Molecular identification of parasitic nematodes (Nematoda: Strongylida) in feces of wild ruminants from Tunisia

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Abstract

In Tunisia and other North African countries, there is a lack of knowledge about parasite biodiversity within threatened wild ruminants and there are not any studies on their gastrointestinal nematodes. Thus the aim of this study was to identify gastrointestinal fauna in the faecal samples of Tunisian wild ruminants. A total of 262 faecal samples were collected from domestic sheep and goat, and wild ruminants (Addax, Barbary sheep, Barbary red deer, Dorcas gazelle, Slender-horned gazelle and Scimitar-horned Oryx) living in protected areas. Samples were examined with floatation (saturated sodium chloride solution), polymerase chain reaction and sequencing of the second internal transcribed spacer region of the rDNA. Microscopic analysis allowed the identification of only *Nematodirus* genus or molecular tools allowed a first identification of five gastrointestinal nematode species in North African wild ruminants: *Chabertia ovina* (1.6%), *Camelostrongylus mentulatus* (1.6%), *Marshallagia marshalli* (4.7%), *Nematodirus helvetianus* (62.5%) and *Nematodirus spathiger* (29.7%). This study reported the first records of *C. mentulatus* and *M. marshalli* in Addax and of *M. marshalli* in Dorcas gazelle and it was the first reported record of *N. helvetianus* and *M. marshalli* in Tunisia.

Introduction

The climate and the ecosystem diversity in the North African countries support the habitat of various wild species but urbanization, desertification, pollution, over exploitation of some wild species and the lack of knowledge on the pathogens of wild animals are under increased threat of wild life. In Tunisia a national strategy has been developed to preserve wild Bovidae and Cervidae, the only forms of wild ruminants in the Artiodactyla order and the most threatened (Anonymous, 2013). These species are classified in the International Union for Conservation of Nature (IUCN) red list of threatened species with different conservation status: the Dorcas gazelle (*Gazella dorcas*) and Barbary sheep (*Ammotragus lervia*) as vulnerable, the Addax (*Addax nasomaculatus*) as critically endangered, the Scimitar-horned Oryx (*Oryx dammah*) as extinct in the wild and the Slender-horned gazelle (*Gazella leptoceros*) as endangered. The Barbary red deer (*Cervus elaphus barbarus*), protected under Appendix III of the Bern Convention, is a rare subspecies of the red deer present only in Northern Africa (Tunisia and Algeria) (Gharaibeh, 1997; Hajji *et al.* 2007) and its population has been increasingly declining (Gharaibeh, 1997; Lovari *et al.* 2016). The national strategy involved the instauration of legislation prohibiting catching and hunting, creation of fenced protected areas, participation in the Convention on Migratory Species (CMS) action plan for the conservation and restoration of sahelo-saharan Antelopes and the cooperation with international organization to reintroduce some wild ruminants that were extinct as the Addax and the Scimitar-horned Oryx (Gilbert and Woodfine, 2008; Woodfine *et al.* 2011). In the protected areas (nature reserves and national parks) only wild animals are present and there is no possible contact with domestic animals, which could be reservoirs of infectious diseases and parasites. Available literature on parasite cross-transmission between domestic and wild host is limited (Walker and Morgan, 2014) but this transmission is possible and even when there is no direct contact between hosts, it could occur from the environment, water, or grazing areas.

The Nematodes play an ubiquitous part of the ecosystem and many of them are generalist (a parasite who survives and reproduces in more than one type of host) (Walker and Morgan, 2014). In livestock, the clinical signs of Nematode-infection are non-specific and dependent on the number of parasites in the host (Brooker, 2010). They strongly affect health, production, population dynamics and susceptibility to other diseases (Perry and Randolph, 1999; Steele *et al.* 2013; Thumbi *et al.* 2013). In fact, wild ruminants are more resistant and often parasite infection are subclinical but this infection can affect body condition, weight gain, fecundity, population density (Arneberg *et al.* 1996; Albon *et al.* 2002; Stien *et al.* 2002; Hughes *et al.* 2009) and has the potential to impede conservation efforts (Dobson and Hudson, 1986).

In Tunisia and most generally in Northern Africa, there is a lack of knowledge on parasite biodiversity of these threatened wild hosts and there are not any studies on their

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gastrointestinal nematodes. Any scientific research in this field will be important to understand the dynamic relationship between populations of threatened hosts and their parasites and to be more effective in the conservation strategy. The study of parasite biodiversity based only on morphology misidentified cryptic species (Hoberg et al. 1999). Thus the use of molecular genetic tools is necessary to unequivocally identify parasite species and improves the understanding of multi-host/multi-parasite systems and parasite distribution (Morgan et al. 2012; Walker and Morgan, 2014).

It is difficult to study nematodes on wild hosts based on sporadic necropsy of naturally dead animals. Thus the aim of the present study was to identify the gastrointestinal nematode fauna present in the Tunisian wild ruminants faecal samples using faecal examination and polymerase chain reaction (PCR).

Materials and methods

Sampling and coprological examination

From May 2015 until February 2016, a total of 262 faecal samples were collected from domestic sheep and goat, and 6 wild ruminant species [Addax (A. nasomaculatus) (Bovidae, Hippotraginae), Barbary sheep (A. lervia) (Bovidae, Caprinae), Barbary red deer (C. e. barbarus) (Cervidae, Cervinae), Dorcas gazelle (G. dorcas) (Bovidae, Antilopinae), Slender-horned gazelle (G. leptoceros) (Bovidae, Antilopinae) and Scimitar-horned Oryx (O. dammah) (Bovidae, Hippotraginae)] from 7 national parks (El Feidja, Bouhedma, Haddaj, Orbata, Dghoumes, Sidi Toui, Djbil) and one nature reserve (Oued Dekouk) (Fig. 1) and from domestic sheep and goat flocks living near these protected areas (Table 1). Samples collected from the wild ruminants were picked from the ground in the grazing areas after looking for each specie's herd, then they were transported in cooling boxes and stored at −20 °C until examination.

Flotation test was used to detect nematodes' eggs in the faecal samples. It was performed using 5 g of feces and saturated sodium chloride solution (specific gravity 1.20 at 20 °C) (Thienpont et al. 1979).

Molecular analyses

DNA isolation and PCR

Genomic DNA was isolated from faecal samples ($n = 262$) after performing the flotation technique using the modified protocol described by Bott et al. (2009). Briefly, 5 g of feces were used instead of 4 g without performing the McMaster step. DNA was purified from the collected eggs using the Wizard Genomic DNA Purification kit (Promega, Madison, USA) according to the manufacturer's protocol. To amplify the complete sequence of the second internal transcribed spacer (ITS2) regions of the ribosomal DNA, three consecutive PCR were performed. In the first PCR (PCR 1), the amplification of the parasites' DNA was performed with the forward (NC5: 5′-GTAGGTGAACCT GCGGAAGGATCATT-3′) and reverse primers (NC2: 5′-TTA GTTTCTTTTCCTCCGCT-3′) corresponding to the conserved 3′–5′ ends of the ITS1-5.8S-ITS2 flanking the 18S and 28S gene regions of the ribosomal DNA (Gasser et al. 1996). PCR mix consisting of 1× PCR buffer (50 mM Tris–HCl, pH 8·5; 50 mM NaCl), 0·2 mM of each dNTP (dATP, dCTP, dGTP, dTTP), 1.5 mM MgCl$_2$, 0.5 mM of each primer mix, 1.25 μL template DNA, 0.024 units Taq DNA polymerase (Vivantis, USA) and sterile distilled water to 25 μL. The PCR was carried out in applied Biosystem, SimpliAmp™ Thermal Cycler using the following cycling conditions: an initial denaturation step at 94 °C for 3 min, 35 cycles (94, 55 and 72 °C for 1 min each) and a final extension step

at 72 °C for 10 min. The second and the third PCR were performed as the protocol of nested PCR described by Sim et al. (2010). In the second PCR (PCR 2), the ITS2 of the rDNA were amplified using 5 μL of the amplicons generated from the PCR 1 as template, 0·8 mM of each NC13aF (5′-ATCGATG AAAAACGCAGC-3′) and NC2 (5′-TTAGTTTCTTTTCCTCC GCT-3′) primers in 25 μL volume containing the same concentrations of PCR buffer, dNTP, MgCl$_2$ and Taq polymerase (Vivantis, USA) as in the PCR 1. The PCR conditions were an initial denaturation step at 95 °C for 5 min, 30 cycles (95, 50 and 72 °C for 1 min each), and a final extension step of 72 °C for 5 min. The PCR products were visualized in 1·5% agarose gels. In the third PCR (PCR 3), all PCR products were amplified using two internal primers, NC14-Fn (5′-GAACGCATAGCG CCGTTGGGTT-3′) and NC2-Rn (5′-TGATATGCTTAAGT TCAGCGGG-3′). The third PCR was conducted as in PCR 2, except that 2 μL of PCR 2 products were used and the annealing temperature was increased to 58 °C (Sim et al. 2010). The final amplification corresponded to the ITS2 region of the rDNA.

DNA sequencing and phylogenetic analysis

All the PCR amplicons (positive) were purified ($n = 92$) and sequenced using NC14-Fn and NC2-Rn primers. A conventional Big Dye Terminator v3·1 cycle sequencing ready reaction kit (Applied Biosystems, Foster City, CA) and an ABI3730XL automated DNA sequencer were used. The chromatograms and sequence alignments were evaluated with the MEGA 6 software program (Tamura et al. 2013) and the network server of the National Center for Biotechnology Information (NCBI) using BLAST. Ninety two sequences were deposited into GenBank database and phylogenetic tree was constructed by the neighbour-joining method (NJ) after 1000 bootstraps (Saitou and Nei, 1987). The evolutionary distances were computed using Kimura 2-parameter method (Kimura, 1980) and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated. Identical sequences in the alignments were treated as single terminal taxa.

Data analysis

The prevalence of positive samples and the standard error (S.E.) were calculated. The differences in prevalence were analysed using χ^2 test and Fisher's exact test with the Statistical Package for Social Sciences (SPSS). Version 20 (IBM SPSS Statistics, Armonk, NY) and Epi Info version 7 (Centers for Disease Control and Prevention, Atlanta, GA, USA). Differences were considered statistically significant at a 0.05 threshold value. The comparison and the evaluation of the agreement between coproscopic and molecular tests were performed using Cohen κ analysis and χ^2 test.

Results

Coprological examination

Using the coproscopic flotation test, the overall prevalence of positive samples from both domestic and wild ruminants was $16 \pm 0.02\%$ (42/262). In wild ruminants, the prevalence of positive samples was $20.96 \pm 0.03\%$ (26/124), while in domestic ones the prevalence was estimated to $11.5 \pm 0.02\%$ (16/138) ($P = 0.038$) (Table 2). All positive samples have a low number of parasite eggs (1 to 3 eggs) by flotation and only Nematodirus eggs were found.
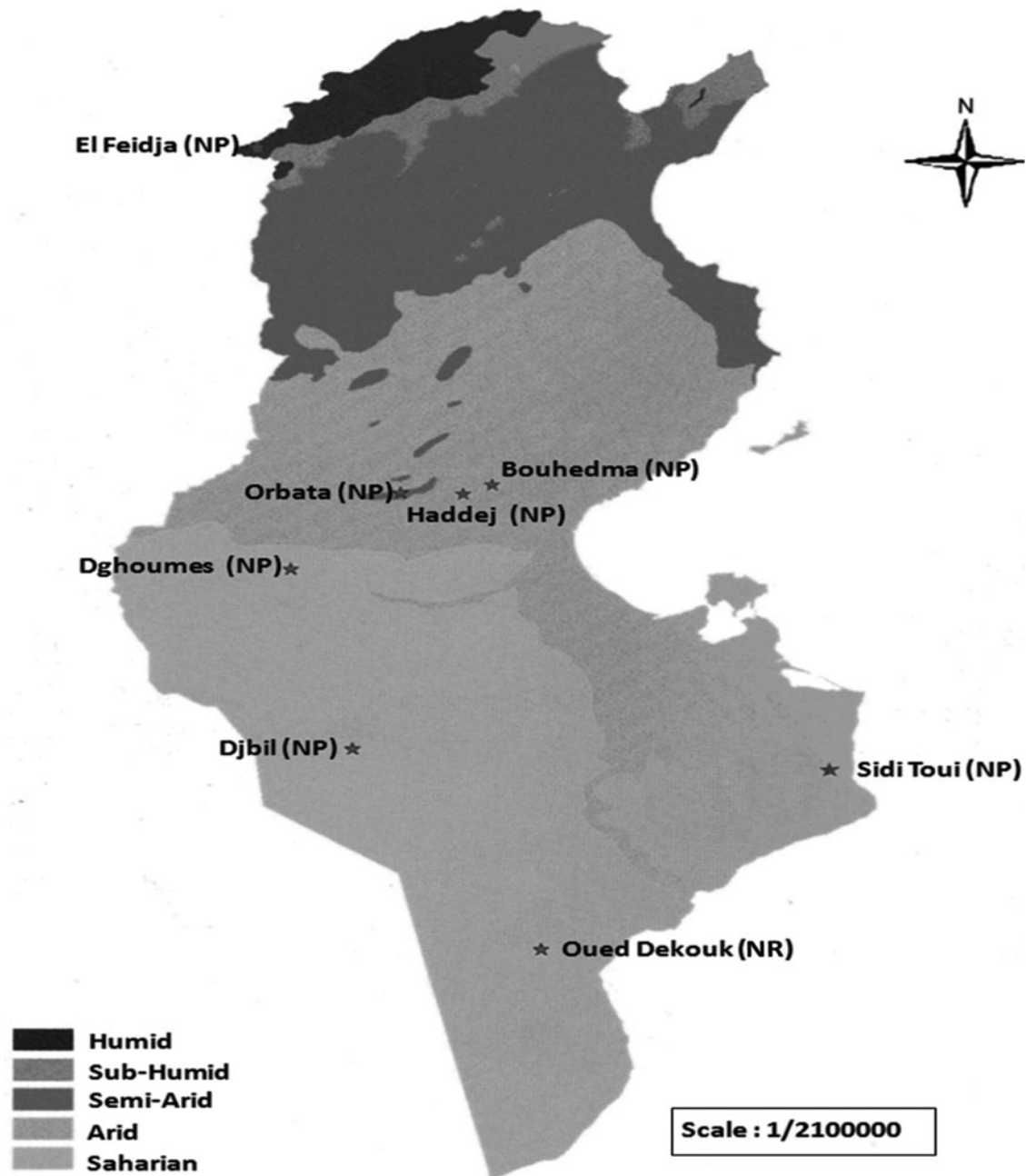
Fig. 1. Bioclimatic map of Tunisia representing studied National parks (NP) and Nature reserve (NR).

Molecular analyses

All the 262 faecal samples were amplified by the PCR. Amplicons were visualized by electrophoresis on a 1.5% agarose gel and produced a single amplification band (≈300 pb in length). The amplification of the ITS2 region of the rDNA only by PCR 2 followed by PCR 3 showed a weak PCR bands on agarose gel. The association of three PCRs allowed to get a higher concentration of PCR products.

The prevalence estimated by PCR (35.1 ± 0.02%; 92/262) was significantly higher than coprological examination ($P = 0.000001$) (Table 2). There was a moderate agreement between the two techniques ($k = 0.52$). The molecular prevalence in wild ruminants (51.6 ± 0.04%; 64/124) was significantly higher than the domestic ruminants (20.28 ± 0.03%; 28/138) ($P = 0.000001$). The infection prevalence in Slender-horned gazelle, Barbary sheep, addax, Scimitar-horned Oryx, Dorcas gazelle and Barbary red deer samples was 83 ± 0.15% (5/6), 81 ± 0.1% (9/11), 63 ± 0.1% (12/19), 48 ± 0.08% (19/39), 43 ± 0.07% (17/39) and 20 ± 0.1% (2/10), respectively.

Nematodes identification

All 92 produced ITS2 sequences were deposited in GenBank (Table 3). The multiple sequences alignment and the comparison with the GenBank database reveal that 66 isolates (71 ± 0.04%) (286 bp length) were *Nematodirus helvetianus*, 21 isolates (22 ± 0.04%) (286 bp length) *Nematodirus spathiger*, 3 isolates (3 ± 0.01%) (291 bp length) *Marshallagia marshalli*, 1 isolate (1 ± 0.01%) (292 bp length) *Camelostrongylus mentulatus* and 1 isolate (1 ± 0.01%) (288 bp length) *Chabertia ovina*. All the sequences showed 100% similarities with those deposited in GenBank database except for *C. mentulatus* and *C. ovina*, which had a similarity of 99.57 and 99.65%, respectively.

Table 1. Faecal samples collected from various animal species and localities

| Bioclimatic stage | National park/ Nature reserve | Geographic coordinates | Wild species (collected faecal samples/estimated population) | | | | | | Domestic species | | Total | Distance between wild and domestic ruminants (km) |
			Gazella dorcas	*Gazella leptoceros*	*Cervus elaphus barbarus*	*Oryx dammah*	*Addax nasomaculatus*	*Ammotragus lervia*	Sheep	Goats		
Humid	El Feidja	40° 53′ N; 6° 58′ E	–	–	10/275	–	–	–	10	10	30	0·2
Arid	Bouhedma	34° 39′ N; 9° 48′ E	8/214	–	–	6/38	6/6	5/4	10	10	45	8
	Haddaj	34° 42′ N; 9° 47′ E	10/40	–	–	7/17	7/47	–	7	10	40	0·3
Semi-Arid	Orbata	34° 22′ N; 9° 03′ E	6/167	–	–	–	–	–	8	6	20	6
	Dghoumes	34° 04′ N; 8° 30′ E	10/172	–	–	10/68	–	–	6	7	33	2
Saharan	Sidi Toui	32° 48′ N; 11° 22′ E	5/8	6/11	–	7/33	–	–	10	10	38	18
	Djbil	33° 14′ N; 9° 26′ E	–	–	–	–	7/36	–	10	10	27	15
	Oued Dekouk (nature reserve)	32° 08′ N; 10° 32′ E	0/16	–	–	9/47	–	6/92	7	7	29	11
Total			39	6	10	39	19	11	68	70	262	

(–): Animal species absent in the park or reserve.

Host and nematodes species

Five nematode species were identified in wild ruminant samples; *N. helvetianus* (62 ± 0.06%; 40/64), *N. sphatiger* (29 ± 0.05%; 19/64), *M. marshalli* (4 ± 0.02%; 3/64), *C. mentulatus* (1 ± 0.01%; 1/64) and *C. ovina* (1 ± 0.01%; 1/64) while only two nematode species were present in sheep and goat samples; *N. helvetianus* (92 ± 0.04%; 26/28) and *N. sphatiger* (7 ± 0.04%; 2/28). In sheep, the infection prevalence by *N. helvetianus* and *N. spathiger* represents 14/15 and 1/15, respectively; while, in goats they represented 12/13 and 1/13, respectively. In addax, five parasite species were identified: *N. helvetianus* (50 ± 0.14%), *N. sphatiger* (25 ± 0.12%), *M. marshalli*, *C. mentulatus* and *C. ovina* (with 8 ± 0.08% for each). In Dorcas gazelle, 3 parasite species were identified; *N. helvetianus* (52 ± 0.1%), *N. sphatiger* (35 ± 0.1%) and *M. marshalli* (11.7 ± 0.07%). In Scimitar-horned Oryx, two parasite species were identified; *N. helvetianus* (73 ± 0.1%) and *N. sphatiger* (26 ± 0.1%). In Barbary sheep, and Barbary red deer only *N. helvetianus* was identified. In Slender-horned gazelle only *N. sphatiger* was identified.

Geographic distribution of nematode species

In the humid zone (National Park of El Feidja), only *N. helvetianus* was identified, in the semi-arid zone (National Park of Orbata) and arid zone (National Park of Bouhedma and Haddaj) *N. helvetianus*, *N. sphatiger* and *M. marshalli* were identified. In the Saharan zone (National Parks: Dghoumes, Djbil, Sidi Toui and the Nature Reserve of Oued Dekouk) four parasite species were identified; *C. mentulatus*, *C. ovina*, *N. helvetianus* and *N. sphatiger*.

Camelostrongylus mentulatus and *C. ovina* were identified only in the samples collected from Djbil National Park and *M. marshalli* only in Bouhedma and Orbata National Parks. *Nematodirus sphatiger* was absent in Dghoumes, El Feidja National Parks and Oued Dekouk Nature Reserve.

Intraspecific variation

The genetic analysis of 66 *N. helvetianus* amplicons from different hosts and geographic regions in Tunisia showed 100% of similarity. Ninety per cent (19/21) of *N. spathiger* isolated from different hosts and geographic regions were identical. Two out of 21 *N. spathiger* amplicons showed a low intraspecific variation (0·34%) by single point mutation that causes a nucleotide base substitution (A199C) for sequence KY930439 in Slender-horned gazelle (Sidi Toui National Park) and (A211G) for sequence KY930440 in goats (Bouhedma National Park) (Table 4). All eggs isolated from Slender-horned gazelle faecal samples were *N. spathiger*. The majority (4/5) of the isolates were genetically identical to *N. spathiger* isolated from various wild and domestic ruminants in the different locations, only one (1/5) amplicon showed an intraspecific variation. All the *M. marshalli* amplicons were 100% identical. The comparison of *C. mentulatus* amplicon from addax (KY930444) with an Australian amplicon from dromedary (*Camelus dromedarius*) (Y14819) showed 99.57% of similarity and a low intraspecific variation (0.42%) represented by a single point mutation (T47A) (Table 4). The comparison of *C. ovina* amplicon from addax (KY930445) with other sequences available in GenBank showed an intraspecific variation varying from 0.3 to 0.9%. It showed a high (99.65%) intraspecific similarity with *C. ovina* isolated from goat in China (KF913466). This genetic variation is a single nucleotide insertion (279insA) (Table 4).

Phylogenetic analyses

NJ tree of *N. helvetianus* isolated from Tunisian wild and domestic ruminants showed that they clustered in the same clade with

Table 2. Faecal samples' examination results by laboratory technique, animal species and localities

Bioclimatic stage	National park/ Natural reserve	Technique	Wild species (positive/examined)						Domestic species		
			Gazella dorcas	Gazella leptoceros	Cervus elaphus barbarus	Oryx dammah	Addax nasomaculatus	Ammotragus lervia	Sheep	Goats	Total
Humid	El Feidja	Fotation	–	–	0/10	–	–	–	0/10	0/10	0/30
		PCR	–	–	2/10	–	–	–	0/10	0/10	3/30
Arid	Bouhedma	Fotation	1/8	–	–	2/6	0/6	0/5	1/10	0/10	4/45
		PCR	3/8			3/6	4/6	5/5	1/10	1/10	17/45
	Haddaj	Fotation	0/10	–	–	1/7	2/6	–	2/7	0/10	5/40
		PCR	2/10			5/7	4/6	–	3/7	1/10	15/40
Semi-Arid	Orbata	Fotation	3/6	–	–	–	–	–	1/8	2/6	6/20
		PCR	5/6	–	–	–	–	–	3/8	3/6	11/20
	Dghoumes	Fotation	0/10	–	–	1/10	–	–	3/6	2/7	6/33
		PCR	2/10			2/10			4/6	4/7	12/33
Saharian	Sidi Toui	Fotation	4/5	4/6	–	3/7	–	–	0/10	0/10	11/38
		PCR	5/5	5/6		5/7			0/10	0/10	15/38
	Djbil	Fotation	–	–	–	–	0/7	–	0/10	0/10	0/27
		PCR	–	–	–	–	4/7		0/10	0/10	4/27
	Oued Dekouk	Fotation	–	–	–	2/9	–	3/6	2/7	3/7	10/29
	(natural reserve)	PCR	–	–	–	4/9	–	4/6	4/7	4/7	16/29
Total		Fotation	8/39	4/6	0/10	9/39	2/19	3/11	9/68	7/70	42/262
		PCR	17/39	5/6	2/10	19/39	12/19	9/11	15/68	13/70	92/262

(–): Animal species absent in the park or reserve.

Table 3. Host, geographic origins and GenBank accession number of the complete ITS2 region of the rDNA sequences of parasite species isolated from Tunisian wild and domestic ruminants' faecal samples

Parasite family	Parasite species	Host (geographic origin ([a]Accession number))							
		Addax nasomaculatus	*Ammotragus lervia*	*Cervus elaphus barbarus*	*Gazella dorcas*	*Gazella leptoceros*	*Oryx dammah*	Goats	Sheep
Strongylidae	*Chabertia ovina*	Djbil (KY930445)							
Trichostrongylidae	*Camelostrongylus mentulatus*	Djbil (KY930444)							
	Marshallagia marshalli	Bouhedma (KY930441)			Orbata (KY930442, KY930443)				
	Nematodirus helvetianus	Bouhedma (KY926477, KY926478, KY926479), Haddaj (KY926480), Djbil (KY926481, KY926482)	Bouhedma (KY926445, KY926446, KY926447, KY926448, KY926449), Oued Dekouk (KY926450, KY926451, KY926452, KY926453)	El Feidja (KY926483, KY926484)	Bouhedma (KY926454, KY926455), Haddaj (KY926456), Orbata (KY926457, KY926458, KY926459), Dghoumes (KY926460, KY9264601), Sidi Toui (KY926462)		Bouhedma (KY926463, KY926464), Haddaj (KY926465, KY926466), Dghoumes (KY926467, KY926468), Oued Dekouk (KY926469, KY926470, KY926471, KY926472), Sidi Toui (KY926473, KY926474, KY926475, KY926476)	Haddej (KY926510), Orbata (KY926507, KY926508, KY926509), Dghoumes (KY926503, KY926504, KY926505, KY926506), Oued Dekouk (KY926499, KY926500, KY926501, KY926502)	Bouhedma (KY926498), Haddaj (KY926495, KY926496, KY926497), Orbata (KY926493, KY926494), Dghoumes (KY926489, KY926490, KY926491, KY926492), Oued Dekouk (KY926485, KY926486, KY926487, KY926488)
	Nematodirus spathiger	Haddaj (KY930426, KY930427, KY930428)			Bouhedma (KY930420), Haddaj (KY930425), Sidi Toui (KY930429, KY930431, KY930432, KY930433)	Sidi Toui (KY930434, KY930435, KY930436, KY930437, KY930439)	Bouhedma (KY930421), Haddaj (KY930422, KY930423, KY930424), Sidi Toui (KY930430)	Bouhedma (KY930440)	Orbata (KY930438)

[a]GenBank Accession number.

Table 4. Fixed differences distinguishing on the ITS2 region of the rDNA sequences of *Chabertia ovina* (KY930445), *Nematodirus spathiger* (KY930434, KY930435, KY930436, KY930437, KY930439 and KY930440) and *Camelostrongylus mentulatus* (KY930444) extracted from wild and domestic ruminants' faecal samples in Tunisia.

Parasites species	Host	Origin	[a]Accession number	Nucleotides (alignment positions)					
C. ovina	Goat	China	KF913466	– (118)	– (119)	T (197)	– (200)	– (201)	– (280)
	Addax nasomaculatus	Djbil, Tunisia	KY930445	– (118)	– (119)	T (197)		– (201)	A (280)
	Sheep	New Zealand	KC998760	C (118)	A (119)	T (197)	– (200)	– (201)	– (280)
	Sheep	New Zealand	KC998759	– (118)	– (119)	A (197)	T (200)	A (201)	– (280)
N. spathiger	Sheep	New Zealand	KC998746	A (199)	A (211)				
	Gazella leptoceros	Sidi Toui, Tunisia	KY930434, KY930435, KY930436, KY930437	A (199)	A (211)				
	Goat	Bouhedma, Tunisia	KY930440	A (199)	G (211)				
	Gazella leptoceros	Sidi Toui, Tunisia	KY930439	C (199)	A (211)				
C. mentulatus	*Camelus dromedarius*	Melbourne zoo, Australia	Y14819	T (47)					
	Addax nasomaculatus	Djbil, Tunisia	KY930444	A (47)					

Nucleotides: T, Thymine; C, Cytosine; G, Guanine; A, Adenine.
[a]GenBank Accession number.
(–): Gap in the sequence alignment.

isolates from China (sheep) (KC580751 and KC580752) (bootstrap value = 78%) (Fig. 2). A phylogenetic tree of *N. spathiger* showed that all the isolates from various Tunisian ruminant species clustered in the same clade with the New Zealander (KC998746, KC998748) and American (AF194143) variants (bootstrap value = 87%). One isolate from goats (Bouhedma National Park) (KY930440) formed an innermost clade with two other amplicons from New Zealand (KC998747) and USA (AF194144) (bootstrap value = 66%) (Fig. 3).

Phylogenetic analysis of the three identical isolates of *M. marshalli* from two Dorcas gazelle (KY930442 and KY930443) and one addax (KY930441) in Tunisia clustered with *M. marshalli* from Iranian sheep (HQ389231) and *M. marshalli* from Russian sheep (JQ863403). Two *M. marshalli* amplicons from Uzbekistan (KT428384) and Russia (JQ839283) form a separate innermost clade in the first one. *Marshallagia marshalli* from France (AJ577469) and *M. marshalli* from Svalbard (Norway) (AH010211) clustered separately in a third clade (Fig. 4). *Camelostrongylus mentulatus* isolated from addax (KY930444) clustered in the same clade with the Australian amplicon from one dromedary (Y14819) with a high bootstrap support value (100%) (Fig. 4). *Chabertia ovina* (KY930445) clustered with amplicons from New Zealander sheep (KC998759 and KC998760) and goats from China (KF913466, KF913467 and KF913470).

Discussion

This study revealed a diversity in nematode species infecting the Tunisian wild ruminants. The addax nematode fauna was the most varied with five parasite species; *N. helvetianus*, *N. sphatiger*, *M. marshalli*, *C. mentulatus* and *C. ovina*. From all identified nematodes only *N. sphatiger* and *C. ovina* have been found or reported previously from domestic sheep and goat in Tunisia,

which reflects the loss of species specific of these nematodes. On a worldwide basis, a comparison of the helminth fauna in a number of wild ruminants species indicated that cross-transmission with domestic hosts is common and achieved by a multi-host parasites (Dunn, 1969; Sharhuu and Sharkhuu, 2004; Walker and Morgan, 2014).

In Scimitar-horned Oryx only two parasite species (*N. helvetianus* and *N. sphatiger*) were identified in Tunisia. Despite the two antelopes, Scimitar-horned Oryx and addax, shared the same grazing areas in Bouhedma National Park, *M. marshalli* was found only in the addax. The absence of this generalist nematode species in Scimitar-horned Oryx can be probably due (i) to the small number of collected samples compared with the number of the estimated population (6/38) or (ii) to a low-level parasitic burden of *M. marshalli* in the grazing areas especially since this parasite has not been reported previously in Tunisia. Pauling *et al.* (2016) revealed the presence of more diversified parasite fauna in two captive herds of Scimitar-horned Oryx, in Fossil Rim Wildlife Center (FRWC) and Kansas City Zoo (KCZ), in the USA.

Three parasite species were found in Dorcas gazelles (*N. helvetianus*, *N. sphatiger* and *M. marshalli*), and only a single species (*N. helvetianus*) from Barbary sheep and Barbary red deer. All parasites isolated from Slender-horned gazelle fecal samples were *N. spathiger*, which is in agreement with previous studies showing that *N. spathiger* was the most frequent species in Arabian sand gazelle (*Gazella subgutturosa marica*) and Arabian mountain gazelle (*Gazella gazella*) in Saudi Arabia (Mohammed *et al.* 2007).

The report of few nematode species per host can be related to the number of samples and to the low density of wild population. The estimated population of the studied wild ruminants is about only 1000 animals or the host–parasite relationship are ineluctably
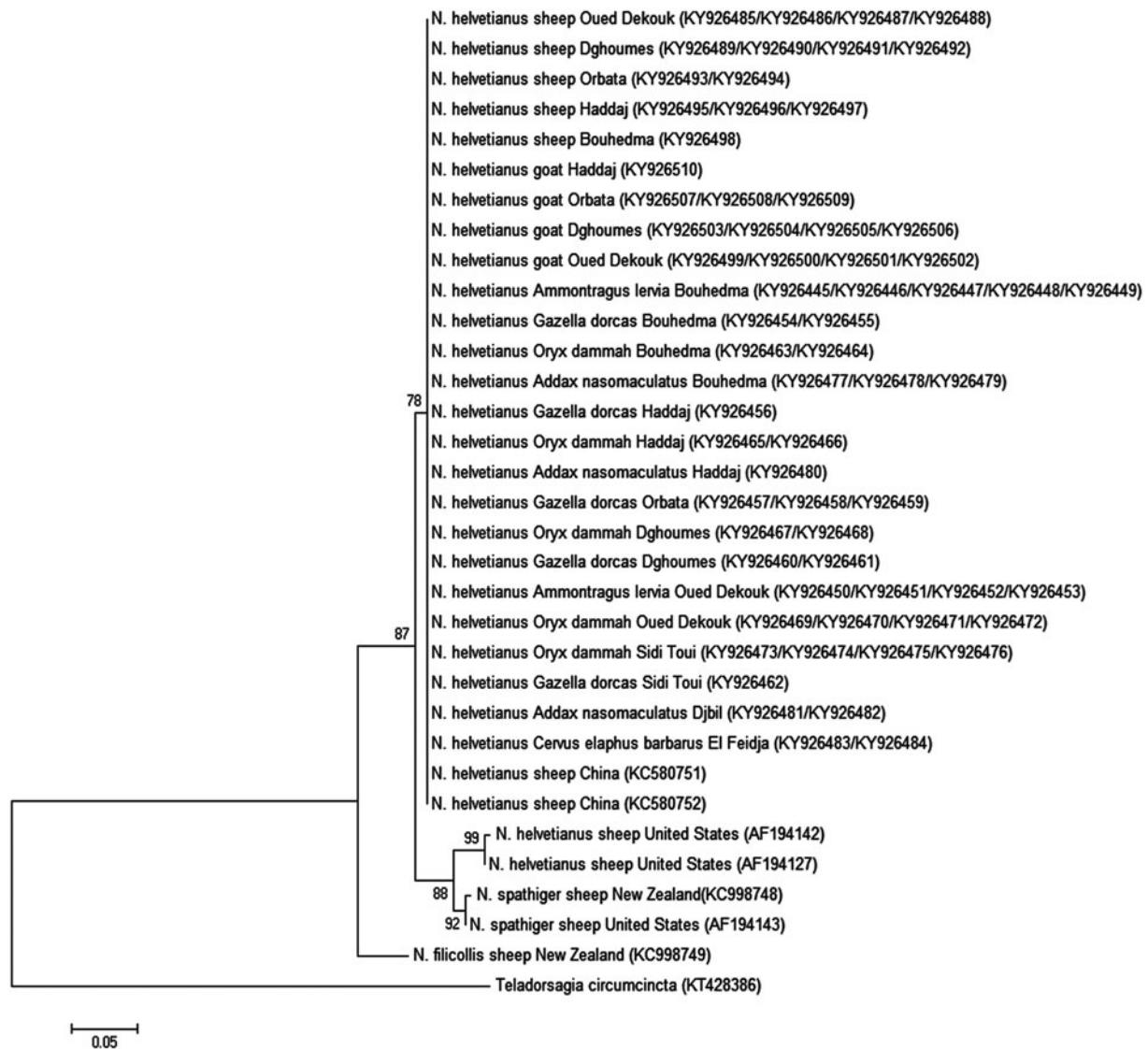
Fig. 2. Phylogenetic tree of *Nematodirus helvetianus* based on ITS2 sequences of the rDNA. The ITS2 sequences were aligned with Mega 6. Neighbour-joining (NJ) tree represent phylogenetic relationships for 66 sequences from wild and domestic ruminants from Tunisian's national parks and nature reserves and 8 sequences from Genbank database. The scale bar represents 0.05 substitution per site. Numbers associated with nodes represent the percentage of 1000 bootstrap supporting the nodes (only percentages higher than 70% were represented).

linked to host-population density and some localized parasite loss can occur when a host population density falls below a minimal threshold for parasite transmission (Lyles and Dobson, 1993).

Regarding the different bioclimatic zones, a richer parasite diversity was found in the Saharan zone, which may be related to the presence of four protected areas in this zone and to a higher sampling. In fact, our results support that certain parasite species have evolved and acquired a high capacity of adaptation to resist to desiccation, and to low and very high temperature characterizing this area.

Marshallagia marshalli, parasite of domestic and wild ruminants, was isolated from addax and Dorcas gazelle living in two national parks (Bouhedma and Orbata) located in semi-arid and arid zones of Tunisia, which is in agreement with the study of Meradi *et al.* (2011) showing that this species is more commonly found in the arid zones (Steele *et al.* 2013). In addition, the occurrence of this nematode species in addax and Dorcas gazelle was not reported previously in the literature in spite of its occurrence in different other wild Bovidae species.

Nematodirus spathiger has not been isolated in humid (El Feidja National Park) and Saharan zones (Djbil and Dgoumes National Parks and Oued Dekouk Nature Reserve). A previous study on goats from the northwest of Tunisia (humid zone) revealed the presence of *N. spathiger* and *N. filicollis* in this region (Azizi, 1985). Therefore, the absence of this species in our study can be probably due to the small number of examined samples and the regular anthelmintic treatments administered to sheep and goats.

Among the five isolated parasites species, *N. helvetianus* was the most prevalent in domestic sheep and goat and wild ruminants, present in all studied bioclimatic zones and not reported previously in Tunisia. This result is in agreement with the observations of Meradi *et al.* (2011) who reported a very high prevalence of *N. helvetianus* in sheep in Algeria. This nematode is a common parasite species of cattle (Anderson, 2000) or its large distribution in different bioclimatic areas and different host shows that the co-evolution of parasites with their hosts and host switching can have profound effects on host specificity.

Previous studies on Tunisian domestic ruminants reported the presence of only *N. spathiger* and *N. filicollis* (Hmidi, 1982; Azizi, 1985). Thus, it can be concluded that there are at least 3 species of *Nematodirus* in Tunisian ruminants as in Algeria (Meradi *et al.* 2011).
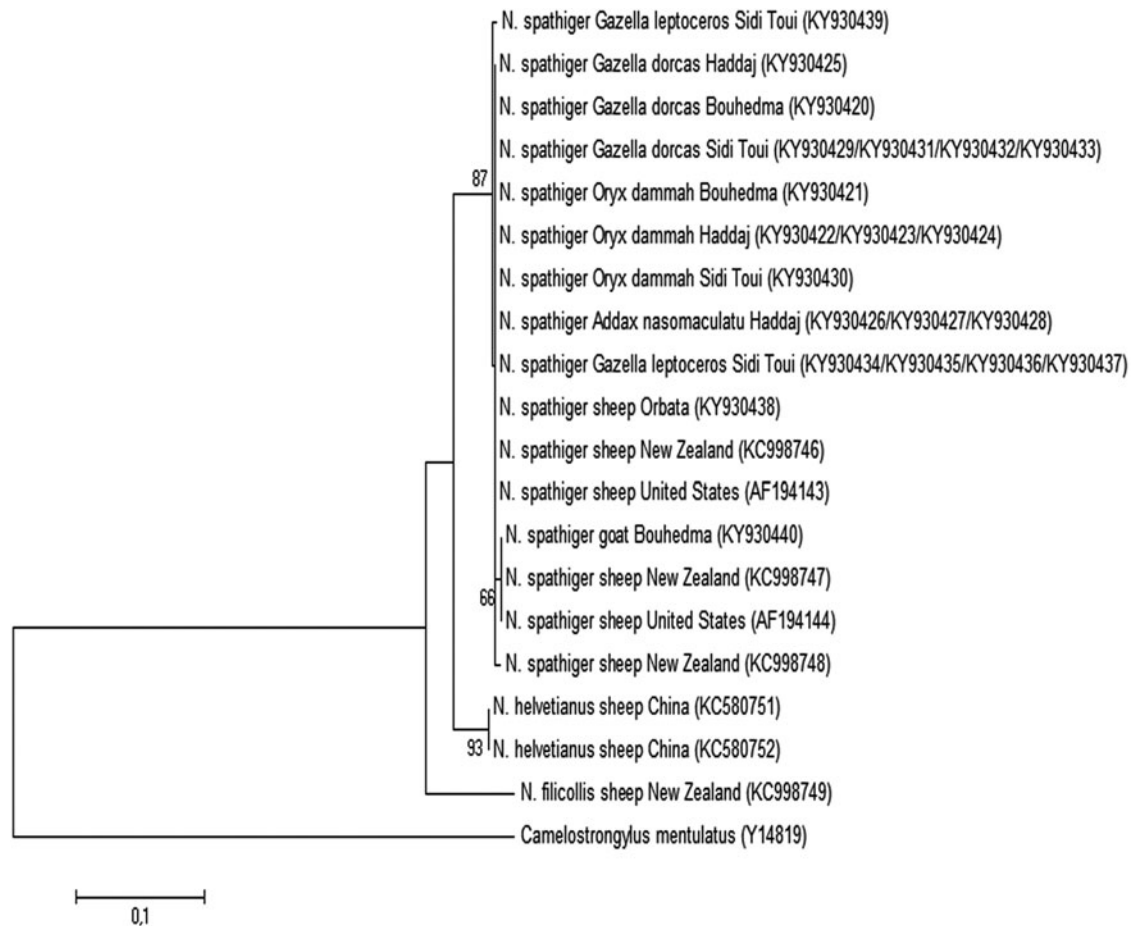
Fig. 3. Phylogenetic tree of *Nematodirus spathiger* based on ITS2 sequences of the rDNA. The ITS2 sequences were aligned with Mega 6. Neighbour-joining (NJ) tree represent phylogenetic relationships for 21 sequences from wild and domestic ruminants from Tunisian's national parks and nature reserve and 9 sequences from Genbank database. The scale bar represents 0.1 substitution per site. Numbers associated with nodes represent the percentage of 1000 bootstrap supporting the nodes (only percentages higher than 65% were represented).

In this study, the occurrence of *C. mentulatus* is reported for the first time in the addax and in a Tunisian wild host. Currently, this pathogenic nematode is not considered as a common parasite but it was reported in various domestic and wild hosts in several regions of the world, such as sheep, goats, camels, Arabian oryx (*Oryx leucoryx*), Slender-horned gazelle and giraffes (Lichtenfels and Hoberg, 1993; Fukumoto *et al.* 1996; Ruiz de Ybáñez *et al.* 2003; Goossens *et al.* 2005). In Tunisia, *C. mentulatus* was reported only in dromedary by Mabrouk (1988). The presence of *C. mentulatus* in addax living in Djbil National Park may be related to the presence of dromedary herds living near this park and to the fact that this protect area was previously a grazing area of this animal species before the reintroduction of addax. In fact, this explanation support that parasite transmission could have occurred from environment, water, or grazing areas even when there is no direct contact between hosts. The identification of this parasite species in a wild critically endangered species is very important and requires vigilance to ensure their survival. In the literature this nematode species seemed to cause lethal infection in wild hosts such as in Blackbuck, Dorcas gazelle and Thomson's Gazelle (*Eudorcas thomsonii*) (Flach and Sewell, 1987; Ortiz *et al.* 2006) and in domestic hosts only cattle were apparently refractory to infection (Thornton *et al.* 1973*a*,*b*).

All identified nematodes in this study, except *C. mentulatus*, are multi-host species and two of them (*N. helvetianus* and *N. sphatiger*) were confirmed to be concurrently found in domestic hosts living around the protected areas. The results of this study are in agreement with the results of Walker and Morgan (2014) that reported that generalist parasites contribute more than specialists to the diversity of parasites in both wild and domestic species and that cross-transmissions between domestic and wild hosts are variable and depending on the regions.

Fifty positive samples by PCR were negative with the flotation technique. PCR method detected animals with very low infection levels because of the very high PCR sensitivity. PCR is not used as a routine screening laboratory tool but is suitable to detect asymptomatic infections or animals with low infection levels. This status is frequent in wild animals since they are more resistant and express low or no clinical signs. Due to the low parasite loads in the fecal samples only the association of three PCRs allowed to get a higher concentration of PCR products.

No intraspecific variation has been recorded in *N. helvetianus* isolated from various animal species and geographic localities as reported previously by Heise *et al.* (1999). Only 10% of isolated *N. spathiger* represented a low intraspecific variation by a single nucleotide difference (0·34%). The observed number of variation site is in agreement with the proportion of polymorphic ITS2 sites (0–0·9%) in *Nematodirus* (Audebert *et al.* 2000) but less than that reported by Nadler *et al.* (2000) within *N. spathiger*, *N. filicollis* and *N. battus* (12; 15 and 19 sites, respectively).

Even though only five nematode species were reported in this study, this parasite biodiversity have a great value and could serve as a baseline to understand the multi-host/multi-parasite systems and parasite distribution in wild hosts. To the best of our knowledge, this is the first molecular identification of five gastrointestinal
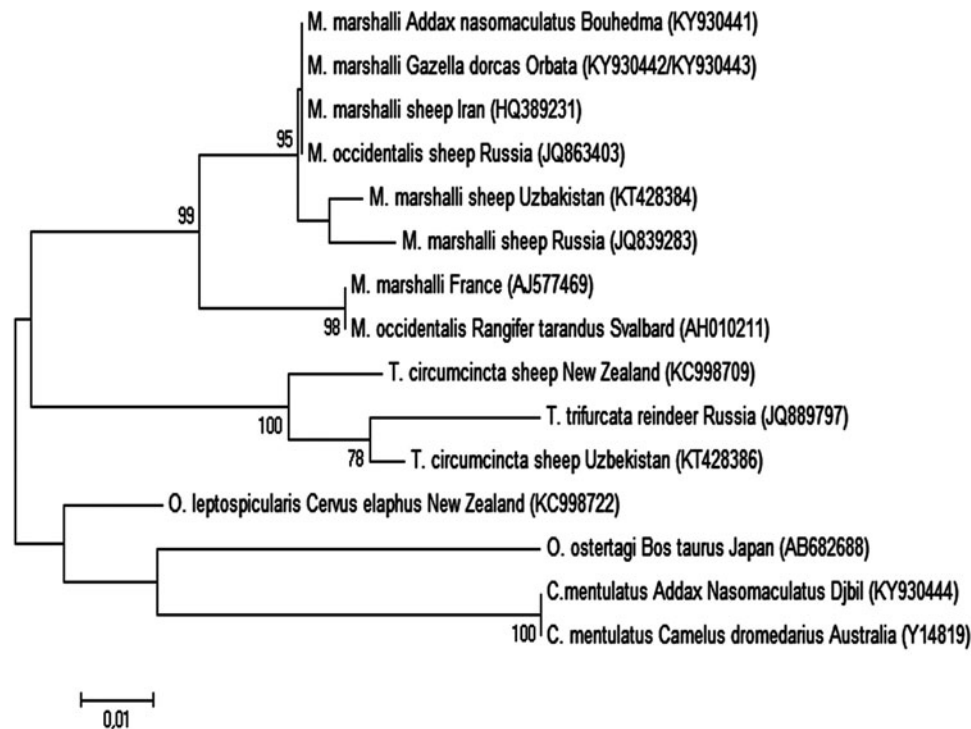
Fig. 4. Neighbour-joining (NJ) tree, based on ITS2 sequences of the rDNA, represents phylogenetic relationships of *Marshallagia marshalli* (3 sequences), *Camelostrongylus mentulatus* (1 sequence) isolated from Tunisian Wild ruminants and 12 sequences from the trichostrongylidae family from GenBank database (*Marshallagia occidentalis*, *Marshallagia marshalli*, *Ostertagia ostertagi*, *Ostertagia leptospicularis*, *Teladorsagia circumcincta* and *Teladorsagia trifurcata*). The scale bar represents 0.01 substitution per site. Numbers associated with nodes represent the percentage of 1000 bootstrap supporting the nodes (only percentages higher than 70% were represented).

nematode species (*C. ovina*, *C. mentulatus*, *M. marshalli*, *N. helvetianus* and *N. spathiger*) of North African wild ruminants and the first report of *C. mentulatus* and *M. marshalli* in addax and of *M. marshalli* in Dorcas gazelle. Also two new records of nematode species in Tunisia, *N. helvetianus* and *M. marshalli* were reported in this study, which extends the knowledge on the biodiversity of nematode parasites of wild ruminants in Tunisia.

References

Albon SD, Stien A, Irvine RJ, Langvatn R, Ropstad E and Halvorsen O (2002) The role of parasites in the dynamics of a reindeer population. *Proceedings of the Royal Society of London. Series B, Biological Sciences* **269**, 1625–1632.

Anderson RC (2000) *Nematode Parasites of Vertebrates: Their Development and Transmission*. UK: Commonwealth Agricultural Bureau International (CABI Publishing).

Anonymous (2013) *Forêt Tunisienne Habitat pour la Faune et la Flore sauvage*. Tunisia: Direction Générale des forêts, Ministère de l'Agriculture.

Arneberg P, Folstad I and Karter AJ (1996) Gastrointestinal nematodes depress food intake in naturally infected reindeer. *Parasitology* **112**(Pt 2), 213–219.

Audebert F, Durette-Desset MC and Chilton NB (2000) Internal transcribed spacer rDNA can be used to infer the phylogenetic relationships of species within the genus *Nematodirus* (Nematoda: molineoidea). *International Journal for Parasitology* **30**, 187–191.

Azizi A (1985) *Les strongles gastro-intestinaux de la chèvre : identification et étude épidémiologique dans la region de Ain Draham*. DVM thesis, National School of Medecine Veterinary of Sidi Thabet, Tunisia.

Bott NJ, Campbell BE, Beveridge I, Chilton NB, Rees D, Hunt PW and Gasser RB (2009) A combined microscopic-molecular method for the diagnosis of strongylid infections in sheep. *International Journal for Parasitology* **39**, 1277–1287.

Brooker S (2010) Estimating the global distribution and disease burden of intestinal nematode infections: adding up the numbers – a review. *International Journal for Parasitology* **40**, 1137–1144.

Dobson AP and Hudson PJ (1986) Parasites, disease and the structure of ecological communities. *Trends in Ecology and Evolution* **1**, 11–15.

Dunn AM (1969) The wild ruminant as reservoir host of helminth infection. *Symposia of the Zoological Society of London* **24**, 221–248.

Flach EJ and Sewell MMH (1987) Gastrointestinal nematodiasis in Blackbuck (*Antilope cervicapra*) at Edinburgh Zoo. *Journal of Zoo and Wildlife Medicine* **18**, 56–61.

Fukumoto S, Uchida T, Ohbayashi M, Ikebe Y and Sasano S (1996) A new host record of *Camelostrongylus mentulatus* (Nematoda; Trichostrongyloidea) from abomasum of a giraffe at a zoo in Japan. *Journal of Veterinary Medical Science* **58**, 1223–1225.

Gasser R, Nansen P and Guldberg P (1996) Fingerprinting sequence variation in ribosomal DNA of parasites by DGGE. *Molecular and Cellular Probes* **10**, 99–105.

Gharaibeh BM (1997) *Systematics, distribution, and zoogeography of mammals of Tunisia*. PhD theses, Faculty of Texas Tech University, Lubbock, Texas, USA.

Gilbert T and Woodfine T (2008) *The Reintroduction of Scimitar-Horned Oryx Oryx Dammah to Dghoumes National Park, Tunisia, Report to Members of the European Endangered Species Programme for Scimitar-Horned Oryx*. Winchester: Marwell Preservation Trust.

Goossens E, Dorny P, Boomker J, Vercammen F and Vercruysse J (2005) A 12-month survey of the gastro-intestinal helminthes of antelopes, gazelles, and giraffids kept at two zoos in Belgium. *Veterinary Parasitology* **127**, 303–312.

Hajji GM, Zachos FE, Charfi-Cheikrouha F and Hartl GB (2007) Conservation genetics of the imperilled Barbary red deer in Tunisia. *Animal Conservation* **10**, 229–235.

Heise M, Epe C and Schnieder T (1999) Differences in the second internal transcribed spacer (ITS-2) of eight species of gastrointestinal nematodes of ruminants. *Journal of Parasitology* **85**, 431–435.

Hmidi A (1982) *Contribution a l'etude des strongyloses digestives du moutons: caracteres epidemiologiques dans la region de Sousse.* DVM thesis, National School of Medecine Veterinary of Sidi Thabet, Tunisia.

Hoberg EP, Monsen KJ, Kutz S and Blouin MS (1999) Structure, biodiversity, and historical biogeography of nematode faunas in holarctic ruminants: morphological and molecular diagnoses for *Teladorsagia boreoarcticus* n. sp. (Nematoda: Ostertagiinae), a dimorphic cryptic species in muskoxen (*Ovibos moschatu*) *Journal of Parasitology* **85**, 910–934.

Hughes J, Albon SD, Irvine RJ and Woodin S (2009) Is there a cost of parasites to caribou? *Parasitology* **136**, 253–265.

Kimura M (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution* **16**, 111–120.

Lichtenfels JR and Hoberg EP (1993) The systematics of nematodes that cause ostertagiasis in domestic and wild ruminants in North America: an update and a key to species. *Veterinary Parasitology* **46**, 33–53.

Lovari S, Lorenzini R, Masseti M, Pereladova O, Carden RF and Brook SM (2016) *Cervus elaphus.* The IUCN Red List of Threatened Species 2016: e.T55997072A22155320. http://dx.doi.org/10.2305/IUCN.UK.2016-2.RLTS.T55997072A22155320.en.

Lyles AM and Dobson AP (1993) Infectious disease and intensive management: population dynamics, threatened hosts, and their parasites. *Journal of Zoo and Wildlife Medicine* **24**, 315–326.

Mabrouk T (1988) *Contribution à l'étude des helminthes gastro-intestinaux du dromadaire en Tunisie.* DVM thesis, National School of Medecine Veterinary of Sidi Thabet, Tunisia.

Meradi S, Bentounsi B, Zouyed I and Cabaret J (2011) The steppe species of gastrointestinal nematodes of small ruminants, with a focus on Marshallagia: climate as a key determinant. *Parasite: Journal de la Société Française de Parasitologie* **18**, 261–269.

Mohammed OB, Omer SA and Sandouka MA (2007) The efficacy of ivermectin and levamisole against natural *Nematodirus spathiger* infection in the Arabian sand gazelle (*Gazella subgutturosa marica*) and the Arabian mountain gazelle (*Gazella gazella*) in Saudi Arabia. *Veterinary Parasitology* **150**, 170–173.

Morgan ER, Clare EL, Jefferies R and Stevens JR (2012) Parasite epidemiology in a changing world: can molecular phylogeography help us tell the wood from the trees? *Parasitology* **139**, 1924–1938.

Nadler SA, Hoberg EP, Hudspeth DS and Rickard LG (2000) Relationships of *Nematodirus* species and *Nematodirus battus* isolates (Nematoda: Trichostrongyloidea) based on nuclear ribosomal DNA sequences. *Journal of Parasitology* **86**, 588–601.

Ortiz J, Ruiz de Ybáñez R, Abaigar T, Goyena M, Garijo M, Espeso G and Cano M (2006) Output of gastrointestinal nematode eggs in the feces of captive gazelles (*Gazella dama mhorr*, *Gazella cuvieri* and *Gazella dorcas neglecta*) in a semiarid region of southeastern Spain. *Journal of Zoo and Wildlife Medicine* **37**, 249–254.

Pauling CD, Oller AR and Jackson V (2016) Fecal parasite identification by microscopy and PCR in scimitar-horned oryx, *Oryx dammah*, managed at two sites. *International Journal for Parasitology: Parasites and Wildlife* **5**, 312–320.

Perry BD and Randolph TF (1999) Improving the assessment of the economic impact of parasitic diseases and of their control in production animals. *Veterinary Parasitology* **84**, 145–168.

Ruiz de Ybáñez MR, Garijo MM, Carpintero M, Martínez-Carrasco C and Ortiz JM (2003) *Camelostrongylus mentulatus* in domestic goats from the Iberian Peninsula. *Journal of Helminthology* **77**, 371–372.

Saitou N and Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* **4**, 406–425.

Sharhuu G and Sharkhuu T (2004) The helminth fauna of wild and domestic ruminants in Mongolia – a review. *European Journal of Wildlife Research* **50**, 150–156.

Sim KA, Hoar B, Kutz SJ and Chilton NB (2010) Amplification of the second internal transcribed spacer ribosomal DNA of individual trichostrongylid nematode larvae by nested polymerase chain reaction. *Journal of Veterinary Diagnostic Investigation* **22**, 433–437.

Steele J, Orsel K, Cuyler C, Hoberg EP, Schmidt NM and Kutz SJ (2013) Divergent parasite faunas in adjacent populations of west Greenland caribou: natural and anthropogenic influences on diversity. *International Journal for Parasitology: Parasites and Wildlife* **2**, 197–202.

Stien A, Irvine RJ, Ropstad E, Halvorsen O, Langvatn R and Albon SD (2002) The impact of gastrointestinal nematodes on wild reindeer: experimental and cross-sectional studies. *Journal of Animal Ecology* **71**, 937–945.

Tamura K, Stecher G, Peterson D, Filipski A and Kumar S (2013) MEGA6: molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution* **30**, 2725–2729.

Thienpont D, Rochette F and Vanparijs OF (1979) *Diagnosing Helminthiasis Through Coprological Examination*, 18th edn. Beerse, Belgium: Janssen Research Foundation.

Thornton JE, Galvin TJ, Bell RR and Ramsey CW (1973a) Parasites of the blackbuck antelope (*Antilope cervicapra*) in Texas. *Journal of Wildlife Diseases* **9**, 160–162.

Thornton JE, Galvin TJ, Bell RR and Ramsey CW (1973b) Transmissibility of gastrointestinal nematodes from blackbuck antelope to cattle, sheep, and goats. *Journal of the American Veterinary Medical Association* **163**, 554–555.

Thumbi SM, Bronsvoort BMDC, Poole EJ, Kiara H, Toye P, Ndila M, Conradie I, Jennings A, Handel IG, Coetzer J A W, Hanotte O and Woolhouse MEJ (2013) Parasite co-infections show synergistic and antagonistic interactions on growth performance of East African zebu cattle under one year. *Parasitology* **140**, 1789–1798.

Walker JG and Morgan ER (2014) Generalists at the interface: nematode transmission between wild and domestic ungulates. *International Journal for Parasitology: Parasites and Wildlife* **3**, 242–250.

Woodfine T, Chetoui A, Zahzah K and Gilbert T (2011) Re-introduction of scimitar-horned oryx to Dghoumes National Park, Tunisia. In Soorae PS (ed.). *Global Re-Introduction Perspectives: 2011, More Case Studies from Around the Globe.* IUCN Publication, pp. 190–193.