Genetic Structure of *Xiphinema pachtaicum* and *X. index* Populations Based on Mitochondrial DNA Variation

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ABSTRACT

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The dagger nematodes *Xiphinema pachtaicum* and *X. index* are two of the most widespread and frequently occurring *Xiphinema* spp. co-infesting vineyards and other crops and natural habitats worldwide. Sexual reproduction is rare in these species. The primary objective of this study was to determine the genetic structure of *X. pachtaicum* and *X. index* populations using eight and seven populations, respectively, from different “wine of denomination of origin (D.O.) zones” in Spain and Sardinia (Italy), by studying mitochondrial (cytochrome oxidase c subunit I or *COI*) and nuclear (D2-D3 expansion segments of 28S rDNA)

markers. Both *Xiphinema* spp. showed low intraspecific divergence among *COI* sequences, ranging from 0.2% (1 base substitution) to 2.3% (10 substitutions) in *X. pachtaicum* and from 0.2% (1 base substitution) to 0.4% (2 substitutions) in *X. index*. Population genetic structure was strong for both species. Nevertheless, molecular differences among grapevine-growing areas were not significant, and intrapopulation diversity was very low. It is hypothesized that this genetic homogeneity in the nematode populations reflects their predominant parthenogenetic reproduction mode and low dispersal abilities. Our results also show that *X. pachtaicum* populations in Spain have possibly been established from two different populations of origin. Results also demonstrated that the two DNA regions studied are suitable diagnostic markers for *X. index* and *X. pachtaicum*.

Dagger nematodes of the genus *Xiphinema* are phytopathogenic species that damage a wide range of wild and cultivated plants through direct feeding on root cells and transmission of some plant viruses (50). This transmission is governed by a marked specificity between plant viruses and their *Xiphinema* spp. vectors. In fact, only 9 of the ≈250 known species of *Xiphinema* have been shown to transmit nepoviruses (genus *Nepovirus*, family *Comoviridae*) (11). The *Xiphinema americanum* group, which comprises a complex of ≈50 cosmopolitan species, seems to be one of the most difficult *Xiphinema* groups for diagnosis (9) and it includes the vectors of several important plant viruses, including *Tobacco ringspot virus*, *Tomato ringspot virus*, *Cherry rasp leaf virus*, and *Peach rosette mosaic virus*.

X. pachtaicum belongs to the *X. americanum* group and is the most widespread and frequently occurring *Xiphinema* sp. in agricultural land, shrub land, forest, and permanent pastures worldwide (27). In Spain, it is the most abundant *Xiphinema* sp. in vineyards and stone-fruit orchards (1,19). This species has fewer ecological requirements and a wider range of habitats and hosts than the other *Xiphinema* spp. (5,33). Reports of *X. pachtaicum* outside the Mediterranean Basin and Central Asia have been considered to be introductions by human activities, as was shown for those from the United States, Australia, and Chile (26,27,39,41).

Similarly, *X. index* is also a worldwide-distributed nematode in grapevine-growing areas (50). This species is thought to have been dispersed from the Middle East into other grapevine-growing regions of the world in soil accompanying propagation material and, today, has become an invasive agricultural soilborne pathogen (4,52). *X. index* severely damages grapevines by direct parasitism and as a vector of *Grapevine fanleaf virus*. Grapevine fanleaf is the most severe virus-induced diseases of grapevines worldwide and can cause important economic losses by reducing yield ≤80% and affecting fruit quality (29). In Spain, *X. index* is widespread in grapevine-growing areas in a wide range of soil textures and pH, even in soils with high a percentage of carbonates (2).

The prevalence of *X. pachtaicum* and the importance of *X. index* as a virus vector, together with the possibility of the latter being an introduced species and occupying a more restricted habitat than *X. pachtaicum*, make them interesting nematode species for the study of population genetics of plant-parasitic nematodes. Results from a recent phylogeographic study of *X. index* using the cytochrome B gene (*cytB*) showed that all haplotypes were shared among populations from Cyprus, Israel, Palestine, and Iran. Conversely, nematode populations from America (Chile and the United States) and Europe (France, Greece, Italy, and Spain) shared only one haplotype, suggesting that the haplotype distribution in *X. index* identified by *cytB* may be paralleled to the history of grapevine cultivation (53). Several studies have been conducted to determine the genetic variability and structure of soilborne plant pathogens, including bacteria, fungi, plant-parasitic nematodes, and viruses (32,35,40). Identification of biotypes or strains of plant pathogens (i.e., population variants) has important implications for understanding evolutionary processes, such as development of parasitic races, adaptation to host defenses, and development of resistance to pesticides

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(45). Knowledge of the genetic structure of plant pathogens may offer insights into the future evolutionary potential of the pathogen populations (30), as well as provide insights for their management using resistant cultivars (37). In plant-parasitic nematodes, intra- and interpopulation genetic variability as well as transfer of genetic material between populations by dispersal followed by successful reproduction (gene flow) at various spatial scales have been scarcely studied (37). However, recent population genetic studies have been conducted in amphimictic species such as *Heterodera schachtii* (37), *Globodera pallida* (36), and *Bursaphelenchus xylophilus* (54). Asexually reproducing root-knot nematodes (i.e., some *Meloidogyne* spp.) (17,24), and mainly parthenogenetic virus-vector nematodes (i.e., *X. index*) (52) do not undergo regular genetic recombination, and genetic variation results mainly from accumulation of mutations.

Mitochondrial DNA analysis has proven to be a powerful tool for assessing intraspecific phylogenetic patterns in plant-parasitic nematodes (24). The protein-coding mitochondrial gene, cytochrome oxidase c subunit 1 (*COI*), has been shown to be applicable for population-based studies in a range of invertebrates (22), for phylogeny of plant-parasitic nematodes (34), and for genetic barcoding (14). In contrast, nuclear genes such as the D2-D3 expansion segments of the 28S ribosomal DNA have a low mutation rate and have been successfully used for species diagnosis and resolving phylogenetic relationships among closely related nematode species (46,47). The application of molecular methods for plant-parasitic nematode population structure and systematics has revealed that long-time assumed single species are, in fact, complexes of cryptic species (i.e., species that are genetically distinct but share morphological similarities) (19,20,46).

The objectives of this research were to determine the population genetic structure and history of *X. pachtaicum* and *X. index*, and to investigate whether the molecular markers used can reliably identify both species. Both nematode species have a widespread distribution in grapevine-growing areas despite low dispersal abilities. Sampling design was performed on areas of leading grapevine-growing areas in Spain and Sardinia (Italy), and within a commercial grapevine orchard in southern Spain, to determine the potential gene flow between grapevine areas and the genetic diversity of the populations within these areas. Furthermore, both species are very difficult to distinguish using morphological features, and their identification requires sufficient expertise and time. Therefore, a molecular tool may greatly enhance the accuracy and speed of species-level identification.

MATERIALS AND METHODS

Field sampling and nematode populations. Eight populations of *X. pachtaicum* were sampled at eight locations in Spain (Table 1; Fig. 1). Seven populations of *X. index* were sampled at five locations in Spain and two locations at southern Sardinia Island, Italy; these two samples were kindly provided by V. A. Prota, Università degli Studi, Sassari, Italy. In Spain, all samples but two were from vineyards and every province represented a “wine denomination of origin (D.O.) zone,” including Condado de Huelva D.O. (Huelva province), Montilla-Moriles D.O. (Córdoba province), Jerez-Xérès-Sherry and Manzanilla-Sanlúcar de Barrameda D.O. (Cádiz province), Málaga D.O. (Málaga province), and Rioja D.O. (Logroño province). Córdoba-IAS and Córdoba-Aulaga populations were sampled on bitter orange at the Agricultural Research Station of the Institute of Sustainable Agriculture-CSIC (Córdoba) and at a fallow soil near Córdoba, respectively (Table 1). Rioja (Rioja D.O.) and Frigiliana (Málaga D.O.) samples are well separated geographically from the other samples by ranges of mountains ($\approx 1,500$ m high), while the remaining locations do not have obvious geographical barriers. For each sampled point, the geographic coordinates were recorded (Tables 1 and 2) by using a GPS II plus (Garmin Corporation, United Kingdom).

Additionally, the sample Moriles-393 (Córdoba province) was subsampled in three separate points of the commercial vineyard (at distances of 50 to 200 m) to determine the genetic diversity within this field. Studied points in this field were selected according to the higher abundance of the targeted nematode species (*X. pachtaicum* or *X. index*) (P. Castillo, C. Gutiérrez-Gutiérrez, J. Navas-Castillo, M. Teixeira Santos, B. B. Landa, and J. A. Navas-Cortés, unpublished data). In this field, for each subsample, the same number of specimens was collected ($n = 9$ for *X. pachtaicum* and $n = 13$ for *X. index*).

Nematode extraction and identification. Nematodes were extracted from soil samples by the sieving method (18). Ten specimens from every location were used for correct species identification by light microscopy using morphology and morphometrics (19). For that, nematodes were killed by gentle heating, fixed in a solution of 4% formaldehyde + 1% propionic acid, and processed to pure glycerin using Seinhorst's (43) method. Specimens were examined using a Zeiss III compound microscope with Nomarski differential interference contrast at powers up to $\times 1,000$ magnification. Measurements were made using a drawing tube attached to a light microscope.

TABLE 1. *Xiphinema pachtaicum* distribution of mitochondrial DNA *COI* haplotypes and D2-D3 sequences among sampling locations

Population localities	Coordinates	Accession numbers ^a		COI haplotypes						<i>n</i> ^b	<i>h</i> ^b	π ^b
		COI	D2-D3	PA	PA1	PB	PC	PD	PE			
Córdoba-IAS (Córdoba province, Spain)	37°51'37.28" N, -4°47'3.27" W	HM921369 (PA) HM921370 (PB)	HM921389 (PA) (Pach7) HM921390 (PB) (Pach5)	8	—	4	—	—	—	12	0.702	4.364
Moriles-393 (Córdoba province, Spain) ^c	37°27'21.99" N, -4°36'42.52" W	HM921371 (PC)	HM921391 (PC) (Pach3)	—	—	—	27	—	—	27	0	0
Frigiliana (Málaga province, Spain)	36°47'38.09" N, -3°54'43.87" W	HM921372 (PA)	HM921392 (PA) (pach7)	3	—	—	—	—	—	3	0	0
Jerez de la Frontera-401 (Cádiz province, Spain)	36°22'55.19" N, -6°11'18.89" W	HM921373 (PA) HM921374 (PD)	HM921393 (PD) (Pach2)	4	—	—	—	1	—	5	0.497	1.200
Jerez de la Frontera-500 (Cádiz province, Spain)	36°43'5.27" N, -6°13'3.94" W	HM921375 (PE)	HM921394 (PE) (Pach4)	—	—	—	—	—	12	12	0	0
Trebujena-506, (Cádiz province, Spain)	36°51'27.87" N, -6°11'31.22" W	HM921376 (PA)	HM921395 (PA) (Pach1)	8	—	—	—	—	—	8	0	0
Bollullos par del Condado-484 (Huelva province, Spain)	37°19'46.85" N, -6°32'27.35" W	HM921378 (PA1)	HM921396 (PA1) (Pach6)	—	8	—	—	—	—	8	0	0
La Guardia-Rioja (Logroño province, Spain)	42°32'29.6" N, -2°34'4.25" W	HM921379 (PC)	HM921397 (PC) (Pach7)	—	—	—	13	—	—	13	0	0
Total	...	...	...	23	8	4	40	1	12	89	...	...

^a GenBank accession numbers. Parentheses showed the correspondence of one specific *COI* haplotype and the corresponding D2-D3 sequence from the same individual and the specific haplotype for D2-D3 region.

^b Haplotype diversity (*h*) and nucleotide diversity (π) for each location; *n* = number of individuals analyzed.

^c Population sampled in three places with high infestation levels in the same field.

DNA extraction and polymerase chain reaction. Nematode genomic DNA was extracted from single adult females of each of the 15 *Xiphinema* populations. DNA extraction was performed as previously described (7). The portion of the *COI* gene was amplified as described by Derycke et al. (12) using primers JB3 (5'-TTTTTGGGCATCCTGAGGTTTAT-3') and JB4.5 (5'-TAAA GAAAGAACATAATGAAAATG-3') for *X. pachtaicum* and primers COI1XIPFW (5'-GATTCTTCGGACATCCTGATG-3') and COI1XIPRV1 (5'-CGAATACAGCTCCTATA-3') for *X. index*. These latter primers were designed by the authors using *X. index* expressed sequence tag sequence from the GenBank database

(CV581361) and amplified the same *COI* region as the first primer set but yielded a slightly shorter fragment (491 versus 456 bp, including primers). We additionally analyzed the D2-D3 region of 28S rDNA from nematode specimens having distinct *COI* haplotypes (except for sample Jerez de la Frontera-401) (Table 1), using primers D2A (5'-ACAAGTACCGTGAGGGA AAGTTG-3') and D3B (5'-TCGGAAGGAACCAGCTACTA-3') and amplification conditions described by Castillo et al. (7).

Single-strand conformation polymorphism analysis. In all, 3 to 39 *COI* polymerase chain reactions (PCRs) were processed for each *Xiphinema* population for single-strand conformation poly-

Fig. 1. Map of Iberian Peninsula and southern Europe showing geographic location and mitochondrial DNA haplotype frequencies of the populations of *Xiphinema pachtaicum* and *X. index*. Circles were divided proportionally to the number of different haplotypes found. Population codes: 116 = Montemayor-116; IAS = Córdoba-IAS; AUL = Córdoba-Aulaga; 393 = Moriles-393; RIO = La Guardia-Rioja; NR4 = Parteolla-NR4; VRM = Trexenta-VRM; FRI = Frigiliana; 506 = Trebujena-506; 499 = Jerez de la Frontera-499; 500 = Jerez de la Frontera-500; 401 = Jerez de la Frontera-401; 474 = Rociana-474; and 484 = Bollullos par del Condado-484. Dot symbol refers to *X. pachtaicum*, while star symbol refers to *X. index* populations.

TABLE 2. *Xiphinema index* distribution of mitochondrial DNA *COI* haplotypes and D2-D3 sequences among sampling locations

Population localities	Coordinates	Accession numbers ^a		COI haplotypes				<i>n</i> ^b	<i>h</i> ^b	π ^b
		COI	D2-D3	IA	IB	IC1	IC2			
Moriles-393	37°27'21.99" N, (Córdoba province, Spain) ^c	HM921380 (IA)	HM921398 (IA) (Index1)	39	—	—	—	39	0	0
Montemayor-116	37°38'32.57" N, (Córdoba province, Spain)	HM921381 (IA)	HM921399 (IA) (Index1)	3	10	—	—	13	0.465	0.769
Córdoba-Aulaga	37°53'36.66" N, (Córdoba province, Spain)	HM921382 (IB)	HM921400 (IB) (Index1)	13	—	—	—	13	0	0
Jerez de la Frontera-499	36°42'15.95" N, (Cádiz province, Spain)	HM921383 (IA)	HM921401 (IA) (Index1)	13	—	—	—	13	0	0
Rociana-474	37°19'24.13" N, (Huelva province, Spain)	HM921384 (IA)	HM921402 (IA) (Index2)	13	—	—	—	13	0	0
Parteolla-NR4	39°22'55.07" N, (Sardinia, Italy)	HM921385 (IA)	HM921403 (IA) (Index1)	13	—	—	—	13	0	0
Trexenta-VRM	39°03'40.38" W, (Sardinia, Italy)	HM921386 (IA)	HM921404 (IA) (Index1)	13	—	—	—	13	0	0
	39°31'32.42" N, (Sardinia, Italy)	HM921387 (IC1)	HM921405 (IC1) (Index1)	—	—	6	7	13	0.873	1.077
	—	HM921388 (IC2)	HM921406 (IC2) (Index1)	—	—	6	7	13	0.873	1.077
Total	...	...	...	94	10	6	7	117	...	...

^a GenBank accession numbers. Parentheses showed the correspondence of one specific *COI* haplotype and the corresponding D2-D3 sequence from the same individual and the specific haplotype for D2-D3 region.
^b Haplotype diversity (*h*) and nucleotide diversity (π) for each location; *n* = number of individuals analyzed.
^c Population sampled in three places with high infestation levels in the same field.

morphism (SSCP) analysis. Each *COI* PCR reaction was conducted using a single adult female as template. For SSCP analysis, 5 µl of *COI* PCR reaction was mixed with 5 µl of loading dye (99% formamide, 1% NaOH, and 1 M bromophenol blue), denaturalized for 10 min at 94°C, and immediately placed on a cooler block until loading on a nondenaturing MDE Gel Solution (Cambrex, Rockland) as described by the manufacturer (0.5 mm and 5% glycerol). Electrophoretic separations were carried out at 80 V and 4°C for 12 h. After electrophoresis, haplotypes were visualized by DNA silver staining following the protocol described by Caetano-Anollés and Gresshoff (6). Nematodes from each *Xiphinema* population were tested in different gels. The commercial molecular DNA marker O'GeneRuler (Fermentas, Burlington, ON, Canada) and a nematode sample (reference haplotype) were included in each gel to compare band positions amongst all gels.

DNA sequencing. *COI* and 28S rDNA PCR products were purified with the GeneClean turbo gel extraction kit (Q-BIOgene SA, Illkirch Cedex, France) according to the manufacturer's instructions and quantified using a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE). Two samples from nematodes showing different *COI* SSCP patterns were cloned into the pGEM-T Easy vector and transformed into JM109 High Efficiency Cells (Promega Corporation, Madison, WI). Purified PCR products of the 28S rDNA from individuals with different haplotypes were used for direct sequencing. Sequencing reactions were conducted in both directions using the same amplification primers for 28S rDNA and vector universal primers for clones containing *COI* sequences. A terminator cycle sequencing-ready reaction kit (BigDye; Perkin-Elmer Applied Biosystems) was used according to the manufacturer's instructions. The resulting products were purified and run on a DNA multicapillary sequencer (Model 3130XL genetic analyzer; Applied Biosystems, Foster City, CA) at the STABVIDA sequencing facilities (Monte da Caparica, Portugal). The sequences of partial *COI* and 28S rDNA of *X. pachtaicum* and *X. index* were deposited in the GenBank database under accession numbers HM921369 to HM921406 (Tables 1 and 2).

Data analysis. 28S rDNA and *COI* sequences from our *Xiphinema* populations and sequences deposited in GenBank were used for phylogenetic tree construction. *X. diffusum* was used as outgroup (AM086701 and AY601600). *COI* and D2-D3 sequences from GenBank from *X. index* (AY601628) and *X. pachtaicum* (GU222424, AY601607, and AY601606) were included in the analysis. The sequences for each gene were aligned with default parameters and manually edited using BioEdit (21). The best fit model of DNA evolution was obtained using the program jModeltest version 0.1.1 (38) and the Akaike Information Criterion (AIC) and was subsequently used in the phylogenetic analyses. The best models were TIM2 + I and TIM3 + G for *COI* and D2-D3, respectively. Phylogenetic analysis of the sequence data sets were performed with neighbor-joining (NJ) and maximum likelihood (ML) using PAUP* 4b10 (48), and Bayesian inference (BI) using MrBayes 3.1.2 (23). Support for each node was calculated using a bootstrap analysis with 1,000 and 100 replicates using a heuristic search for NJ and ML, respectively. BI analysis was initiated with a random starting tree and was run with four chains for 500×10^3 generations. The Markov chains were sampled at intervals of 100 generations. Two runs were performed for each analysis. The topologies were used to generate a 50% majority rule consensus tree.

Nucleotide (π) and haplotype diversity (h), were calculated for *COI* sequences using ARLEQUIN version 3.11 (15). The genetic structure of *X. pachtaicum* and *X. index* populations was analyzed using analysis of molecular variance (AMOVA) as implemented in ARLEQUIN, using the Tamura model to calculate the distance matrix. This model was most similar to that determined by jModeltest. AMOVA calculates the molecular variance and Φ

statistics among and within populations, and the significance of the variance components is tested by permuting haplotypes among populations (16). Population pairwise Φ_{ST} values were calculated using Tamura distances and corrected for multiple comparisons. Subsequently, we investigated whether grouping localities according to D.O. zones (group 1: Córdoba-IAS + Moriles-393; group 2: Jerez de la Frontera-500 + Trebujena-506 + Jerez de la Frontera-401) or to a north-south division (group 1: Córdoba-IAS + Moriles-393 + La Guardia-Rioja; group 2: Bollullos par del Condado-484 + Jerez de la Frontera-500 + Trebujena-506 + Jerez de la Frontera-401 + Frigiliana) would contribute significantly to the observed genetic variation for *X. pachtaicum*. For *X. index*, grouping was done according to country, D.O. zones (group 1: Moriles-393 + Montemayor-116 + Córdoba-Aulaga; group 2: Parteolla-NR4 + Trexenta-VNR) or to the north-south axis (group 1: Montemayor-116 + Córdoba-Aulaga + Moriles-393; group 2: Rociana-474 + Jerez de la Frontera-499). Finally, a statistical parsimony haplotype network was constructed using the program TCS version 1.21 (8) to visualize intraspecific relationships between *COI* haplotypes.

RESULTS

Sequence analysis. In total, 89 individuals of *X. pachtaicum* were analyzed for sequence variation within the 443-bp mitochondrial *COI* gene amplicon (removing primers), yielding six different haplotypes (Table 1). Pairwise divergences between the *COI* sequences ranged from 0.2% (1 base substitution) to 2.3% (10 substitutions), most of them being third-base transversions. No insertions or deletions occurred between the sequences from *X. pachtaicum*. For *X. index*, 117 individuals were analyzed for sequence variation within the 418-bp mitochondrial *COI* gene amplicon (removing primers), yielding four different haplotypes (Table 2). For *X. index*, pairwise divergences between the *COI* sequences ranged from 0.2% (one base substitution) to 0.4% (two substitutions), most of them being third-base transversions. Also, no insertions or deletions occurred between the sequences of *X. index*.

For the D2-D3 expansion segment, the differences showed a pattern similar to that for *COI*. The maximum differences for *X. pachtaicum* ranged from 0.1% (one base substitution) to 0.9% (seven substitutions) for an alignment of 760 bp whereas, for *X. index*, the differences consisted of only one nucleotide difference in position 682 for the population Jerez de la Frontera-499. However, nucleotide position 127 presented an ambiguous nucleotide (C/T) in the majority of the studied populations.

Phylogenetic analysis. All haplotypes were grouped according to their respective *Xiphinema* sp. (Fig. 2). For *X. pachtaicum*, *COI* sequences were grouped into two low-supported clades, one with haplotypes PC and PB and another with haplotypes PA, PA1, PD, and PE (Fig. 2). Surprisingly, *X. pachtaicum* (GU222424) was highly differentiated (23.3%, 106 to 107 substitutions) from our *X. pachtaicum* sequences. *COI* haplotypes of *X. index* were pooled into one highly supported clade (Fig. 2). The nuclear D2-D3 region nicely depicted each species, with one well-supported clade for *X. pachtaicum* and one for *X. index*.

Population genetic structure. AMOVA showed a pronounced structuring among populations in both species (*X. index*: $\Phi_{ST} = 0.66$, $P < 0.0001$; *X. pachtaicum*: $\Phi_{ST} = 0.88$, $P < 0.0001$). Populations of both species in most cases always contained a single haplotype (Fig. 3). Pairwise population comparisons for *X. pachtaicum* showed that most populations were significantly differentiated from each other, except for population Córdoba-IAS, which was strongly differentiated from Moriles-393 and La Guardia-Rioja but not from the other populations (Table 3). No significant amount of variation could be explained by grouping *X. pachtaicum* populations according to D.O. zones (38.53%, $F_{CT} = 0.385$, $P = 0.09$). In contrast, a large amount of variation could be

explained by grouping the populations according to a north-south axis (61.43%, $F_{CT} = 0.614$), albeit at a borderline nonsignificant level ($P = 0.053$). For *X. index*, pairwise population comparisons showed that the strong differentiation was caused by Montemayor-116 and Trexenta-VRM populations, which were highly differentiated from each other and from all other populations (Table 4). Interestingly, the second Sardinian population (Parteolla-NR4) was not differentiated from the other Spanish populations. In general, population differentiation was less pronounced in this species than in *X. pachtaicum*. Grouping *X. index* populations according to D.O zones, country, or north-south axis did not result in significant F_{CT} values (data not shown).

The intrafield variability study for both *Xiphinema* spp. (locality Moriles-393) (Tables 1 and 2) also showed a high homogeneity, because only one haplotype was found for each species: haplotypes PC and IA for *X. pachtaicum* and *X. index*, respectively.

Demographic history. The maximum parsimony network of *X. pachtaicum* shows that haplotypes PB and PC have accumulated mutations compared with the other haplotypes. These two haplotypes almost exclusively occur in the northern populations. However, one individual from Bollullos par del Condado (Huelva province), not included in this study because only a single individual was sampled, also contained the same haplotype (PC). The other four haplotypes differ in only one or two mutations, and were found in the southern populations. One exception is haplotype PA, which also occurred in the Córdoba-IAS population. For *X. index*, only one to two mutations occurred between the haplotypes, and haplotype IA was found in almost all populations sampled.

DISCUSSION

For plant-parasitic nematodes, intra- and interpopulation genetic diversity, as well as gene flow at various spatial scales, are poorly

known (37) and differ between nematodes and sampled areas. *G. pallida* showed a high genetic structure coupled with isolation by distance in the Andean Cordillera but absence of genetic structure in agronomic areas (36). Another study found low genetic differentiation for *H. schachtii* between fields and regions (37). Research on some *Meloidogyne* spp. using different markers and sampling scales indicated a low differentiation in mitochondrial haplotypes (24) or a panmictic structure using amplified fragment length polymorphism markers (17). These different genetic structures preclude general conclusions for plant-parasitic nematode species and warrant individual studies for each species. A comparative population genetic structure between closely related species, as in our study, could help to better understand species distributions, connectivity between regions, and human influences in the actual species distribution.

The primary objective of this study was to determine the genetic variation and genetic differentiation in *X. pachtaicum* and *X. index*, two nematode species that are widespread in grapevine-growing areas worldwide. These two species probably have different geographical origins, predominantly reproduce by parthenogenesis, and show low dispersal abilities. The strong genetic structuring observed in both *Xiphinema* spp. is in agreement with these biological characteristics. Nevertheless, the two species showed different levels of genetic diversity, with *X. index* exhibiting fewer haplotypes and mutations, despite the larger geographical sampled area. *X. index* probably originated in the Middle East and was later introduced in different Mediterranean areas (4,51). If *X. index* has been introduced to Spain, then this introduction was widespread and most likely originated from the same source population, because the same haplotype (IA) was shared among almost all populations. In contrast, *X. pachtaicum*, one of the most widely distributed *Xiphinema* spp. in Europe (4), showed a large number of mutations between haplotypes PB and PC on the one hand and the other four haplotypes on the other hand. This,

Fig. 2. Bayesian inference (BI) phylogenetic trees based on mitochondrial DNA *COI* and D2-D3 expansion segments of 28S for haplotypes detected in *Xiphinema pachtaicum* and *X. index* populations. Support values are represented on the tree as neighbor-joining (NJ), BI, or maximum likelihood (ML). Haplotype names are as in Tables 1 and 2. Names between parentheses are the individuals with a specific *COI* haplotype.

The low diversity of haplotypes suggests that both *X. pachtaicum* and *X. index* have a recent origin in Spain. Their widespread distribution across Spain may then be the result of passive dispersal through agricultural activities (infested soil or infected plant-propagating material). This would explain the high similarity between Frigiliana and Rioja populations and some other

populations that are separated by high ranges of mountains and long distances ($\approx 1,000$ km north to south). Consequently, human activities (49) or other passive dispersal agents (animals, birds, wind, and so on) (31) may be an important factor for the observed geographical distribution of these nematode species. None of the genetic differences among the grapevine-growing areas could be explained by extensive and continuous grapevine cultivation in the sampled geographic areas or by inadequate sanitation practices during long periods of time.

The low overall genetic variability within populations and strong genetic subdivision between *Xiphinema* populations is likely to be caused by the parthenogenic mode of reproduction, which is expected to reduce variation within and increase variation between populations. In the absence of gene flow, local selective sweeps will remove variation within populations but will not remove variation between populations (42). The pronounced genetic structure indicated by AMOVA suggests an overall low gene flow among our *Xiphinema* populations. This result agrees with other *X. index* phylogeographic studies using more variable markers (microsatellites), in which no isolation by distance was observed at the grapevine field scale but a strong differentiation among sampling points within the same grapevine field was detected (52). Villate et al. (52) suggest that this pattern is consistent with the clonal reproductive mode and the limited capacities of active dispersal of *Xiphinema* spp. leading to a patchy spatial distribution within grapevine fields (52,53). The lack of intrafield variability in our study is probably the result of using a mitochondrial gene instead of highly variable microsatellites, as were used by Villate et al. (52). Mitochondrial markers (*CytB*) used in other studies also showed large homogeneity in Western Europe (51).

Next to clonality and low dispersal, a strong genetic structuring among populations may also be explained by extinction and recolonization events with few individuals. These colonization-extinction dynamics may produce distinct genetic differentiation at very small geographical scales, as has been demonstrated in free-living rhabditid nematodes inhabiting ephemeral resources (3,10,13,44). However, the *Xiphinema* populations here are not ephemeral and, in view of their limited dispersal ability and widespread distribution across large geographic distances, the effects of metapopulation dynamics will probably be unimportant. Additionally, an extinction-recolonization model would be difficult to reconcile with the mix of highly differentiated haplotypes (PA and PB) in Córdoba.

Our second aim was to investigate the molecular identification of both species using the *COI* and D2-D3 rDNA fragments. Molecular discrimination among species in the *X. americanum* group is very important because some species are known to be vectors of plant viruses (50). The correspondence of tree topologies from mitochondrial and nuclear genes has been used to delineate cryptic nematode species (12). In our findings, both *Xiphinema* spp. are well separated by the two molecular markers, which is in agreement with a broader phylogeny of the family *Longidoridae* (19). Although *COI* did not show enough resolution to distinguish among the majority of the *X. americanum* species complex, it was useful in separating *X. simile* (AM086708, AM0867113), *X. pachtaicum* (AM086710), and *X. americanum* from Portugal (AM086709) (28). Nevertheless, sequence AM086710 of *X. pachtaicum* was considered to be a pseudogene (indicated in the National Center for Biotechnology Information) and clearly differed from all the sequences in the present study. Surprisingly, *COI* sequences obtained by Kumari et al. (25) from specimens collected in the Czech Republic differ in as much as 106 to 107 nucleotides with our *COI* sequences. In our study, *X. pachtaicum* was identified using morphological, morphometrical, and molecular data for each nematode population. All our data agree with measurements from original descriptions as well as with other molecular markers such as D2-D3 deposited in

GenBank (AY601606). Additionally, internal transcribed spacer (ITS)1 sequences from some of our populations also agree with the ITS1 sequences deposited in the GenBank (AY430178) (19). In addition, another D2-D3 sequence (AY601607) assigned to *X. pachtaicum* from Eastern Europe (Moldavia) suggests that close morphometrical species may be present in this region and, for this reason, additional sequences from the same population may clarify *X. pachtaicum* species definition. In view of the possible problems with *COI* (pseudogenes and low resolution), the addition of the D2-D3 region of the 28S rDNA may provide a reliable tool to correctly identify *X. pachtaicum* and differentiate it from *X. index*.

In summary, this work represents the first population genetic study of two worldwide distributed plant-parasitic nematode species co-infesting similar crops and habitats using the *COI* gene. Several conclusions can be drawn from a phytopathological point: (i) both *Xiphinema* spp. showed low levels of molecular diversity at the intrapopulation level; however, this diversity was lower in *X. index* than *X. pachtaicum*; (ii) *X. pachtaicum* populations in Spain have two distinct pools of origin; (iii) populations of both *Xiphinema* spp. had a strong genetic structure which is most likely the result of their clonal reproduction; and (iv) *COI* and 28S regions are suitable diagnostic markers for both *Xiphinema* spp.

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