted on the tree, resulting in an estimation of species diversity. The ultrametric tree was produced in BEAST v1.10.4 (Drummond et al., 2018) without outgroups and duplicated haplotypes were excluded from the dataset using FaBox 1.5 (Villesen 2007). For the 28S rRNA dataset, the set priors were: substitution model = GTR; base frequencies = estimated; site heterogeneity model = gamma; length of chain = 5 × 10^7 generations. For *coxI* dataset, substitution model = GTR; base frequencies = estimated; site heterogeneity model = invariant site; Length of chain = 1×10^7 generations and different codon positions were regarded as different partitions. For both molecular markers, the uncorrelated lognormal relaxed clock and constant size coalescent prior were used as clock type and tree model, respectively. Tracer v1.7.1 (Rambaut et al., 2018) was used to check for effective sample size values (ESS > 200). TreeAnnotator v1.10.4

(Drummond et al., 2018) was used to obtain consensus trees, using a burn-in of 10%. The ultrametric tree produced by BEAST were submitted to R using packages 'ape' (Paradis and Schliep 2018) and 'splits' (Ezard et al. 2017).

We relied on a conservative consensus approach similarly as described in Hauquier et al. (2017) in order to maximize the reliability of species boundaries using the different species delimitation methods. More specifically, we recognized species clades that: 1) received high nodal support (at least 75% bootstrap support in the ML tree and 90% PP in the BI phylogeny), 2) showed compatible patterns based on statistical parsimony, ABGD and GMYC analyses, 3) formed concordant clades in the trees inferred from nuclear and mitochondrial markers and/or expressed different morphological characteristics.

RESULTS

New distribution areas, hosts and soil infestation levels. In this research, we have identified 28 new populations of the reniform nematode *R. macrosoma* in several European countries and crops, additional to those published by Palomares-Rius et al. (2018) (Table 1). These data significantly increase the distribution of this species in Europe and constitute the first report of this nematode in eight European countries (*viz.* Czech Republic, France, Germany, Hungary, Italy, Romania, Serbia and Portugal) (Table 1). In all these countries, except for Portugal, we studied more than one population per country, Hungary being the most studied country, with 7 populations (Table 1). Soil infestation densities ranged from 3 to 1760 individuals per 100 cm^3 soil. Four new host-plants including corn, pea, wheat and almond-peach hybrid rootstock were added to the already studied host-plants, such as bean, chickpea, hazelnut, peanut, soybean, wild and cultivated olives trees (Table 1). Samples with high population densities and the presence of abundant number of eggs and mature sedentary females were found. In all the samples, morphometrics and morphology agreed well to previous *R. macrosoma* populations (Fig. 2).

Molecular variability and phylogeography. *coxI* fragment was sequenced (336 bp) from 210 individuals and 16 haplotypes were identified. There were 64 variable sites without any insertions or deletions. From those individuals with different *coxI* haplotype or from different populations the 28S rRNA fragment was sequenced and 13 haplotypes were found out of 46 sequenced studied (Table 1). The alignment and study of mutations in the *coxI* coding region

showed that the mutations were in the 1st and 3rd position in the codon (Fig. S3). The mutations in the 1st codon position were distributed in two regions, while the 3rd position was distributed along the alignment, showing that these sequences likely proper coding regions. Three stop codons (TAG) were found in haplotypes coi-H1, coi-H4, coi-H9-coi-H12 and coi-H14-coi-H16 in three variable positions (12, 68 and 109 in the amino acid alignment). All these stop codon positions were coincident with a Tyrosine (Y) with the other sequences (Fig. S4). The alignment of D2-D3 expansion segments of 28S rRNA showed some position with heterozygosity when the chromatograms were analyzed, while for the same position other haplotypes showed a clear and unique nucleotide (data not shown).

The number of studied individuals for *coxI* reached a saturation point using a rarefraction approach (Fig. S5). The number of *coxI* and D2-D3 region haplotypes per population was from 1 to 3, or from 1 to 2, respectively (Table 1). The distribution of haplotypes per country (Fig. 4A and Table 2) showed a major diversity of haplotypes in Eastern countries (Greece, Hungary, Romania, and Serbia), while in Western Europe the number of these haplotypes was either lower or with only one haplotype was detected in some countries, even with many populations sequenced (i.e. France). Haplotype network showed three clades of related haplotypes (Fig. 4B): i) Clade I, with haplotypes coi-H1, coi-H2, coi-H8 and coi-H9 detected in Greece, Spain and Portugal; ii) Clade II, with haplotypes coi-H3, coi-H7 and coi-H13 detected in Czech Republic, France, Germany, Hungary, Italy, Serbia, and Spain); and iii) Clade III, with the rest of haplotypes (coi-H4, coi-H5, coi-H6, coi-H10, coi-H11, coi-H12, coi-H14, coi-H15 and coi-H16) localized in Hungary, Romania and Serbia. Each group showed different and characteristic patterns depending of the country. For example, Crete (Greece) as an island showed specific haplotypes not shared in other countries and with important molecular differences compared to the other haplotypes in continental Europe. Clade II contains the most prevalent and widespread haplotypes such as coi-H3 and coi-H7, while Clade III has the major number of different haplotypes with small differences in nucleotides and grouped in a restricted area (central Europe) being detected in only three countries. In some countries, the presence of prevalent and rare haplotypes was also found (i.e. Czech Republic, Hungary, Serbia, and Spain). However, within the same nematode population, the presence of prevalent haplotypes and rare haplotypes was only found in Bagamer (Hungary) and Reus (northern Spain), while the presence of the most prevalent haplotypes (coi-H3 and coi-H7) together in the same population were found in Bonyhad (Hungary), Bagamer (Hungary), Moretta (Italy) and Ancona (Italy) (Table 1).

The summary statistics Tajima´s D and Fu´s Fs for populations with at least 3 sequenced individuals are shown in Table S1. Only the population from Bonyhad (Hungary) had a *P* significant for Tajima's D statistic and any population was significant for the Fu's Fs test and all values were higher than 0 in the population when it was possible to calculate (Table S1). The analysis of the whole dataset as one group also showed significant results (Tajima's D = 2,306, $P < 0.05$; Fu's Fs = 31,052, $P = < 0.0001$). In this case, these tests reject the null hypothesis of demographic stability. Population pairwise Fst using as distance method Kimura 2P are showed in Table S2, and using different geographical entities (country) in Table 3. In both cases, at population and country levels both showed an important population genetic structure, being the majority of them as large (0.15-0.25) to very large (above 0.25) following Wright's division (Wright 1978). The majority of the comparisons were significant after 1023 permutations. Only 4 pairwise Fst values comparisons were not significant (Spain *vs*. France, France *vs*. Italy, France *vs*. Serbia, and Hungary *vs*. Serbia). We also found an important genetic structure in *R. macrosoma* populations using a hierarchical AMOVA for the *coxI* marker across 23 populations (n ⩾ 3 reports) and 9 countries (Table 4). When all populations were considered, the majority of the molecular variation was associated among populations (97.3%), while the variation within the populations was minimal (2.53%) and showing an important genetic structure. When countries were used as groups, the majority of the variation was related to differences among countries (69.71%), followed by among population within countries (27.95%). All comparisons inside these groupings were significant (Table 4). We found that the separation of the populations within two groups (Western *vs* Eastern in Europe) was smaller than the variation in comparison to the variation among populations (72.49%).

The studied populations showed a significant Isolation by Distance (IBD) using the *coxI* marker with a correlation of 0.3579 and p-value of 0.0027 (Fig. S6). We found a major consistent cloud of points with several minor patches using the package MASS (Fig. S6). These patches could be associated to the distribution of correlated points in the graphs, in which three major lines of genetic distances were observed mainly related with distant and differentiated populations (i.e. Cretan, Greek populations) and a major presence of these species in central Europe with important genetic differences (Fig. S6).

Phylogeny, species delimitation and phylogeographic hypothesis. The *coxI* phylogeny (Fig. 5) showed a basal position for haplotypes coi-H1 and coi-H8 (both unique to Crete, Greece). Main continental European haplotypes formed a well-supported clade where haplotypes coi-H2 and coi-H9 (from Spain and Portugal) occupied a basal position. These

haplotypes are unique to the Iberian Peninsula (Fig. 4). Other prevalent haplotypes as coi-H3 and coi-H7 formed a well-supported clade, including coi-H13, a haplotype unique to Spain. The other haplotypes formed a clade (clade III), and they came from Serbia, Romani and Hungary (central Europe). By contrast, D2-D3 expansion segments of 28S rRNA phylogeny formed not well-supported clades at exception of the basal clade from Crete (Greece). Several D2-D3 haplotypes have more than one haplotype correspondence with *coxI* sequences, as it is the case for 28S-H5, 28S-H9 and 28S-H8. There is a congruent phylogenetic relationship of clades between *coxI* and D2-D3 expansion segments of 28S rRNA haplotypes, as was showed between Greek and Iberian Peninsula unique haplotypes. D2-D3 expansion segments of 28S rRNA showed just a few nucleotides with phylogenetic information for the inference of phylogenies, and only haplotypes with important differences showed a good congruence between both phylogenies.

The species delimitation study showed congruence for some clades in both markers *coxI* and D2-D3 expansion segments of 28S rRNA with high congruence between clades of haplotypes in Crete populations (coi-H1 and coi-H8 haplotypes and 28S-H1 and 28S-H2), but with low-moderate PP support and separation of these two haplotypes in the ML analysis (Fig. S7 and S8). The species delimitation methods used (GMYC and ABGD) also pointed these two haplotypes as a new species, at exception of the GMYC single and ABGD with 5 species. However, the clade of coi-H1 and coi-H8 was well-supported in the ultrametric phylogenetic tree used with additional species of the genus *Rotylenchulus* for the two different species separation methods. Morphological differences of these populations were already discussed in Palomares-Rius et al. (2018). In this case, minor morphometric differences of these populations from original description and those reported in Spain were found in body length (428-526 *vs* 520-640, 432-520 µm), stylet (15-21 *vs* 18-22, 16-20 µm), a ratio (26.1-31.6 *vs* 30-38, 27.6-32.1), c' ratio (2.6-4.0 *vs* 3.7-5.0, 2.6-4.0), V ratio (58-65 *vs* 63-68, 59-66), o ratio (105-156 *vs* 139-188, 116-156) (Palomares-Rius et al. 2018).

Two different hypothetical glacial refuges were tested (Mediterranean *vs* central Europe) (Fig. S1). The first scenario Mediterranean glacial refugee was more probable than the European scenario using three groups (posterior probability of 0.9930 *vs* 0.0070). This is also congruent with the phylogenetic analysis of *coxI* haplotypes, where South Mediterranean haplotypes (Crete and some haplotypes in the Iberian Peninsula) occupied a basal position in the phylogenetic tree).

DISCUSSION

The new geographical distribution of *R. macrosoma* in Europe changes the former consideration of this nematode as a Mediterranean species (Palomares-Rius et al. 2018). Additionally to the presence and description of this nematode species in Israel (Dasgupta et al. 1968; Qing et al. 2019), it has been reported in six Mediterranean countries (Castillo et al. 2003; Van den Berg et al. 2016; Palomares-Rius et al. 2018; Sikora et al. 2018). Although we are aware that accurate prevalence of this nematode in Europe cannot be established with the present data, the prevalence can be estimated to be higher than currently perceived. The presence in localities in Northern Europe (with colder winters and humid soil conditions) showed an important flexibility of ecological existence and probably survival strategies. As mentioned in the introduction, *R. reniformis* has a great survival potential by the use of several strategies (anhydrobiotic form, egg survival, etc.), which may be shared by *R. macrosoma*. The soil density levels found in some sampling points could indicate the possibility of damage in plants. However, this point is difficult to assess as the samples were not studied for plant damage and some of them came from farmers with limited plant data. A damage threshold of 16 individuals per 200 cm^3 of soil has been reported for *R. reniformis* in cotton using pot tests (Sud et al. 1984). Other data suggest significant increases in cotton after nematicide application when initial densities are in the range of 100-250 nematodes/100 cm^3 of soil (Davis et al. 2018). No data for threshold damage in corn or wheat with *R. reniformis* or *R. macrosoma* are available. In our data, 13 sampling points were above 100 nematodes per 100 cm^3. However, this data only gives an idea of samples at the middle of the crop season, when the majority of the samples came and not at the beginning of the crop. We also identified new hosts for *R. macrosoma* being corn, pea, wheat and almond-peach hybrid rootstock, additional to the already cited hosts (olive, peanut, bean, banana and hazelnut) (Dasgupta et al. 1968; van den Berg et al. 2012; Castillo et al. 2003; Palomares-Rius et al. 2018). While cotton (*Gossypium barbadense*), pepper (*Capsicum annuum*), winter wheat (*T. aestivum*) and sour orange (*Citrus aurantinum*) were non-host of this species (Cohn and Mordechai 1988; Robinson et al. 1997). However, in our case, we found three out of 37 sampling points with wheat as host crop.

Molecular diversity and population genetic structure of *Rotylenchulus macrosoma*. Sequencing of more individuals from populations studied in Palomares-Rius et al. (2018) and new populations in other European countries showed the presence of some stop codon (TAG)

in some specific haplotypes of *R. macrosoma* using alternative flatworm mitochondrial genetic code (coi-H1, coi-H4, coi-H9, coi-H10, coi-H11, coi-H12, coi-H14, coi-H15, coi-H16) (Fig. S4). This has not been detected in other sequenced species of this genus such as *R. reniformis, R. macrosomoides* and *R. macrodoratus*, even with the limited amount of sequenced individuals, and these species translate correctly using the alternative flatworm code (Palomares-Rius et al. 2018; van den Berg et al. 2016). We believe that this stop codon (TAG) codifies a Tyrosine (Y) because this alignment position has been found in other haplotypes for *R. macrosoma*, and in other species of this genus, or as far as phylogenetically related nematode species such as *Globodera pallida* or *Rotylenchus magnus*, among others using a blastn in NCBI. Additionally, the mutations occurred in the first and third codon positions, which showed that this gene could be functional inside the mitochondrial genome, even with this stop codon in some haplotypes. We used the same primer set for the other populations and species, having high resolution in chromatograms from Sanger sequencing and being a coding sequence in the rest of haplotypes or outside this stop codon position. However, without all mitochondrial sequence and with the short fragment of the gene sequenced it is difficult to corroborate and demonstrate that it is not a pseudogene. Jacob et al. (2009) found in *Radopholus similis* and *R. arabocoffeae* an exception of the code in Nematoda, where the codon (TAA) codifies also for Y.

The results of several analyses of the mtDNA data support a strong genetic structure in *R. macrosoma* populations found in Europe with a predominant number of haplotypes in *coxI* and D2-D3 expansion segments of 28S rRNA. The summary statistics Tajima´s D and Fu´s Fs reject the null hypothesis of selective neutrality and demographic stability when all the data were combined. However, when populations were studied separately, this result was not detected. In this case, the number of haplotypes per population is low, probably by random events of introduction in places where those nematodes had not originated or selection for particular genotypes, which is quite often seen when using resistant/susceptible cultivars or other factors as sublethal concentrations of nematicides (Young and Hartwig, 1988; Meher et al., 2009). The positive number in Tajima's D and Fu's Fs statistics could suggest that this species may have suffered a recent bottleneck (Tajima 1989; Fu 1997). In our case, we have two dominant haplotypes in the *coxI* dataset (coi-H1 and coi-H3) and the expansion of soil organisms in the case of soil crop pathogens that could be related to infested plant material or soil adhered to machinery, as major processes of expansion in PPN among fields. Therefore, the bottleneck effect in our populations could be important in the explanation of these results. In our case, additionally to these two prevalent haplotypes in Europe, coi-H3 and coi-H7, there

are other separated haplotypes with important molecular differences, but restricted to specific areas in Europe (clade I and clade II haplotypes, Fig. 4B). Interestingly, we detected some areas in Central Europe with a high number of different haplotypes with a small variability among them (clade III, Fig. 4B).

AMOVA analysis showed that the majority of the variation was among countries, showing a strong genetic structure. However, the separation between Western countries, with a lower number of haplotypes, in comparison to the Eastern countries did not give more variation than that the variation found among populations within these two groups. Even with the IBD test correlated with geographical distances, the maximum genetic distances did not correlated with the maximum separation between populations. In this case, the important genetic differences and the patching distribution of this species with a high number of fields with positive samples in Central Europe could influence this result. Some papers pointed that the IBD could be influenced by habitat configuration and maximum migration distance (van Strien et al. 2015). In our case, with the limited dispersal of soil PPN, the dispersion by human activities could even change more the natural dispersal pattern of this species.

Species delimitation. These methods and the stringent criteria used to detect more than one species in the dataset showed that Cretan populations were genetically separated from the others, but not reaching all our strict criteria commented in Material and Methods section to be considered a separated species. Different species delimitation methods outputs and Bayesian phylogenetic analysis pointed to the separation among Cretan populations as a different species, but in a conservative point of view these populations have just a few morphological differences in comparison to the continental populations and all the analyses were not completely congruent with the species separation. In this regard, the cryptic speciation in PPN is a frequent phenomenon with almost indistinguishable morphology, but with important genetic differences (Palomares-Rius et al. 2014). Cretan populations could have been isolated for a long time without the introduction of other populations from continental Europe or Middle East, and the morphological differences may be a result of geographical intraspecific variability.

Hypothesis about the phylogeography of *Rotylenchulus macrosoma* in Europe. The phylogenetic analysis of *coxI* and D2-D3 expansion segments of 28S rRNA markers showed that some haplotypes were closely related to the ancestral haplotype for this species (Fig. 5). coi-H1 and coi-H8 haplotypes from Crete, Greece were almost as outgroup for both

phylogenetic trees and coi-H2 and coi-H9 are unique to the Iberian Peninsula and closely related to Cretan haplotypes in the TCS haplotype network (Fig. 4B), while others occupied an intermediate position as coi-H3, coi-H13 and coi-H7. This prompted us that Iberian Peninsula and Crete (Greece) are two putative refugia during the Last Glacial Maximum (LGM) (Fig. 6). After the last period of glaciation, Crete populations became isolated while populations from Iberian Peninsula gave place to haplotype coi-H3 and other haplotypes as coi-H13 and coi-H7, which are widely present in all continental Europe. Areas of more recent expansion could be central Europe with a recent expansion of this species in these areas, keeping a wide number of recent haplotypes (rest of haplotypes). This scenario is also accepted as more plausible than the European as a refugee scenario and is supported by our ABC analysis. This could support our idea that the nematode survived in those areas during glacial periods, since this area is considered as a glacier refuge (Hewitt 2001; Gomez and Lunt 2006). Although our data are limited in natural environments, the only one available sample is from wild olive in Southern Spain (Vejer). However, the human activities such as agricultural management and agriculture trade could be an important factor in their expansion and the movement of *R. macrosoma* population's across Europe in forward-reverse dispersion routes as described for other pathogens (McDonald and Stukenbrock 2016). On the other hand, some areas such as continental Greece or areas where this species has been previously cited were not sampled and therefore need to be studied in further studies. These areas could have different haplotypes and possibly different scenarios for location of glacial refuges, which could support our hypothesis. Additionally, the current distribution of *R. macrosoma* could expand to new areas in Europe due to the effect of global climate change on niche shifts of this species. .

Conclusions and Perspectives. This study showed the extension of *R. macrosoma* in several European countries, in some cases close to pathogenicity levels. The complexity of phylogeographic patterns in PPN could be related to rare events of occurrence in soil (not widely and homogenously distributed over a region), the possibility of human population movement related to agricultural practices and the scarce ecological parameters studied in some species (mutation rates, generations per year, etc). This study also suggests the Mediterranean region as a putative refuge during glacial periods for PPN. Additionally, more studies need to be carried out to clarify the morphological and ecological requirements for *R. borealis* and *R. macrosoma*. These studies must be complemented with molecular data of

different populations of these two species. In this regard, the use of topotypes could help to resolve this question not included in this article.

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Figure legends

Fig. 2. Light micrographs of *R. macrosoma* females parasitizing corn roots.

Fig.4. Haplotype analysis of *R. macrosoma*. A) Haplotype map distribution of the sampled populations. B) TCS network analysis with geographic data.

Fig. 5. Comparison of different haplotype phylogenies using Bayesian Inference and Maximum Likelihood of *R. macrosoma* haplotypes for *coxI* and D2-D3 region of 28S rRNA. Lines showed related populations with their respective haplotypes.

Fig. 6. Hypothesis of putative phylogeographic expansion of *R. macrosoma* after the Last Glacial Maximum with the different haplotypes. Small box the phylogenetic tree of *coxI* using a Bayesian Inference approach. Map using the Last Maximum Glacial expansion and the land mass was created using ArcGIS 10.2 (www.esri.com/arcgis) and adobe illustrator CS6 (www.adobe.com/products/illustrator.html).

Supplementary figure legends

Fig. S1. Phylogeographic hypotheses for genetic lineages of *R. macrosoma* explicitly tested using diyabc. Scenarios 1 and 2 correspond to gradual diversification from an ancient population. Pop 1: Crete Greece, Pop 2: Iberian Peninsula, Pop 3: Rest of Europe.

Fig. S3. Distribution of SNPs in the *coxI* region studied in *R. macrosoma*.

Fig. S4. Alignment of amino acid sequences from coxI haplotypes in *R. macrosoma*.

Fig. S5. Rarefraction analysis of *coxI* haplotypes.

Fig. S6. Isolation by distance using *coxI*. A) Plots showing the correlation between genetic distance as Edwards' distance (Euclidean) *vs* geographical distances using great-circle distances; B) MASS plot; C) Mantel test results using adegenet package.

Fig. S7. Molecular species-delimitation analysis of the *R. macrosoma* using the *coxI.* Two methods were used: generalized mixed Yule coalescent model (GMYC) and automatic barcode gap discovery (ABGD). Delimitation results are visualized as bars on an ultrametric Bayesian maximum clade credibility tree of the *coxI* gene. For the ABGD analysis, columns correspond to the 6 and 8 species groupings, respectively, as recovered for different prior intraspecific divergence assumptions. Bayesian posterior probabilities are indicated on the branches. GMYC was studied with using single and multiple type analysis.

Fig. S8. Molecular species-delimitation analysis of the *R. macrosoma* using the D2-D3 expansion segments of 28S rRNA. Two methods were used: generalized mixed Yule coalescent model (GMYC) and automatic barcode gap discovery (ABGD). Delimitation results are visualized as bars on an ultrametric Bayesian maximum clade credibility tree of the D2-D3 expansion segments of 28S rRNA. For the ABGD analysis, columns correspond to the 6 and 8 species groupings, respectively, as recovered for different prior intraspecific divergence assumptions. Bayesian posterior probabilities are indicated on the branches. GMYC was studied with using single and multiple type analysis.

Table 1. Nematode populations of *Rotylenchulus macrosoma* sampled and sequenced in this study.

Reference					Haplotype	
Populations number	**Sample code**	**Host-plant**	**Host-plant, locality, province**	***R. macrosoma* individuals/ 100 cm^3 soil**	**coxI***	**D2D3****
1	186105	corn	Njegosevo, Vojvodina, Serbia	231	coi-H3 (9)	28S-H5 (1)
2	186111	corn	Bečej, Vojvodina, Serbia	483	coi-H4 (1)	28S-H6 (1)
3	186107	corn	Novi Bečej, Vojvodina, Serbia	18	coi-H14 (5)	28S-H6 (1)
					coi-H15 (3)	28S-H12 (2)
						28S-H13 (2)
4	186231	corn	Nadlac, Arad, Romania	21	coi-H5 (3)	28S-H6 (1)
					coi-H6 (2)	28S-H10 (2)
5	186365	corn	Mircea Voda, Romania	91	coi-H11 (10)	28S-H6 (1)
6	186256	corn	Hajdúböszörmény, Hungary	104	coi-H4 (9)	28S-H6 (1)
7	184190	corn	Bonyhad, Tolna, Hungary	75	coi-H3 (8)	28S-H5 (1)
					coi-H7 (1)	
8	197260	corn	Bonyhad, Tolna, Hungary	62	coi-H3 (1)	-
9	197156	corn	Létavertes, Hajdú-Bihar, Hungary	480	coi-H10 (2)	28S-H8 (1)
10	197349	corn	Bagamer, Hajdú-Bihar, Hungary	629	coi-H3 (1)	28S-H4 (1)
					coi-H7 (1)	
					coi-H11 (1)	
11	197979	corn	Kondoros, Békés, Hungary	14	coi-H16 (5)	28S-H8 (2)
12	172745	wheat	Peregu Mare, Romania	620	-	28S-H9 (1)
13	185614	corn	Asola, Mantova, Italy	280	coi-H7 (1)	28S-H5 (1)
14	185343	corn	Roccabianca, Parma, Italy	479	coi-H3 (1)	28S-H5 (1)
15	185721	corn	Moretta, Cuneo, Italy	1760	coi-H3 (2)	28S-H5 (1)
					coi-H7 (7)	
16	ANT04	olive	Ancona, Ancona, Italy	321	coi-H3 (9)	28S-H5 (1)
					coi-H7 (2)	
17	185719	corn	Gaden, Bavaria, Germany	38	coi-H7 (11)	28S-H5 (1)
18	197227	corn	Möckmühl, Heilbronn, Germany	344	coi-H3 (1)	-
19	185601	corn	St. Padron de Conques, Aveyron, France.	4	coi-H3 (1)	28S-H5 (1)
20	186293	corn	Laurac, Languedoc-Rosellón, France	5	coi-H3 (1)	28S-H5 (1)
21	185733	corn	Neyron, Auvergne-Rhône-Alpes, France	22	coi-H3 (1)	28S-H5 (1)
22	184392	corn	Le Sen, Landes, France	313	coi-H3 (8)	28S-H5 (1)
23	172687	pea	Santarem, Santarem, Portugal	36	coi-H9(1)	28S-H7 (1)
24	184525	wheat	Bzenec, Moravia, Czech Republic	38	coi-H7 (7)	28S-H5 (1)
25	OLI087	olive	Istro, Crete, Greece	3	coi-H8 (8)	28S-H2 (2)
26	OLI038	olive	Hersonisos, Crete, Greece	13	coi-H1 (10)	28S-H1 (2)
27	OLI040	olive	Hersonisos, Crete, Greece	3	coi-H1 (11)	28S-H1 (1)
28	OlLI117	olive	Limnes, Crete, Greece	3	coi-H1 (9)	28S-H1 (2)
29	OLI119	olive	Limnes, Crete, Greece	8	coi-H1 (13)	28S-H1 (1)

30	ST079	olive	Huevar del Aljarafe, Sevilla, Spain	28	coi-H2(10)	28S-H3 (2)
31	J096	olive	Jerez de la Frontera, Cádiz, Spain	175	coi-H3(8)	28S-H5 (3)
32	AVER	hazelnut	Reus, Tarragona, Spain	24	coi-H3(7) coi-H13(3)	28S-H4 (1)
33	BAET	wild olive	Vejer de la Frontera, Cádiz, Spain	3	coi-H3(1)	28S-H5 (2)
34	ZARA	almond x peach	Montañana, Zaragoza, Spain	620	coi-H3(3)	-
35	185586	corn	Grenade, Haute-Garonne, France	21	coi-H3 (1)	28S-H5 (1)
36	197691	wheat	Mihail Kogalniceau, Romania	1711	coi-H11 (10)	-
37	197352	corn	Tépe, Hajdú-Bihar, Hungary	26	coi-H12 (2)	28S-H11 (2)

* coxI haplotype accessions: MT075822 (coi-H1); MT075823 (coi-H2); MT075824 (coi-H3); MT075825 (coi-H4); MT075826 (coi-H5); MT075827 (coi-H6); MT075828 (coi-H7); MT075829 (coi-H8); MT075830 (coi-H9); MT075831 (coi-H10); MT075832 (coi-H11); MT075833 (coi-H12); MT075834 (coi-H13); MT075835 (coi-H14); MT075836 (coi-H15); MT075837 (coi-H16). Number between parentheses indicate individuals detected in each haplotype.
** 28S haplotype accessions: MT084013 (28S-H1); MT084014 (28S-H2); MT084015 ((28S-H3); MT084016 (28S-H4); MT084017 (28S-H5); MT084018 (28S-H6); MT084019 (28S-H7); MT084020 (28S-H8); MT084021 (28S-H9); MT084022 (28S-H10); MT084023 (28S-H11); MT084024 (28S-H12); MT084025 (28S-H13). Number between parentheses indicate individuals detected in each haplotype.

Table 2, Overview of the frequency of the different coxI haplotypes found for *R, macrosoma* in different countries in Europe. Only included populations with ≥ 3 individuals sequenced.

	coi-H1	coi-H2	coi-H3	coi-H4	coi-H5	coi-H6	coi-H7	coi-H8	coi-H9	coi-H10	coi-H11	coi-H12	coi-H13	coi-H14	coi-H15	coi-H16	n	Pop[1]	*h*	*π*
Portugal									1								1	1	-	-
Spain		10	19										3				32	5 (1)	0,568 ± 0,063	0,032 ± 0,005
France			12														12	5 (4)	0,000 ± 0,000	0,000 ± 0,000
Italy			12				10										22	4 (2)	0,515 ± 0,052	0,011 ± 0,001
Germany			1				11										12	2 (1)	0,000 ± 0,000	0,000 ± 0,000
Hungary			10	9			2			2	1	2				5	31	7(1)	0.818 ± 0.039	0.035 ± 0.003
Czech Republic							7										7	1	0,000 ± 0,000	0,000 ± 0,000
Romania					3	2					20						25	3	0.353 ± 0.112	0,006 ± 0.002
Serbia			9	1										5	3		18	3 (1)	0,640 ± 0,080	0,034 ± 0,003
Crete, Greece	43							7									50	5	0,0219 ± 0,071	0,004 ± 0,001
Total	43	10	63	10	3	2	30	7	1	2	21	2	3	5	3	5	210			

The total number of specimens, populations (Pop), the haplotype diversity h and nucleotide diversity π per country are stated
h = haplotype diversity; *π* = nucleotide diversity
[1]Between parenthesis populations with only one individual sequenced because no other available individuals

Table 3. Pairwise F_{ST} values for the coxI dataset for *Rotylenchulus macrosoma* populations grouped by country of origin

	Spain	France	Italy	Germany	Hungary	Romania	Serbia	Greece
Spain	-							
France	0.16774	-						
Italy	0.22701*	0.30763	-					
Germany	0.43497*	1.00000*	0.44924*	-				
Hungary	0.24829*	0.33585*	0.28005*	0.33733*	-			
Romania	0.65936*	0.92049*	0.85808*	0.92274*	0.45543*	-		
Serbia	0.20877*	0.31067	0.33748*	0.49094*	0.01381	0.51145*	-	
Crete, Greece	0.88051*	0.96983*	0.95101*	0.96898*	0.87190*	0.96157*	0.90624*	-

*Comparisons were significant between the two pairs of countries ($P < 0.01$) indicating significant genetic differentiation

Table 4. *Rotylenchulus macrosoma*. Results of a hierarchical AMOVA for the coxI marker across 23 populations with at least 3 individuals studied (n ⩾ 3 reports) and 9 countries.

Source of variation[3]	d. f.	Sum of squares	Variance components	% of variation		*P*
All sequences						
Among populations	22	2387.5	12.745 (Va)	97.47		
Within populations	173	57.2	0.330 (Vb)	2.53		
Fixation Indices						
F_{ST} (within populations)	0.975				Va and F_{ST}	< 0.0001[a]
Countries[1]						
Among countries	8	1926.1	9.862 (Va)	69.71		
Among populations within countries	14	461.5	3.954 (Vb)	27.95		
Within populations	173	57.2	0.330 (Vc)	2.34		
Fixation Indices						
F_{CT} (among countries)	0.697				Va and F_{CT}	< 0.0001[b]
F_{SC} (among populations within countries)	0.923				Vb and F_{SC}	< 0.0001[c]
F_{ST} (within populations)	0.977				Vc and F_{ST}	< 0.0001[a]
Europe (Western *vs* Eastern countries)[2]						
Among groups	1	444.8	3.809 (Va)	25.31		
Among populations within groups	21	1942.7	10.909 (Vb)	72.49		
Within populations	173	57.2	0.330 (Vc)	2.20		
Fixation Indices						
F_{CT} (among groups)	0.253				Va and F_{CT}	0.0196[b]
F_{SC} (among populations within groups)	0.971				Vb and F_{SC}	< 0.0001[c]
F_{ST} (within populations)	0.978				Vc and F_{ST}	< 0.0001[a]

[1]Countries: Spain, France, Germany, Italy, Czech Republic, Romania, Hungary and Crete, Greece.
[2]Countries: Spain, France, Germany and Italy vs. Czech Republic, Romania, Hungary, and Crete, Greece
[a] Probability of obtaining equal or lower F value determined by 1,023 randomizations by permuting haplotypes among populations among countries or groups.
[b] Probability of obtaining equal or lower F value determined by 1,023 randomizations by permuting populations among countries or groups.
[c] Probability of obtaining equal or lower F value determined by 1,023 randomizations by permuting haplotypes among populations within countries or groups.

Supplementary tables

Table S1. Tajima-D and Fu-Fs tests for populations.

Table S2. Comparison of pairs of population for the Fst.

Table S3. Tajima-D and Fu-Fs tests for country.