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Characterisation of populations of *Longidorus orientalis* Loof, 1982 (Nematoda: Dorylaimida) from date palm (*Phoenix dactylifera* L.) in the USA and other countries and incongruence of phylogenies inferred from ITS1 rRNA and *coxI* genes

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Summary – Needle nematode populations of *Longidorus orientalis* associated with date palm, *Phoenix dactylifera*, and detected during nematode surveys conducted in Arizona, California and Florida, USA, were characterised morphologically and molecularly. The nematode species most likely arrived in California a century ago with propagative date palms from the Middle East and eventually spread to Florida on ornamental date palms that were shipped from Arizona and California. This is the first validated continental record of this needle nematode species in the USA and the Americas. The USA populations of *L. orientalis* contained a small number of males that were not reported in the original description and are herein described. *Longidorus orientalis* was able to survive for at least 4 years at very low numbers in the warm and humid environment of Florida on date palms imported from California and Arizona. Association of *L. orientalis* with *L. africanus* was observed in all of the surveyed sites, indicating that date palm is a host of both nematodes. Phylogenetic relationships of *L. orientalis* with closely related *Longidorus* species, in addition to relationships between populations of *L. orientalis* from the USA, Greece, Iran and Spain, were inferred from the analyses of D2-D3 of 28S rRNA, ITS1 rRNA and partial *coxI* gene sequences. The PCR-D2-D3 expansion segments of 28S rDNA-RFLP diagnostic profile is provided. *Longidorus orientalis* populations display a high level of intraspecific variation (up to 15.5%) in *coxI* mtDNA sequences. Analysis of phylogenetic relationships of nematode populations revealed incongruence of the ITS1 rRNA and *coxI* mtDNA gene trees, which might be the result of selective introgression of mtDNA through gene flow between previously isolated populations introduced simultaneously into new geographical regions.

Keywords – 28S rRNA gene, Arizona, California, *coxI* of mtDNA, Florida, Greece, Iran, ITS1 rRNA gene, *Longidorus africanus*, morphology, mtDNA introgression, needle nematode, PCR-RFLP, phylogeny, Spain.

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In Florida, needle nematodes associated with date palm, *Phoenix dactylifera* L., have been detected since 1989 in palm shipments originating from California. Some of these needle nematodes were identified as *Longidorus africanus* Merny, 1966, which is a serious parasite of vegetable crops in Southern California (Lehman, 2002). However, other populations, morphologically different from *L. africanus*, have been found alone or in a mixture with *L. africanus* in palm shipments from California (Lehman, 2002). Females of these populations had a conoid lip region and hemispherical tails unlike the rounded lip region and elongated-conoid tails of *L. africanus* and were ascribed, in the records of the Florida Department of Agriculture and Consumer Services (Lehman, 2002), to different taxa, including *L. belloi* Andrés & Arias, 1988, *L. belondiroides* Heyns, 1966 and *L. orientalis* Loof, 1982. These identifications were recorded, in Florida, for many years without any diagnostic validation using molecular and supplemental morphological analyses. Among these tentatively identified species, only *L. orientalis* has been reported as a potential parasite of date palm. This needle nematode species, native to the Middle East, was originally described from declining date palms in Saudi Arabia and grapevine (*Vitis* sp.) in Iraq (Loof, 1982). Subsequently, this species was reported to be associated with citrus (*Citrus* sp.), date palms and fig (*Ficus* sp.) trees in Iran (Noruzi & Barooti, 2005), unidentified graminaceous plants and grapevines in Spain (Palomares-Rius *et al.*, 2010) and, recently, with grapevines in Greece (Crete) (Tzortzakakis *et al.*, 2014).

The intensification of the date palm trade between Arizona, California and Florida and an increase in federal funding for the inspection of plant shipments entering Florida have resulted in a greater number of detections of needle nematodes and other nematode species at Florida inspection stations. The bill of lading documents that accompanied the date palms shipped to Florida and found to be infested with needle nematodes, indicated that the centres of origin of these needle nematode populations in the USA are located in commercial date palm orchards and nurseries in Riverside and Imperial Counties, California, and Yuma County, Arizona. In California, many studies (Lamberti, 1968; Radewald *et al.*, 1969; Kolodge *et al.*, 1987; Ploeg, 1998) have been conducted on *L. africanus*, a needle nematode native also to Middle East. However, this nematode species has not been reported on date palm in California and its association with other *Longidorus* species on California date palms has remained unnoticed for decades. It is possible, that these needle nematodes arrived in California as early as 1912 with shipments of potted date palm offshoots imported from the Middle East (Morton, 1987). Nevertheless, there is a need to ascertain the taxonomic status of date palm-associated needle nematodes other than *L. africanus* in California and Arizona and to obtain more information on the ability of these nematodes to survive in geographical areas climatically different from the arid conditions of Riverside and Yuma counties in California and Arizona, respectively. Herein, we present the results of a morphological and biological study on date palm-associated needle nematode populations from Arizona, California and Florida in order to: *i*) ascribe needle nematode populations, other than *L. africanus*, from date palms in California and Arizona to the correct taxon, using morphological and morphometric analysis; *ii*) provide molecular characterisation of these populations of *Longidorus* species using sequences of the D2-D3 expansion segments of the 28S nuclear ribosomal RNA, the ITS1 of rRNA and partial *coxI* of mtDNA genes; and *iii*) analyse phylogenetic relationships within the American (California and Arizona) populations and other molecularly and morphologically similar populations from distant geographical areas using rRNA and *coxI* gene sequences and their congruence with morphological characters.

Materials and methods

Nematode populations

During a survey initiated in 2013, populations of *Longidorus* spp. were obtained from soil samples collected from the rhizosphere of date palms grown in commercial date palm orchards located in Riverside and Imperial Counties, California, and in Yuma County, Arizona. Major date palm commercial farms are located in these counties. In these farms, large date palms at the end of their productive cycle are sold as landscape trees and shipped mainly within the USA. In 2014, a total of seven rhizosphere soil samples were collected at two sites in Bard and Thermal in Imperial County, California (Table 1). An additional sample was collected in Coachella Valley, Riverside County, California. Three samples in the Bard area comprised loamy soil and four in the Thermal area were sandy (three) or loamy (one) soils. No field samples were collected from any localities in Arizona. However, *Longidorus* spp. were obtained from soil and roots samples removed from the root balls of imported Arizona and Cali-

Table 1. Populations of *Longidorus orientalis* used in the present study.

Locality	Host	Sample code	GenBank accession number			Collector or identifier
			D2-D3 of 28S rRNA	ITS-rRNA	*coxI*	
Greece, Crete, Heraklion Province, P. Elias	*Vitis vinifera*	O44	KJ802877	KP406935	KP406963	E.A. Tzortzakakis
Iran, Khuzestan Province, Ahvaz, sample 2	*Phoenix dactylifera*	CD1562	–	KP406948	KP406962	Z. Tanha Maafi
Iran, Khuzestan Province, Ahvaz, sample 3	*P. dactylifera*	CD1563	–	KP406946	KP406964	Z. Tanha Maafi
Iran, Khuzestan Province, Ahvaz, sample 5	*P. dactylifera*	CD1564	–	KP406947	KP406965	Z. Tanha Maafi
Spain, Murcia Province, Alhama de Murcia	*V. vinifera*	P28	KP406931	KP406936	KP406951	P. Castillo
Spain, Huelva Province, Bollullos par del Condado	*V. vinifera*	N10	KP406933	–	KP406949, KP406950	P. Castillo
Spain, Huelva Province, El Rocío	Gramineae	R44	GU001823	GU001821, GU001822	KP406961	P. Castillo
USA, Arizona, Yuma County, Somerton and Yuma (detected in Florida)	*P. dactylifera*	CD1550, N13-01176-77	–	KP406939	KP406954	R.N. Inserra; J.D. Stanley
USA, Arizona, Yuma County, Yuma (detected in Florida)	*P. dactylifera*	CD1553, N14-00082	–	KP406940	KP406952	R.N. Inserra; J.D. Stanley
USA, Arizona, Yuma County, Yuma (detected in Florida)	*P. dactylifera*	N14-00509	–	–	–	R.N. Inserra; J.D. Stanley
USA, California, Imperial County, Bard	*P. dactylifera*	CD1437	KP406934	KP406938	–	A. Ploeg
USA, California, Riverside County, Coachella Valley	*P. dactylifera*	CD1537	–	KP406943, KP406944	KP406957, KP406958	A. Ploeg
USA, California, Imperial County, Bard (detected in Florida)	*P. dactylifera*	CD1379, N13-01062	KP406932	KP406941	KP406955	R.N. Inserra; J.D. Stanley
USA, California, Imperial County, El Centro (detected in Florida)	*P. dactylifera*	CD1661, N14-01143	–	KP406937, KP406942, KP406945	KP406956, KP406959, KP406960	R.N. Inserra; J.D. Stanley
USA, California, Imperial County, El Centro (detected in Florida)	*P. dactylifera*	CD1671, N14-01225	–	–	KP406953	R.N. Inserra; J.D. Stanley

fornia date palms intercepted at the Florida inspection stations.

During a survey in 2013-2014, a total of 258 soil samples were collected from the rhizosphere of date palms that had been imported from Arizona and California and transplanted in central and south-western Florida for at least 2-4 years. The survey was conducted in order to monitor the introduced *Longidorus* spp. populations previously found associated with the imported date palms. Soil samples from California and Florida were collected from the upper 10-40 cm of soil around roots of date palms, using a shovel or sampling tube. At the Florida inspection stations, soil and roots were removed from the root balls of date palms using a trowel or sampling tube. Nematodes were extracted from samples of variable size (400-1200 cm^3 of soil) by the Cobb (1918) decanting and sieving method followed by rapid centrifugal-flotation method (Jenkins, 1964). Nematodes from samples collected in California were picked directly from the water suspension obtained after decanting and sieving the soil. Nematode densities were expressed as specimens in 100 cm^3 of soil. Specimens from Arizona and Imperial County, California were permanently mounted in anhydrous glycerin and used for morphological and molecular analyses. Heat-killed specimens extracted from soil samples collected from date palms transplanted in Florida for at least 2-4 years were morphologically identified in water using a compound microscope.

Specimens of *L. orientalis* from the populations collected in Riverside County, California, and those originating from Imperial County, California, and extracted from samples collected at Florida inspection stations were used only for molecular analyses. The population from Thermal, California, was not characterised molecularly and identified only morphologically. The *L. africanus* sample collected from date palms imported from Arizona, Yuma County, Yuma, was analysed only molecularly.

Several specimens of needle nematodes identified as *L. orientalis* and morphologically similar to one of the USA populations of *Longidorus* spp. were obtained from Spain, Iran and Greece (Crete) and included in this study (Table 1). These additional populations, already characterised morphologically, were compared with the USA populations. They also were included in additional molecular analyses and phylogenetic studies along with the populations from Arizona and California.

Morphological analysis

Specimens for morphological analysis and light microscopy (LM) were killed by gentle heat, fixed in a solution of 4% formaldehyde + 1% propionic acid or FPG (Netscher & Seinhorst, 1969), processed to pure glycerin using Seinhorst's (1962) method and mounted on permanent slides. Light micrographs were taken with an automatic Infinity 2 camera attached to a compound Olympus BX51 microscope equipped with a Nomarski differential interference contrast and an automatic MicroPublisher 5.0 RTV camera attached to a compound Leica microscope. Measurements were made with the same microscope and a compound Leitz Ortholux II research microscope equipped with a drawing tube/camera lucida or an ocular micrometer.

DNA extraction, PCR assays and sequencing

DNA was extracted from individual nematodes. Protocols of DNA extraction with proteinase K, PCR and sequencing were described by Tanha Maafi *et al.* (2003). The following primer sets were used for amplification: D2-D3 expansion segments of 28S rRNA gene with forward D2A (5′-ACA AGT ACC GTG AGG GAA AGT TG-3′) and reverse D3B (5′-TCG GAA GGA ACC AGC TAC TA-3′) primers (Rubtsova *et al.*, 2001), ITS1 with forward rDNA2 (5′-TTG ATT ACG TCC CTG CCC TTT-3′) (Vrain *et al.*, 1992) and reverse rDNA5.8S (5′-ACG AGC CGA GTG ATC CAC CG-3′) primers (Cherry *et al.*, 1997) and *coxI* with either forward COIF (5′-GAT TTT TTG GKC ATC CWG ARG-3′) (He *et al.*, 2005a) or forward L_orient_COIF (5′-GAA GTT TAC ATT YTA ATT YTA CCC-3′) (this study) and either reverse XIPH1 (5′-ACA ATT CCA GTT AAT CCT CCT ACC-3′) (Lazarova *et al.*, 2006) or mixture of reverse COX_LongR1 (5′-AAT ACA ATT CCA GTT AAT CCT CC-3′) with reverse COX_LongR2 (5′-AAW ACA ATR CCA GTA AGA CCT CC-3′) primers (this study). Two μl of the PCR product was run on a 1% TAE buffered agarose gel. PCR products were purified after amplification with QIAquick (Qiagen) gel extraction kit and used for direct sequencing. New sequences of *L. orientalis* and *L. africanus* were deposited in GenBank under accession numbers KP406931-KP406965 and KP711809, respectively.

Sequence and phylogenetic analysis and structure reconstruction for ITS rRNA

The new sequences of D2-D3 of 28S rRNA, ITS1 rRNA and *coxI* of mtDNA genes were aligned using

ClustalX 1.83 with default parameters with their corresponding published gene sequences (Kumari *et al.*, 2009; Palomares-Rius *et al.*, 2010; Gutiérrez-Gutiérrez *et al.*, 2013; Tzortzakakis *et al.*, 2014). Outgroup taxa for each data set were chosen according to the results of previously published data (Gutiérrez-Gutiérrez *et al.*, 2013; Subbotin *et al.*, 2013).

Sequence data sets were analysed with Bayesian inference (BI) using MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001). BI analysis for each gene was initiated with a random starting tree under the GTR + I + G model and was run with four chains for 1.0×10^6 generations. The Markov chains were sampled at intervals of 100 generations. Two runs were performed for each analysis. After discarding burn-in samples and evaluating convergence, the remaining samples were retained for further analysis. The topologies were used to generate a 50% majority rule consensus tree. Posterior probabilities (PP) are given on appropriate clades. The *coxI* sequence alignment was used to construct a phylogenetic network estimation using statistical parsimony with the TSC software (Clement *et al.*, 2000).

Mfold software Version 3 (Zuker, 2003; available online at http://mfold.rna.albany.edu/?q=mfold/RNA-Folding-form) was applied to predict the secondary structures for the D3 expansion region of 28S rRNA and the ITS1 rRNA using the energy minimisation approach. Structures were visualised using VARNA (Darty *et al.*, 2009) and drawn with Adobe Illustrator CS6 and Adobe Photoshop CS6.

PCR-D2-D3-RFLP

The PCR product of D2-D3 of rDNA was digested by one of the following restriction enzymes: *Alu*I, *Hinf*I, *Bsp*143I (*Mbo*I), *Tru1*I (*Mse*I) or *Rsa*I (Fermentas International) (Subbotin *et al.*, 2013) in the buffer stipulated by the manufacturer. Digested DNA was run on a 1.4% TAE buffered agarose gel, stained with ethidium bromide, visualised on UV transilluminator and photographed. The length of each restriction fragment from the PCR products was predicted by a virtual digestion of the sequences using WebCutter 2.0 (available online at http://www.firstmarket.com/cutter/cut2.html).

Results and discussion

Morphological analysis

Morphological examination of Arizona, California and Florida *Longidorus* spp. samples revealed that they consisted of two different species identified as *L. africanus* and *L. orientalis*. *Longidorus africanus* is a well-known, economically damaging needle nematode species in California. The morphological identification of *L. africanus* was based on voucher specimens and the identification key for *Longidorus* spp. by Chen *et al.* (1997) and validated by the results of the molecular analysis. Morphological identification of *L. orientalis* populations was based on the characters originally reported by Loof (1982). For this study, populations of *L. africanus* were not characterised morphologically and molecularly since *L. africanus* is a well-defined species that is common in Southern California. Morphological and molecular analyses of *L. orientalis* populations were conducted as this species was not previously reported in the USA. All *L. orientalis* populations comprised mostly females and juveniles. A few males were found in the Arizona and California populations detected at the Florida inspection stations. Morphological and morphometric characterisation of populations of *L. orientalis* from the USA and from other geographical areas are given in Figures 1 and 2 and Table 2.

Morphological characteristics of Arizona and California *L. orientalis* populations

Female

Morphological variability was observed between the Arizona and California populations and paratypes from the original description of *L. orientalis*. The American populations of *L. orientalis* had a smaller body length similar to the female specimens described from Greece (Tzortzakakis *et al.*, 2014), but differed from the latter by lower c ratio and greater c′ ratio values (Table 2). The range of values of other characters were similar to those of *L. orientalis* reported in the original (Loof, 1982) and subsequent descriptions of the species (Palomares-Rius *et al.*, 2010; Tzortzakakis *et al.*, 2014). The presence of a symmetrically bilobed amphidial fovea and an almost uniformly cylindrical body shape in the Arizona and California populations indicated that they should be considered representatives of *L. orientalis*. The reproductive system was very similar to that illustrated in the original description except that the latter lacked details of the spermatheca and the sphincter between the spermatheca and the uterus. Although these organs were usually obscured in mature females belonging to the Arizona and California populations with well-developed and reflexed ovaries, they were observed in a young and non-gravid female. This female showed a slit-like vulval aperture,

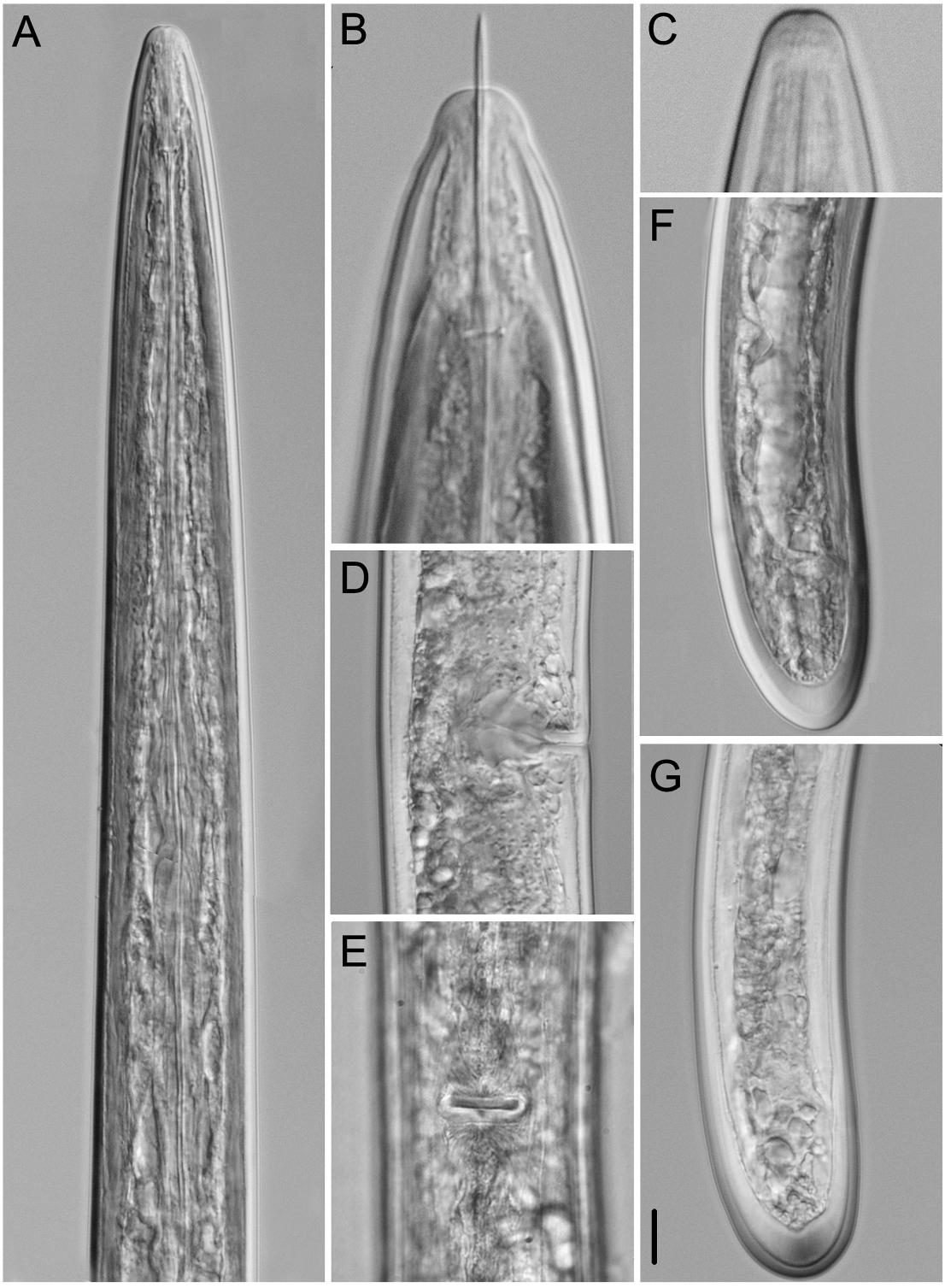

Fig. 1. Light micrographs of *Longidorus orientalis* female from California. A-C: Anterior region; D, E: Lateral and ventral view of vulval region; F, G: Tail shape variation. (Scale bar: A, D, E = 11 μm; B, C = 5.5 μm; F, G = 12 μm.)

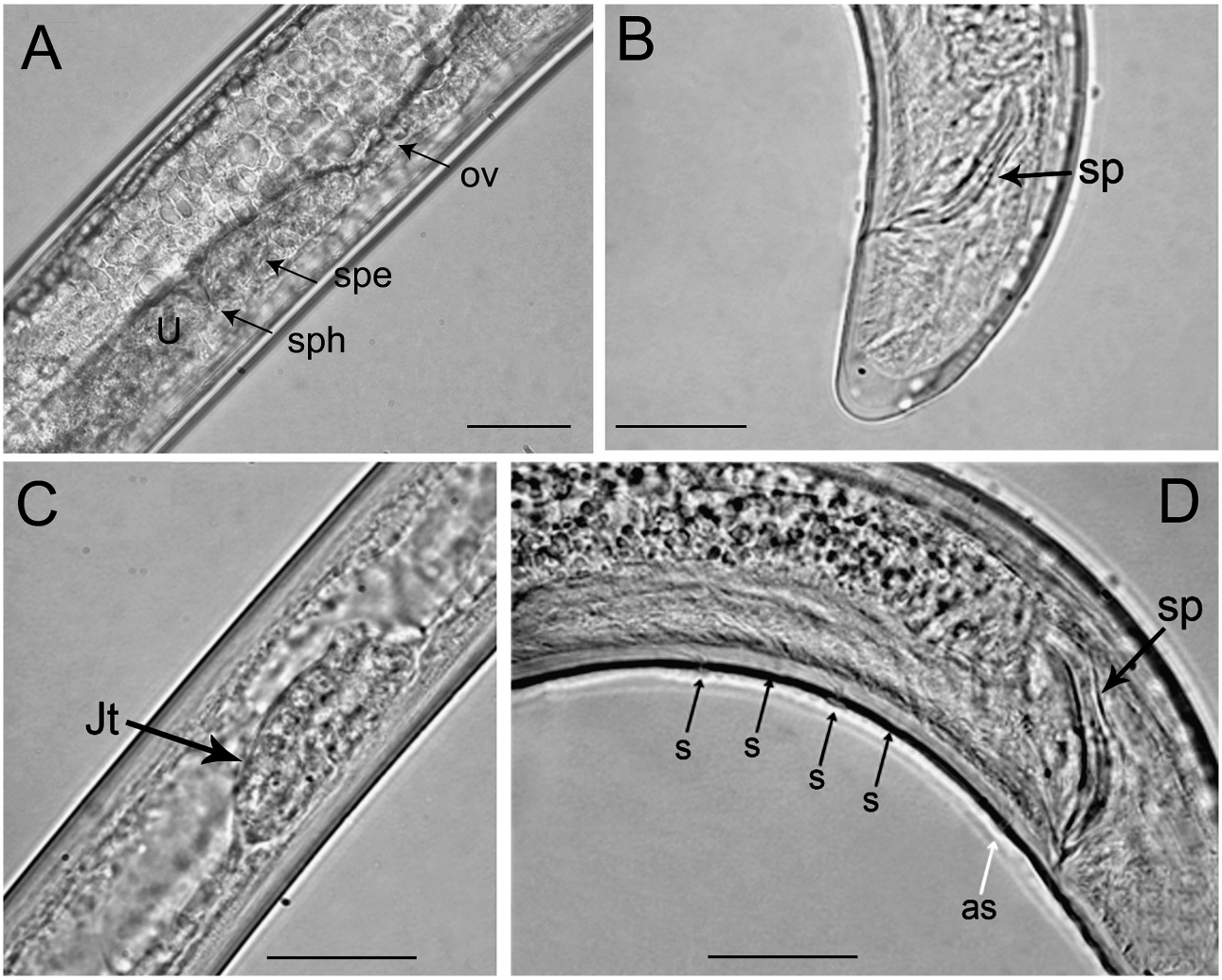

Fig. 2. Light micrographs of *Longidorus orientalis* female and male from Arizona. A: Portion of female gonad showing ovary (ov), a pear-like spermatheca (spe), sphincter (sph) and distal portion of uterus (U); B: Posterior body region of male showing spicules (sp); C: Middle body portion of male showing junction of both testes with *vas deferens* (Jt); D: Posterior body portion of males showing spicules (sp) and adcloacal (as) and four ventro-median pairs of supplements (s) arranged in a staggered row. (Scale bar = 20 μm.)

which was 14.5 μm long and delimited by prominent lips about 2.5 μm in thickness. The posterior branch of the genital tract consisted of a short ovary connected to a long tubular oviduct ending in a pear-shaped spermatheca, 35 μm long and 15 μm wide, containing few nucleate cells resembling sperm. A sphincter joined the spermatheca to a large uterus, which was connected through a short vagina to the vulval opening.

An undescribed single male specimen of *L. orientalis* was reported in Iran (Noruzi & Barooti, 2005). In this study, three males were found in the Arizona population and one in the California population. The ratio of male to females was 1:30. The morphological description of the male is given here.

Male

Body cylindrical, straight for most of its length and ventrally arcuate in distal posterior portion when killed by gentle heat. Cuticle 2.4 μm at mid-body and 3.4 μm thick in posterior portion of body. Lateral chord 13.8 μm wide, *ca* 25% of body diam. Pores not discernible in anterior and central part of body, but six pairs, four subventral and two subdorsal observed on tail. Labial re-

Table 2. Morphometrics of *Longidorus orientalis* from USA and other countries. Measurements are in μm and in the form: mean ± s.d. (range).

Character	Location						
	Arizona, USA	Arizona, USA	California, USA	Greece	Spain	Iran	Saudi Arabia
	N14-0082-84	N14-00509	CD1437	Tzortzakakis *et al.* (2014)	Palomares-Rius *et al.* (2010)	Palomares-Rius *et al.* (2010)	Loof (1982)
n	10 ♀♀	3 ♂♂	17 ♀♀	1 ♀	18 ♀♀	6 ♀♀	10 ♀♀
L	4236 ± 261 (3805-4554)	3567 ± 533 (3210-4180)	4182 ± 411 (3580-4840)	4068	4547 ± 192 (4356-4900)	4833 ± 546 (4150-5600)	4040-5030
a	94.6 ± 7.5 (82.5-106)	86.7 ± 5.5 (81.0-92.0)	91.6 ± 7.9 (79.3-103.3)	80.6	97.9 ± 4.8 (91.8-105.1)	90.7 ± 8.8 (75.4-100)	82-105
b	12.0 ± 0.7 (11.0-13.7)	11.7 ± 3.9 (8.4-16.0)	10.9 ± 1.5 (8.8-13.9)	11.8	13.9 ± 2.0 (12.0-17.9)	9.4 ± 1.23 (8.1-11.1)	9.3-13.5
c	121.0 ± 9.4 (104-134)	97.3 ± 11.0 (90-110)	116.6 ± 12.0 (100.5-145.0)	125.2	158.4 ± 8.2 (145.2-169.0)	193.4 ± 33.3 (135.7-231)	194
c′	1.0 ± 0.04 (1.0-1.1)	1.2 ± 0.08 (1.1-1.2)	1.0 ± 0.09 (0.8-1.1)	0.9	0.8 ± 0.04 (0.8-0.9)	0.7 ± 0.1 (0.6-0.9)	0.7-0.9
V	50 ± 2.42 (46-54)	–	49 ± 1.4 (46-51)	51	50 ± 1.3 (48-52)	52 ± 3.9 (48-59)	45-54
Odontostyle length	91 ± 3.7 (88-97)	82 ± 4.2 (78-86)	90 ± 3.8 (86-98)	94	88 ± 3.0 (83-92)	105 ± 5.3 (105-110)	99-104
Odontophore length	57 ± 3.9 (53-62)	54 ± 2.1 (52-56)	57 ± 6.9 (39-70)	58	56 ± 4.7 (50-63)	58 ± 5.3 (53-65)	59-78
Oral aperture-guiding ring	27 ± 1.5 (24-29)	26 ± 2.2 (24-28)	28 ± 2.6 (25-33)	28	31 ± 0.9 (30-33)	29 ± 2.6 (24-31)	27-33
Lip region diam.	9.4 ± 0.5 (9.0-10.0)	9.8 ± 0.8 (9.0-10.5)	10.4 ± 0.7 (9.0-12.0)	10.5	8.9 ± 0.6 (8.0-10.0)	8.9 ± 0.6 (8.0-10.0)	10-11
Oral aperture-guiding ring/lip region diam.	3.0 ± 0.2 (2.6-3.1)	2.7 ± 0.2 (2.5-2.8)	2.6 ± 0.3 (2.3-3.4)	–	3.5 ± 0.3 (3.1-4.1)	2.8 ± 0.4 (2.4-3.3)	–
G_1	10.9 ± 2.8 (6.6-14.4)	–	9.1 ± 1.3 (6.8-11.2)	–	6.5 ± 0.8 (5.4-7.4)	7.9 ± 1.5 (6.2-9.3)	7-15
G_2	8.8 ± 2.3 (6.1-12.5)	–	8.2 ± 1.2 (6.3-11.1)	–	6.6 ± 0.9 (5.8-7.8)	8 ± 2.5 (5.8-10.8)	6-15
DO	12.8 ± 2.4 (10.5-18)	14.3 ± 2.1 (12-16)	12.7 ± 2.8 (8.6-17.7)	–	10.9 ± 2.1 (8.4-14.0)	15.2 ± 2.5 (12-18)	12-16
DN	24.3 ± 3.7 (20.8-31.3)	23.6 ± 3.2 (20-26)	21.0 ± 5.0 (13.8-34.6)	–	23.7 ± 6.6 (16.5-34.1)	34 ± 5.9 (28-40)	30-38
SN_1 and SN_2	58.5 ± 3.9 (53.6-64.7)	55.6 ± 3.5 (52-59)	47.9 ± 5.7 (38.9-55.4)	–	51.9 ± 1.5 (50.3-53.8)	69.2 ± 10.9 (60-85)	46-57
SO_1 and SO_2	85.1 ± 3.5 (76.1-88.0)	81.7 ± 4.2 (77-85)	81.3 ± 4.8 (71.8-87.6)	–	87.6 ± 4.7 (82.1-93.0)	97.5 ± 5.6 (90-103)	80-88
Pharynx length	354 ± 29.9 (315-410)	315 ± 72.5 (250-393)	384 ± 41.1 (303-475)	–	333 ± 46.0 (247-376)	484 ± 30.2 (451-520)	–
Tail length	35 ± 1.3 (34-38)	37 ± 1.5 (35-38)	36 ± 3.2 (31-43)	32	29 ± 1.4 (27-31)	35 ± 1.9 (32-38)	–
Lateral guiding piece length	–	21 ± 1.7 (20-23)	–	–	–	–	–
Spicule length	–	37 ± 8.3 (30-46)	–	–	–	–	–

gion 9.4 μm broad, rounded, marked by a depression. Amphid fovea symmetrically bilobed. Nerve ring 14 μm wide, located 10 μm posterior to base of odontophore. Cardia semicircular. Posterior testes not discernible. *Vas deferens* discernible in distal portion. Spicules curved, 46.1 μm long. Lateral guiding piece 22 μm long. Supplementary papillae consisting of an adanal pair and four ventro-median pairs arranged in a staggered row. Distance between adanal pair and posteriormost precloacal supplement somewhat greater than that between ventromedian supplements.

Remarks

The USA specimens of *L. orientalis* were compared to *L. belloi* and *L. belondiroides*, the two other species reported in Florida on date palm shipments from Arizona and California. *Longidorus orientalis* populations from Arizona and California morphologically differed from *L. belloi*, by shorter body lengths (3805-4554 μm Arizona, 3580-4840 μm California *vs* 5000-8600 μm) and a symmetrically bilobed *vs* asymmetrically bilobed amphidial fovea. Furthermore, Arizona and California populations differed from *L. belondiroides* by a less tapered *vs* more tapered anterior body and a bilobed *vs* pouch-like, non-bilobed amphidial fovea. This finding confirms an unverified previous identification of *L. orientalis* in Florida made by Esser in 1995 (Lehman, 2002) after examining longidorids in a palm shipment from California and casts doubts on other identifications, such as those of *L. belloi* and *L. belondiroides* as longidorids infesting these palms (Lehman, 2002). This new record validates the occurrence of *L. orientalis* in the USA and the Americas.

Survey of longidorids associated with date palms in California

The population densities of longidorids in California samples ranged from fewer than 1-25 *L. africanus* and fewer than 1-30 *L. orientalis* (100 cm^3 of loamy soil)$^{-1}$ in the Bard area. Lower densities (4 *L. orientalis* (100 cm^3 of soil)$^{-1}$) were found in sandy loam soils at the Thermal area. However, no longidorids were found in sandy soil samples in Thermal. The two species occurred in about 30% of the samples collected. Although the number of samples collected was limited, it appears that *L. orientalis* has a preference for heavy soil types. In this limited survey the population levels of *L. africanus* did not differ from those of *L. orientalis* in sandy loam soils. However, *L. africanus* can occur in high density in sandy soils in Imperial County, since soil texture does not affect its reproductive rate (Radewald *et al.*, 1969). The prevalence of these nematodes in California date palm and other crops and their damage potential for the date palm industry remains to be determined.

Arizona longidorids on imported date palms

A total of 78 samples of coarse sand and roots were collected in 2012-2014 from date palm shipments that originated from Arizona and were intercepted at the inspection stations in Florida. Each shipment consisted of 3-5 trees. *Longidorus africanus* and *L. orientalis* were found in 5 and 43% of these samples, respectively. The longidorid density in these samples was low (fewer than one to a maximum of three specimens in 100 cm^3 of soil). The consistent detection of *L. orientalis* in these sandy soils indicates that populations of *L. orientalis* can inhabit light textured soils. Surveys in Arizona's commercial date palm orchards planted in sandy soils are needed to clarify this aspect of the nematode's biology.

Survey of longidorids on imported date palms transplanted in Florida

The majority of 258 soil and root samples collected from Arizona and California imported date palms established in Florida were free of *Longidorus* species. However, 5.8% of the samples contained longidorids that were identified morphologically as *L. orientalis*. Another 1.2% of the samples were infested with longidorids identified as *L. africanus* in association with *L. orientalis* specimens similar to those samples collected in Riverside County, California. The infested samples from Florida were mainly from counties in the central region of the state. These results provide evidence that these two longidorids are able to survive for at least 2-4 years in the warm and humid Florida environment although at low population levels (fewer than one specimen (100 cm^3 of soil)$^{-1}$). Long-term field observations are needed in order to verify the effect of Florida's humid climate on the ability of *L. orientalis* to persist in native soils. The same considerations apply to *L. africanus*, which in California reproduces quickly on other hosts such as Bermuda grass, a common turf in Florida.

Molecular characteristics of *L. orientalis* from USA and other countries

The results of the molecular analyses reported in the following sections corroborate the morphological

identification of these *L. orientalis* populations from the USA.

PCR-RFLP study

PCR-D2-D3-RFLP profiles generated by five enzymes for *L. orientalis* are given in Figure 3. Lengths of restriction fragments from RFLP for the D2-D3 of the 28S rRNA gene obtained with WebCutter 2.0 were: uncut PCR, 812 bp; *Alu*I, 529, 269, 14 bp; *Hinf*I, 302, 205, 129, 103, 73 bp; *Bsp*143I, 573, 176, 63 bp; *Tru*1I, 812 bp; *Rsa*I, 273, 204, 134, 107, 51, 37, 6 bp. Virtual digestion of the D2-D3 of 28S rRNA gene sequences did not reveal intraspecific variation in the restriction profiles for studied samples.

D2-D3 of the 28S rRNA gene

The alignment contained 25 sequences of *Longidorus* species including seven *L. orientalis* sequences and two outgroups and was 821 bp in length. Intraspecific variation for *L. orientalis* was 0.0-0.3% (0-3 bp). Phylogenetic relationships of *L. orientalis* with several related species, as inferred from BI, is presented in Figure 4. Mfold 3.0 software reconstructed putative secondary structures for the D3 of 28S rRNA as also shown in Figure 5. *Longidorus orientalis* has a sister relationship with *L. andalusicus* Gutiérrez-Gutiérrez, Cantalapiedra-Navarrete, Montes-Borrego, Palomares-Rius & Castillo, 2013 and shared with it a five stem-loop structure of the D3 expansion segment and lack of the D4_1 stem-loop.

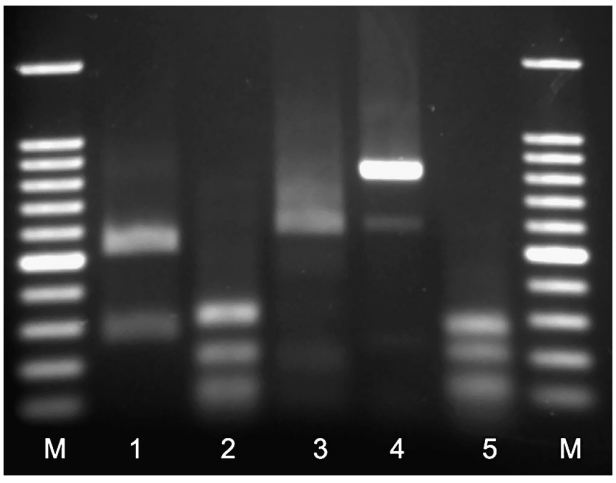

Fig. 3. PCR-D2-D3-28S-RFLP diagnostic profile for *Longidorus orientalis*. M: 100 bp DNA marker (Promega, USA); 1: *Alu*I; 2: *Hinf*I; 3: *Bsp*143I; 4: *Tru*1I; 5: *Rsa*I.

Another related species, *L. oleae* Gutiérrez-Gutiérrez, Cantalapiedra-Navarrete, Montes-Borrego, Palomares-Rius & Castillo, 2013, also has a five stem-loop structure of the D3 expansion segment with the D4_1 stem-loop, but without the D4 stem-loop. Other studied *Longidorus* species have a six stem-loop structure of the D3 expansion segment (Fig. 5).

ITS1 rRNA gene

The alignment contained 18 sequences of *Longidorus orientalis*, and two sequences of *L. iuglandis* Roca, Lamberti & Agostinelli, 1984 and *L. magnus* Lamberti, Bleve-Zacheo & Arias, 1982 used as outgroup taxa, and was 933 bp in length. Intraspecific variation for *L. orientalis* was 0.0-1.6% (0-14 bp). The ITS1 sequences (USA, Iran, Greece and Spain) can be sorted into four types that are distinguished from each other by several unique nucleotides and an insertion/deletion fragment (Fig. 6).

Mfold 3.0 software reconstructed optimal secondary structures for the ITS1 of *L. orientalis*. The ITS1 itself represents three domain structures (Fig. 6A). The ITS1 alignment of a fragment of the longest stem-loop structure for four types is given in Figure 6B. The putative secondary structure of this stem-loop (type I) without an insertion (consensus USA sample) is given in Figure 6C. Short insertion fragments with different lengths for other three types are given in Figure 6D. These insertions are formed by consensus repeats: $(CGA)_n$ or $(CCA)_n$ and $(GUCG)_n$ or $(GUYG)_n$.

Phylogenetic relationships within *L. orientalis* with associated host-plants as inferred from BI is presented in Figure 7A. The ITS1 sequences formed three clades on the tree. All USA samples clustered together, whereas Spanish samples were distributed among two clades. Sequence diversity within Spanish samples was 1.0% (9 bp).

coxI mtDNA gene

The alignment contained 17 sequences of *Longidorus orientalis* and two sequences of *L. uroshis* Krnjaic, Lamberti, Krnjaic, Agostinelli & Radicci, 2000 and *L. poessneckensis* Altherr, 1974 used as outgroup taxa and was 278 bp in length. Intraspecific nucleotide sequence variation for *L. orientalis* was 0-15.5% (0-43 bp). Fifty-nine positions were variable. Seven *coxI* haplotypes were distinguished within *L. orientalis* samples from four

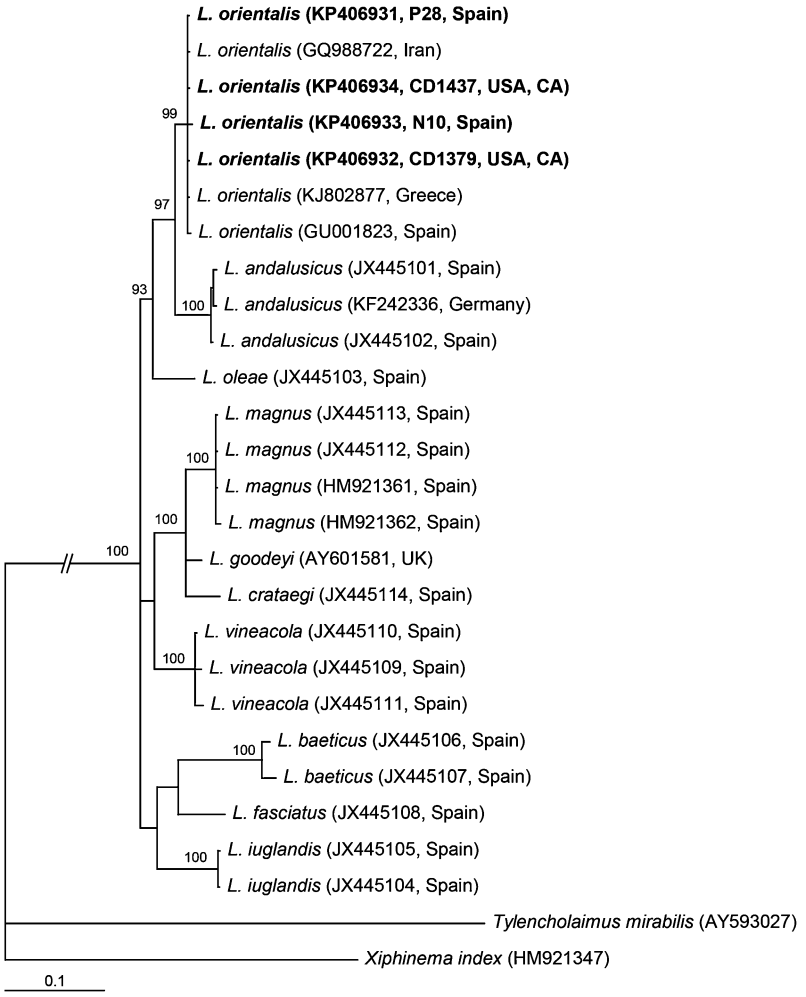

Fig. 4. Phylogenetic relationships of *Longidorus orientalis* with other related species: Bayesian majority rule consensus tree reconstructed under the GTR + I + G model and inferred from the D2-D3 of 28S rRNA gene sequence.

sampling geographical areas: USA (California, Arizona), three haplotypes; Iran, two haplotypes; Greece (Crete), one haplotype; and Spain, two haplotypes (Table 3). The *coxI* haplotype from Greece (Crete) was identical to that from Iran. Nucleotide sequences were also converted into amino-acid sequences and the analysis revealed that intraspecific amino-acid sequence variation for *L. orientalis* was only 1.0% (one amino acid).

Phylogenetic relationships of *L. orientalis coxI* haplotypes as inferred from BI are presented in Figure 7B. The *coxI* sequences of the USA samples were distributed among two main clades. Sequences from Spain were also distributed among two clades, moreover, the sequences of the Spanish samples from grapevine were more similar with sequences of the USA samples from date palm, than from that of the Greece (Crete) sample. Sequence diversity within the USA haplotypes or within Spanish haplotypes was 15.5% (43 bp).

Statistical parsimony network showing the relationships between the *L. orientalis* haplotypes is given in Figure 8. Haplotypes from H1 to H4 were closely related between themselves and distributed in all regions studied (USA, Spain, Greece and Iran). The other three haplotypes (H5-H7) were significantly distinct from each other and from the haplotypes H1-H4.

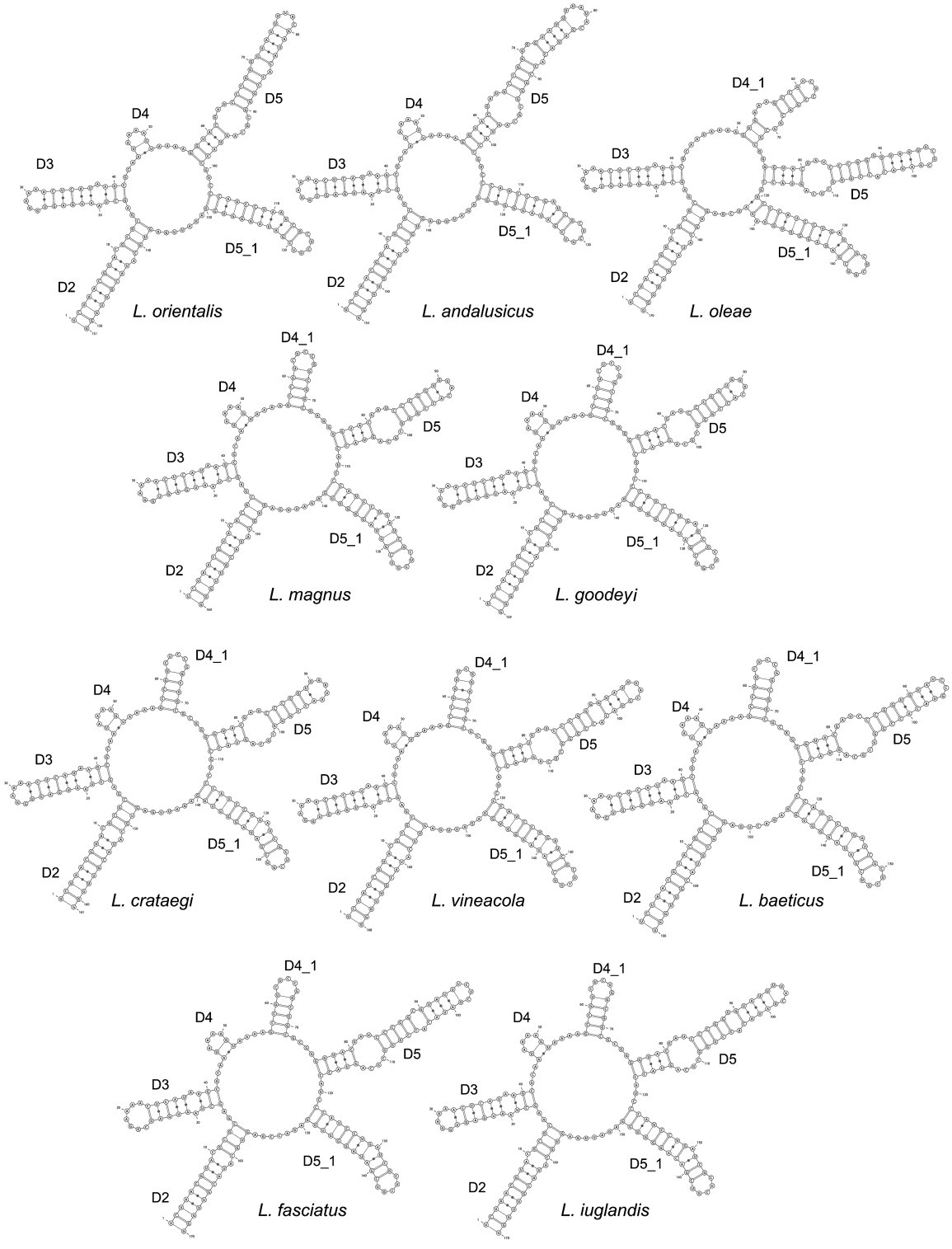

Fig. 5. Putative secondary structures of the D3 expansion segment of 28S rRNA gene for longidorid nematodes. Stem-loop structure codes are given according to He *et al.* (2005b).

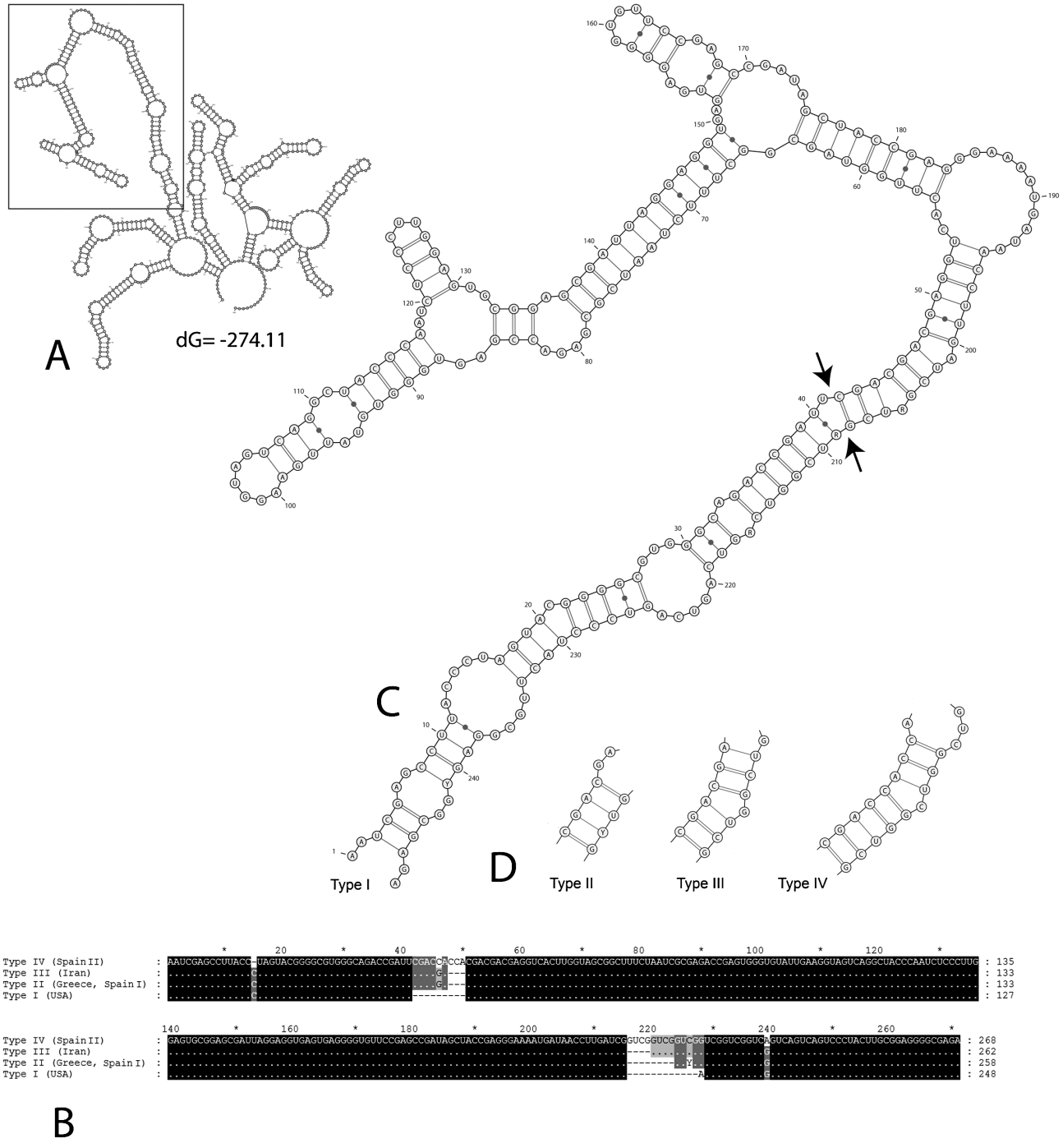

Fig. 6. Putative secondary structures of the ITS1 for *Longidorus orientalis*. A: Putative secondary structure of the ITS1 with indication of a longest stem-loop structure; B: Alignment of sequences of a longest stem-loop structure with insertions/deletions; C: Putative secondary structure of a longest ITS1 stem-loop reconstructed for nematodes from the USA with indication of the position of insertions by arrows; D: Putative secondary structure types (II-IV) of insertions for sequences obtained from nematodes collected from other geographical regions.

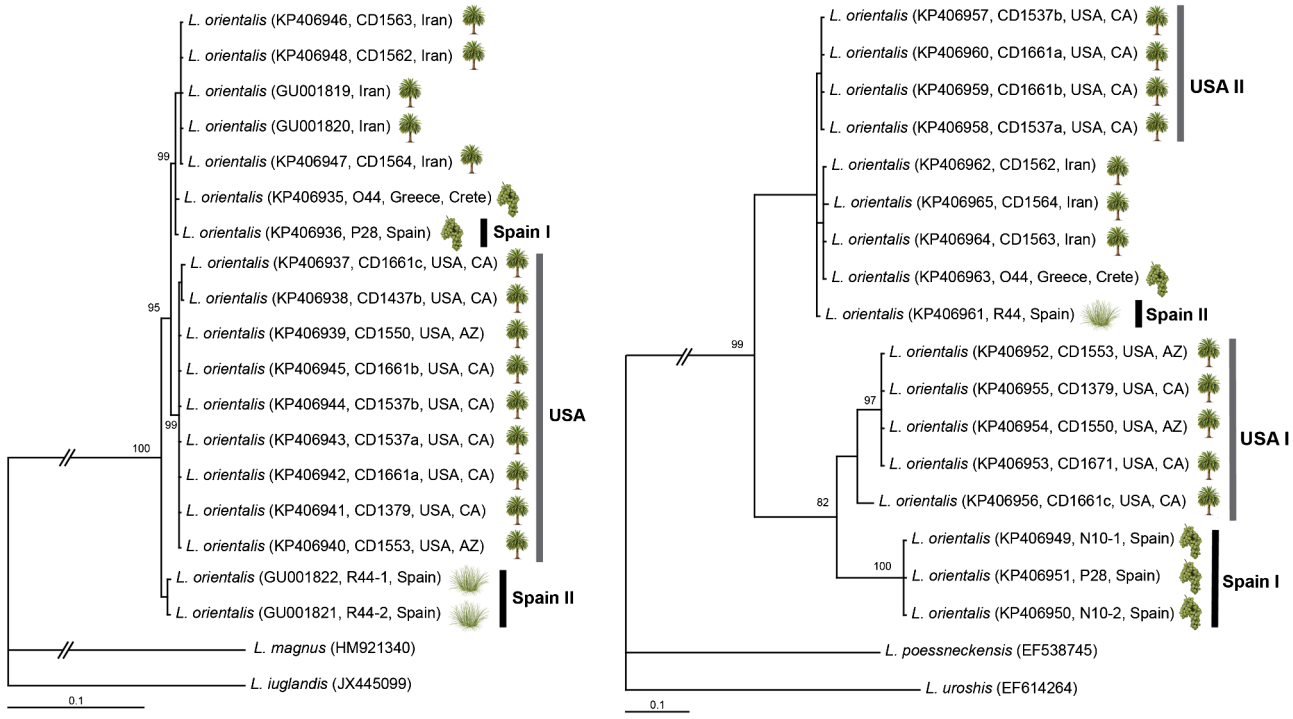

Fig. 7. Phylogenetic relationships between *Longidorus orientalis* populations: Bayesian majority rule consensus tree reconstructed under the GTR + I + G model and inferred from the ITS1 rRNA gene sequences (A) and partial the *coxI* gene sequences (B). This figure is published in colour in the online edition of this journal, which can be accessed *via* http://booksandjournals.brillonline.com/content/journals/15685411.

HIGH INTRASPECIFIC SEQUENCE VARIATION FOR *COXI* GENE AND BARCODING GAP

The mitochondrial *coxI* gene has been proposed as a standard reliable genetic marker to: *i*) elucidate cryptic diversity; *ii*) study phylogeographical relationships; and *iii*) identify and barcode eukaryote species, including representatives of the phylum Nematoda. After the analysis of partial *coxI* gene sequences of free-living marine nematodes, Armenteros *et al.* (2014) concluded that conspecific and interspecific sequence variations of this gene are clearly separated and formed a so called "barcoding gap". The authors noticed that all conspecific genetic distances were <3% of genetic divergence, whereas all interspecific distances (within and among genera) were >14% and, thus, concluded that the *coxI* gene was promising for DNA barcoding. This conclusion is in agreement with several other studies, for example: filarioid nematodes with intraspecific and interspecific sequence variations, 0.5 and 15.5% (Ferri *et al.*, 2009), respectively, *Xiphinema diversicaudatum* (Micoletzky, 1927) Thorne, 1939 with an intraspecific variation of 3.1% (Chizhov *et al.*, 2014) or with *X. index* Thorne & Allen, 1950 and *X. pachtaicum* (Tulaganov, 1938) Kirjanova, 1951 with intraspecific variation ranging from 0.2-0.4% and 0.2-2.3%, respectively (Gutiérrez-Gutiérrez *et al.*, 2013). However, in several organisms, intraspecific variation can exceed this standard level. For example, survey studies of inter- and intraspecific variation across several insect orders has shown that *coxI* sequence differences varied substantially between closely related insect species, with up to 26.0% intraspecific compared with 30.7% interspecific sequence variation (Cognato, 2006). In our study the intraspecific diversity of *coxI* gene sequence for *L. orientalis* was high, 15.5%, a value not previously reported for nematodes.

High sequence divergence in *coxI* gene between samples may be incorrectly estimated, if a pseudogene and a functional gene are compared. Because of the very high similarity in amino acid sequences between all haplotypes, we are confident that the studied sequences repre-

Table 3. Variable positions in the *coxI* haplotypes of *Longidorus orientalis* from different geographical areas and illustrated in Figure 8.

Haplotype	Number of sequences	Variable positions (bp)																													
		3	6	12	15	21	27	30	33	39	42	45	51	54	57	60	63	66	72	75	78	79	87	93	96	102	105	108	120	135	139
H1	1	G	C	C	T	G	C	T	C	A	A	T	A	C	G	T	C	T	T	A	T	C	T	A	G	C	C	A	A	C	C
H2	3	G	C	C	T	G	C	T	C	A	A	T	A	C	G	T	C	T	T	C	T	C	T	A	G	C	C	A	A	C	C
H3	1	G	C	C	T	G	C	T	C	A	A	T	A	C	G	T	C	T	T	C	T	C	T	A	G	C	C	A	A	C	C
H4	4	G	C	C	C	G	C	T	C	A	A	T	G	C	G	T	C	T	T	C	T	C	T	A	G	C	C	A	A	C	C
H5	3	T	T	C	T	G	C	C	T	C	T	C	A	T	A	C	T	T	C	T	T	T	C	G	A	T	T	G	A	C	C
H6	1	A	T	T	T	A	C	C	T	T	A	C	A	T	A	C	C	C	C	C	T	T	C	A	G	C	C	A	A	T	T
H7	4	A	T	T	T	A	T	C	T	T	A	C	A	T	A	C	C	C	C	C	C	T	C	G	A	C	C	A	G	C	T

Haplotype	Number of sequences	Variable positions (bp)																											
		141	147	153	159	162	165	168	174	177	180	183	192	195	198	204	207	210	213	216	219	225	226	234	237	246	256	265	267	273
H1	1	G	C	A	T	A	T	T	T	T	C	G	C	T	C	A	T	G	T	A	G	T	G	A	G	A	T	C	A	G
H2	3	G	C	A	T	A	T	T	T	T	C	G	C	T	C	A	T	G	T	A	G	T	G	A	G	A	T	C	A	G
H3	1	G	T	A	T	A	T	T	T	T	C	G	C	T	C	A	T	G	T	A	G	T	G	A	G	A	T	C	A	A
H4	4	G	T	A	T	A	T	T	T	T	C	G	C	T	C	A	T	G	T	A	G	T	G	A	G	A	T	C	A	A
H5	3	A	T	G	A	A	T	C	T	T	T	A	G	C	T	A	C	C	C	C	A	C	G	C	A	G	C	T	A	A
H6	1	G	C	G	A	G	C	C	T	C	T	A	G	C	T	A	C	A	C	A	A	C	C	A	A	A	T	C	G	A
H7	4	A	C	A	A	A	C	T	C	C	T	A	G	C	T	G	C	A	C	A	A	C	A	A	A	A	C	C	G	A

sent authentic mitochondrial genes and not nuclear paralogues or pseudogenes.

The high mitochondrial variability observed in *L. orientalis* may cast doubt on the reliability of nematode DNA barcoding. Although there are a low number of studies on the intraspecific variability of *coxI* in longidorids species, distinct overlap of intraspecific and interspecific divergence values can complicate the establishment of threshold values to identify new candidate species. There is no standard percent sequence divergence that allows a reliable prediction of species boundaries for nematodes. Set DNA parameters should be used with caution for nematode barcoding. The high variation observed in the *coxI* priming sites can adversely affect the certainty of the nematode identification by barcoding and thus integrative taxonomical approaches are needed for an accurate identification of these and other plant-parasitic nematodes (Gutiérrez-Gutiérrez *et al.*, 2013).

INCONGRUENCE OF THE ITS1 AND *COXI* GENE PHYLOGENIES AND INTROGRESSION OF MTDNA

One of the remarkable results of the present study is discordance in phylogenetic relationships inferred from the ITS1 rRNA and *coxI* gene sequence datasets. While in the ITS1 rRNA tree all USA samples clustered together, they are distributed into two clades in the *coxI* tree. A similar discordance was observed for the Spanish population from grapevine (group I), which in the ITS1 rRNA gene tree was related with the Iranian populations from date palm, whereas in the *coxI* gene tree clustered with the group I of USA populations. Also, in the ITS1 rRNA gene tree, the Spanish population from grasses (group II) occupied a basal position, whereas in the *coxI* gene tree it clustered with populations from Greece (Crete), Iran and those of the group II from the USA.

The most plausible explanation for incongruence in phylogenies is the introduction of nuclear DNA through hybridisation between previously isolated nematode populations and possible selective introgression of mtDNA as a result of gene flow between these genetically diverse populations. The occurrence of males and females with functional spermathecae in some *L. orientalis* populations support the conclusion that mating events occur in this species.

The date palm is adapted to areas with long, hot summers with little rain and low humidity, but with abundant underground water. These conditions are mainly found in river valleys and oases in the arid and subtropical deserts of the Middle East and North Africa

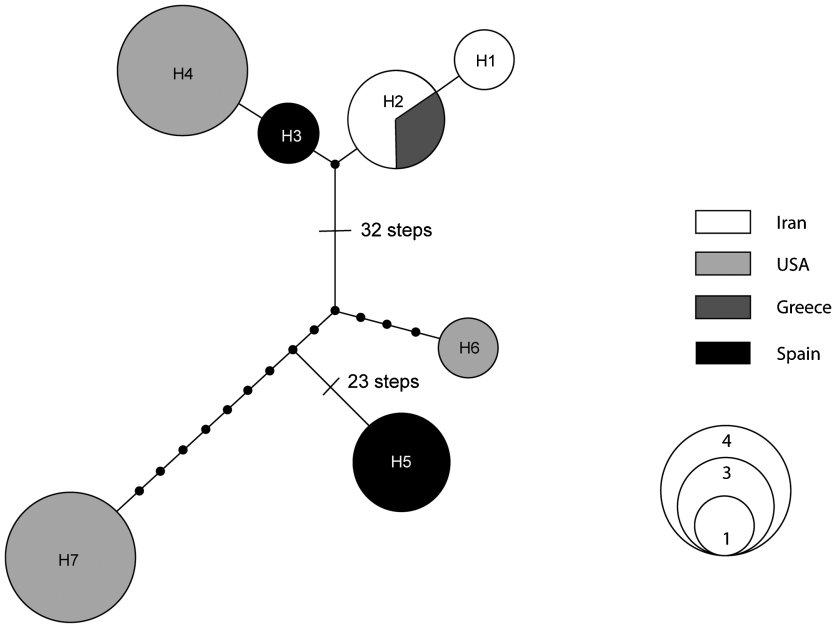

Fig. 8. Statistical parsimony network showing the phylogenetic relationships between *coxI* haplotypes of *Longidorus orientalis*. Small black cycles represent missing haplotypes. Pie chart sizes are proportional to the number of samples with a particular haplotype.

(Jaradat, 2011). It is likely that such isolated areas created ideal conditions for the limitation of a gene flow between associated native parasitic nematode populations and finally led to its rapid genetic diversification. Orchards established from date palms with associated nematodes introduced from different world regions seem to be good arenas for forming new hybrid zones of previously geographically separated nematode populations. Mating of individuals from genetically different populations can take place in these areas. In such hybrid populations a rapid homogenisation of ITS rRNA genes may occur and lead to identical or very similar sequences within individuals, whereas the different types of mtDNA might still persist in a population.

It has been known that mitochondrial DNA is inherited through the maternal line in many organisms. As a result, mtDNA generally has a lower expected coalescence time than most nuclear genes, assuming that no selection is involved (Avise, 2004; Zink & Barrowclough, 2008). However, it has been shown in several studies that maternal mtDNA might be completely replaced as a result of bi-parental inheritance. The mtDNA might be more sensitive to introgression than nuclear DNA, and this introgression can occur despite the levels of gene flow and at low levels of hybridisation between specimens from different populations. As a result, the rest of the genome may tell a very different story and the results from mtDNA may not reflect the typical history of the taxa involved (Ballard & Whitlock, 2004).

The phenomenon of mtDNA introgression is rather common in nature and was first documented in the early 1980s in *Drosophila* flies (Powell, 1983), mice (Ferris *et al.*, 1983) and later in many other organisms (Rheindt & Edwards, 2011). We hypothesise that selective introgression of mtDNA might occur in *L. orientalis* populations. According to this possible scenario, the USA populations originated as a result of the hybridisation of two allopatric populations: one from Iran and Greece (Crete, grapevine) and another one from an unknown location. In agricultural areas, mating of nematodes carrying various mtDNA haplotypes might be rather common and could lead to a complex mtDNA introgression pattern. Mitochondrial introgression and high mutation rates are considered as major problems in DNA barcoding with the *coxI* gene. Thus, the use of several genetic markers is recommended to obtain reliable molecular identification through the DNA barcoding approach and reconstruct phylogeographical patterns of nematodes.

Origin and dispersal of *L. orientalis*

The presently known distributions of *L. orientalis* and other phylogenetically related species indicate that the Mediterranean basin and the Middle East are most probably the regions of origin for this nematode group. From these areas some members (*L. goodeyi* Hooper, 1961, *L. vineacola* Sturhan & Weischer, 1964) of this group were dispersed to central and northern Europe and subsequently, to other world regions. Other species, such as *L. africanus* and *L. orientalis*, spread to the Middle East and North Africa reaching geographically isolated countries such as Greece (Crete) and Spain. Most likely *L. orientalis* was introduced to California from the Middle East and further dispersed in the USA with the importation of date palms.

The Spanish were the first to introduce date palms to America. During the late 17th and early 18th centuries, missionaries planted date palms around the missions of Mexico, and by the latter half of the 18th century, date palm cultivation had spread to the missions of Southern California. During the latter half of the 19th century, date palms were cultivated by American pioneers who planted seeds, presumably from imported Persian Gulf dates. Between 1876 and 1890, a few offshoots were imported from Algeria, Egypt, and Oman, and were planted experimentally in the hot, interior valleys of the Southwest. In 1890, some 447 offshoots, representing 27 varieties were sent from the Algerian Sahara to various areas of the Southwest. Cultivation in California was spurred by the establishment of a USDA date palm experimental station, which was built in the Coachella Valley in 1904 (Lee, 1963; Chao & Krueger, 2007). Many varieties of date palm from Algeria, Iraq, Morocco, Saudi Arabia and Tunisia were introduced to California over several decades. Presently, date palms are grown in Southern California and several other states including Arizona, Florida, Louisiana, Nevada and Texas.

Planting of offshoots is the primary means of propagation of date palms. Offshoots, after several years of attachment to the parental palm, produce roots and can be removed and planted into large pots or into field (Chao & Krueger, 2007). Thus, nematodes inhabiting soil attached to offshoots can be transmitted from a field to a nursery and then again to a new and uninfested field.

Concluding remarks

The characterisation of *L. orientalis* populations from the USA and other geographical areas, conducted in this study, has revealed the high mtDNA variability of this species. These genetically diverse *L. orientalis* populations, alone or in association with *L. africanus*, have spread from the Middle East into the surveyed areas of the USA. The results of the surveys conducted in California and at the inspection stations in Florida confirm that date palm is a susceptible host to both needle nematodes. These data support the hypothesis that probably *L. africanus*, like *L. orientalis*, arrived in California on imported date palms from the Middle East. Our field observations suggest also that so far as we know date palm is the most conducive known host for the development and reproduction of *L. orientalis*. Our data suggest that *L. orientalis*, like *L. africanus*, may thrive in both heavy and light textured soils. Two *L. orientalis* populations used in this study were found associated with grapevine in Greece (Crete) and Spain, indicating that grapevine is another host of the nematode. Finally, the ability of American *L. orientalis* populations to infest hosts other than date palms in Arizona, California and Florida requires further studies.

Acknowledgements

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