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RESEARCH

First morphometric and molecular characterization of *Fasciola* spp. in Northwest Tunisia

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Abstract

The aim of this study was to characterize the Tunisian *Fasciola* spp. flukes by morphometric and molecular analyses. Flukes were collected from livers of sheep slaughtered in Sejnane slaughterhouses (Bizerte gouvernorate, Northwest Tunisia) between January and March 2021.

Five morphometric parameters were determined for all the liver flukes, as follows: (i) total body length (BL), (ii) distance between ventral sucker and the tail (VS-T), (iii) distance between oral sucker and ventral sucker (OS-VS), (iv) abdomen diameter (AD), (v) tail diameter (TD) and the body length to width ratio (BL/BW). Molecular identification of the fluke specimens was carried out by polymerase chain reaction, restriction fragment polymorphism (PCR-RFLP) of a 680 bp sequence of the internal transcribes spacer 1 (ITS1) gene and by amplification, sequencing, and phylogenetic analysis of a 500 bp sequence of the ITS2 gene. Morphometric measurements showed that the mean of the total body length of the adult flukes was 21.1 ± 2.7 mm with minimum and maximum lengths of 13 and 31 mm, respectively. The PCR-RFLP analysis revealed a single profile consisting of three bands of approximately 370, 100, and 60 bp. *Fasciola* sequences described in the present study (GenBank numbers: OQ457027 and OQ457028) showed 99.58–100% identity to *Fasciola hepatica*. In conclusion, the results of this study show that molecular and phylogenetic analyses confirm the presence of a single species of *F. hepatica* in the Sejnane region Northwest of Tunisia. However, further studies are needed to identify the occurrence of *Fasciola* species in other Tunisian regions.

Keywords *Fasciola hepatica* · Sheep · Morphometry · PCR-RFLP · ITS1 · ITS2 · Tunisia

Introduction

Fasciolosis is a worldwide zoonosis caused by *Fasciola* trematode parasites, mainly *Fasciola hepatica* (Linnaeus, 1758) and *Fasciola gigantica* (Cobbold, 1856) (Itagaki et al. 2022).

Liver flukes primarily infect domestic ruminants, although wild herbivores and other mammals, including humans, can be also infected (Mas-Coma et al., 2009; Sabourin et al., 2018; Evack et al., 2020; Omar et al., 2021). Of the two species, *F. hepatica* has a cosmopolitan distribution over the five continents with the exception of Antarctica (Abebe et al., 2010;

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Admassu et al., 2015), while *F. gigantica* is widespread in the tropical and subtropical regions of Africa and Asia (Robinson and John 2009). Moreover, *F. hepatica* and *F. gigantica* could coexist in areas where the climatic and ecological conditions allow the survival intermediate hosts of both species (Mas-Coma et al., 2009; Malatji et al., 2019). Consequently, cases of hybridization between *F. hepatica* and *F. gigantica* have been reported in areas where the geographical distribution of the two *Fasciola* species overlaps, such as in some African countries like Egypt and South Africa (Amer et al., 2011; Haridwal et al., 2021) and in Asia like Japan, Korea, China, and Vietnam (Itagaki et al., 2005a; Ichikawa and Itagaki, 2012; Anh et al., 2018; Itagaki et al., 2022). *Fasciola* hybrids result from hybridization between *Fasciola* species composed of both mixed and introgressive genotypes resulting from interspecific mating between them (Nguyen et al., 2018; Eliza et al., 2020).

It is important to distinguish between the two infections caused by *F. hepatica* and *F. gigantica*, respectively, as they have distinct pathological and epidemiological features and different control approaches. Distinction at the species level can be performed by morphological measurements of adult flukes based on body length and width, including the *ratio* between them (Periago et al., 2006, 2008; Diyana et al., 2020; Shykat et al., 2022), but this method does not allow identification in areas where the two species overlap due to the multiple variations in their morphological characteristics (Mas-Coma and Bargues 1997). *Fasciola* identification to the species level can be performed also by molecular tools. For this purpose, different molecular targets have been used, mainly nucleotide sequences of the first nuclear ribosomal internal transcribed spacer (ITS1), second nuclear ribosomal internal transcribed spacer (ITS2), and 28S ribosomal RNA genes (28S rRNA) (Marcilla et al., 2002; Itagaki et al., 2005a; Anh et al., 2018; Evack et al., 2020; Omar et al., 2021). Moreover, mitochondrial DNA (mtDNA) markers like cytochrome c oxidase 1 (cox1) and the NADH dehydrogenase subunit 1 (NAD1) genes have been developed for the phylogenetic studies and genetic variability of *Fasciola* species (Farjallah et al., 2009; Gupta and Bhardwaj, 2013; Bargues et al., 2017; Laatamna et al., 2021; Shykat et al., 2022). Furthermore, the coprological analyses were not able to differentiate between the eggs of the two *Fasciola* species; however, they only allow a diagnosis at the genus level (Mas-Coma et al., 2005).

Fasciolosis is one of the most serious parasitic infections of small ruminants in Tunisia, particularly in sheep reared in the northwest and southwest regions of the country. The mean prevalence of *F. hepatica* infection in the Sejnane region (Northwest Tunisia) was 70%, 65%, and 60% in tracer lambs, ewes, and lambs, respectively (Akkari et al., 2011), while the prevalence values were about 35% and 44% in sheep in Gafsa and Tozeur regions (southwest Tunisia), respectively (Ayadi et al., 1997; Hammami et al., 2005). Human fasciolosis appears to be under notified and/or diagnosed in Tunisia since only 36 cases were reported between 1940 and 2005 (Hammami et al., 2005). However, there is a lack of molecular studies and genetic characterization of Tunisian liver flukes (Farjallah et al., 2009; Amor et al., 2011). Moreover, morphometric characterization was never performed on Tunisian *Fasciola* populations. Therefore, the present study aimed to perform the first morphological and molecular study on Tunisian *Fasciola* spp. population collected from sheep livers in Sejnane slaughterhouse, Northwest Tunisia, using polymerase chain reaction restriction fragment polymorphism (PCR-RFLP) analysis of the ITS1 and sequence analysis of ITS2.

Materials and methods

Study area and sample collection

Between January and March 2021, monitoring activities were performed at the local sheep slaughterhouse in Sejnane (Northwest Tunisia). The Sejnane district belongs to the governorate of Bizerte, and it is situated in the Northwest of Tunisia at a mean altitude of 92 m (Fig 1). This locality is characterized by a Mediterranean climate with a mean annual rainfall of 507.5 mm and a mean annual temperature ranging from 10.7 to 30 °C during winter and summer, respectively (National Institute of Meteorology, Tunisia 2021). Moreover, this slaughterhouse is the only one in the Sejnane district, which includes the three main endemic regions of fasciolosis in northern Tunisia (Sejnane, Joumine, and Bazina).

A total of 382 adult liver flukes were collected from 66 sheep slaughtered in the study period. The mean number of flukes collected from each liver was 5.78 ± 2.01 with a maximum and minimum of 2 and 24 flukes per liver, respectively. After collection, the liver flukes were thoroughly washed with sterile distilled water and preserved in 70% ethanol at room temperature until further analysis.

Fasciola morphometric measurement

Of the total 382 liver flukes, 335 (87.7%) were subjected to morphometric analysis, as some specimens were damaged during collection. To characterize the Tunisian *Fasciola* liver flukes, several morphometric parameters, expressed in millimeters (mm), were estimated using a stereomicroscope equipped with a calibrated ocular micrometer. Therefore, the morphometric parameters included in the study were as follows: (i) total body length (BL), (ii) distance between ventral sucker and the tail (VS-T), (iii) distance between oral sucker and ventral sucker (OS-VS), (iv) abdomen diameter

Fig. 1 Map of Tunisia showing the study area (Sejnane district, Northwest Tunisia)

(AD), (v) tail diameter (TD) and the body length to width ratio (BL/BW) (Valero et al., 2001; Akhlaghi et al., 2017; Diyana et al., 2020) (Fig. 2, Table 1). The results are given as mean values, standard deviation (SD), and range of values (min and max) of all measured parameters.

To avoid any bias due to specimens' preservation, all morphometric measurements were carried out in the day of flukes' collection.

DNA extraction

A total of 66 liver flukes (i.e., one fluke for each liver) were selected for molecular analyses. DNA was extracted from approximately 50 mg of each fluke specimen, using the Wizard® Genomic DNA Purification Kit (Wizard Genomics DNA Promega, Madison, WI, USA) according to the manufacturer's instructions, and DNA was stored at −20 °C until analysis. The PCR was performed for each sample using a pair of primers targeting the hypervariable regions V1–V3 coding for the 18S rRNA gene to detect the presence of eukaryotic DNA and to assess its quality as described by Wang et al. (2014) (Table 2).

ITS1-PCR and RFLP analysis

PCR was performed for the *Fasciola* spp. ITS1 gene, approximately 680 bp in length, coding for the 18S-5.8S rRNA, according to the protocol of Itagaki et al. (2005b). Briefly,

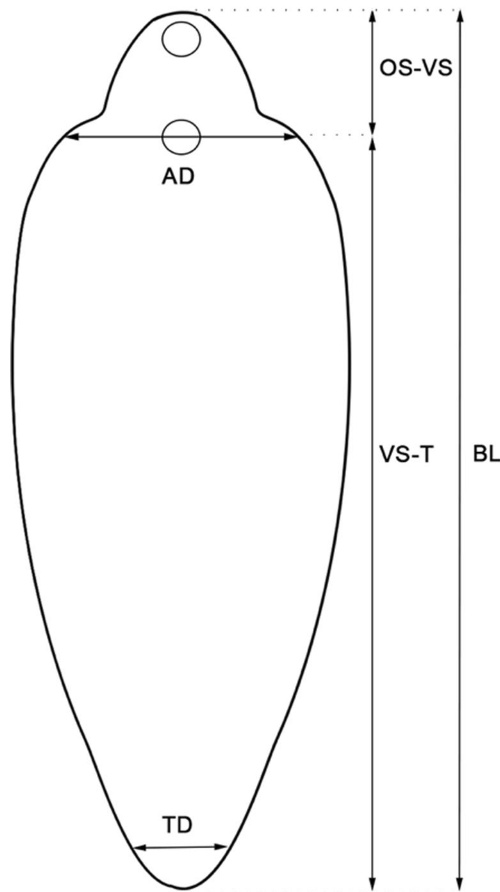

Fig. 2 Schematic representation of morphometric measurements. BL, total body length; VS-T, distance between ventral sucker and the tail; OS-VS, distance between oral sucker and ventral sucker; AD, abdomen diameter; TD, tail diameter and the body length to width ratio (BL/BW)

a total volume of 25 µl containing 1x PCR buffer, 4.5 mM $MgCl_2$, 1.0 mM of each dNTP, 0.75 U of Taq polymerase (Bioron GmbH, Germany), 10 µM of each primer (ITS1-F: TTGCGCTGATTACGTCCCTG and ITS1-R: TTGGCT GCGCTCTTCATCGAC) (Bio Basic, Canada Inc.), and 2 µL DNA sample was prepared A negative control consisting of nuclease-free water was added for each PCR run. The PCR program consisted of an initial denaturation at 94 °C for 10 min, followed by 25 cycles (94; 58 and 72 °C for 90 s each) and a final extension at 72 °C for 10 min (Table 2). PCR products were examined in a 2% w/v agarose gel, stained with ethidium bromide at a concentration of 0.5 µg/ml, and visualized under UV light.

Fasciola species were identified using an RFLP assay with the RsaI restriction enzyme. The reaction mixture consisted of 10 µL PCR product, 2 µl buffer, 1 µL RsaI enzyme, and distilled water for a final volume of 22 µL. Then, the mixture was incubated at 37 °C for 3 h (Nangru et al. 2022). The RFLP products were electrophorized in a 2.5% w/v agarose gel, stained with ethidium bromide at a concentration of 0.5 µg/mL, and then visualized under UV light. ITS1 profiles allowing species identification were obtained from the study by Ichikawa and Itagaki (2010).

ITS2 amplification and phylogenetic analysis

The genetic diversity of Tunisian *Fasciola* specimens was investigated by amplifying approximately a 500 bp of the ITS2 gene coding for the 5.8S and 28S rRNA according to the protocol of Itagaki et al. (2005a). The reaction volume consisted of 12.5 µl master mix (Takara, France), 6.25 pM of each primer (ITS2-F: TGTGTCGATGAAGAGCGCAG and ITS2-R: TGGTTAGTTTCTTTTCCTCCGC) (Eurofins,

Table 1 Morphometric data (mean, SD, and range values) of *Fasciola* flukes isolated from sheep in Tunisia compared to those of *F. hepatica* in Bolivia and Iran and *Fasciola gigantica* in Iran

Morphometric parameters	Mean in mm ± SD (range)			
	Fasciola spp. (Sheep, Tunisia) (Current study)	*Fasciola hepatica* (Sheep, Bolivia) (Valero et al. 2001)	*Fasciola hepatica* (Sheep, Iran) (Yakhchali et al. 2015)	*Fasciola gigantica* (Sheep, Iran) (Yakhchali et al. 2015)
Total body fluke length (BL)	21.1 ± 2.7 (13–31)	16.10 ± 4.80 (4.90–31.11)	21.1 ± 2	34.1 ± 4
Abdomen diameter (AD)	10.14 ± 3.04 (2–18)	7.11 ± 2.27 (1.58–12.55)	12 ± 1.5	9 ± 0.1
Ventral sucker to tail length (VS-T)	18.5 ± 2.6 (11–27)	13.03 ± 4.45 (3.16–27.39)	19 ± 1.8	23.7 ± 4.2
Oral to ventral sucker length (OS-VS)	2.6 ± 0.7 (1–9)	1.51 ± 0.31 (0.06–2.56)	2.8 ± 0.8	2.9 ± 0.3
Tail diameter (TD)	6 ± 2.22 (2–12)	NA	NA	NA
Body length to width ratio (BL/BW)	2.32 ± 0.96 (1.07–5.75)	2.33 ± 0.44 (1.33–4.17)	(1.46 - 2.09)	(3.77–6.28)

SD standard deviation, *NA* not available

Table 2 *Fasciola* spp. PCR primers and conditions

Target DNA	Primer name sequence (5′–3′)	Amplicon size (bp)	Amplification cycle	Reference
18S	1A: AAC CTG GTT GAT CCT GCC AGT 564R: GGC ACC AGA CTT GCC CTC	700	Initial denaturation: 5 min at 94 °C Amplification: 25 cycles at 94; 59 and 72 °C for 50 s each Final extension: 72 °C for 10 min	Wang et al. (2014)
ITS1	ITS1-F: TTGCGCTGATTACGTCCCTG ITS1-R: TTGGCTGCGCTCTTCATCGAC	680	Initial denaturation: 10 min at 94 °C Amplification: 25 cycles at 94; 58 and 72 °C for 90 s each Final extension:72 °C for 10 min	(Itagaki et al. 2005b)
ITS2	ITS2-F: TGTGTCGATGAAGAGCGCAG ITS2-R: TGGTTAGTTTCTTTTCCTCCGC	500	Initial denaturation: 10 min at 95 °C Amplification: 35 cycles at 94 °C for 60 s, 53 °C for 90 s, and 72 °C for 60 s Final extension: at 72 °C for 10 min	(Itagaki et al. 2005a)

Germany), 5 µl of DNA, and distilled water to a volume of 25 µL. The amplification conditions were as follows: an initial denaturation at 95 °C for 10 min, followed by 35 cycles (94 °C at 60 s, 53 °C at 90 s, and 72 °C at 60 s) and a final extension at 72 °C for 10 min (Table 2). PCR products were electrophorized in a 1% w/v agarose gel, stained with ethidium bromide at a concentration of 0.5 µg/ml, and visualized under UV light.

All amplicons were purified using the Zymo Research DNA Clean & Concentration clean up kit (Genomics DNA Clean & Concentrator®, USA) according to the manufacturer's instructions and sequenced in the forward and reverse directions with the same primers used for amplification. The chromatograms were evaluated and analyzed with 4peaks software (version 1.8). Multiple sequence alignment was performed using MEGA XI software (version 11.0.11) (Kumar et al., 2018). The NCBI Basic Local Alignment Search Tool (BLAST) was used to evaluate the level of identity with other reported sequences deposited in the GenBank database (Altschul et al. 1990). Phylogenetic trees of Tunisian *Fasciola* spp. were constructed using the neighbor-joining method (Saitou and Nei 1987) implemented in the software MEGA XI following 1000 bootstrap replications. Moreover, *F. gigantica* (GenBank accession number DQ385828) was used as an outgroup root.

Results

Fluke's morphometric measurement

The morphometric characteristics of the isolated flukes from sheep are summarized in Table 1. The results showed that the mean of the total body length of the adult flukes was 21.1 ± 2.7 mm and the mean ratio length/width of the fluke body was 2.32 ± 0.96 mm. Moreover, the morphometric assessment revealed that the mean of abdomen diameter and the tail diameter of the *Fasciola* specimens were 10.14 ± 3.04 mm and 6 ± 2.22 mm, respectively. The morphological parameters of the adult *Fasciola* specimens isolated from sheep in Tunisia did not differ from those of *F. hepatica* isolated from sheep in Bolivia and Iran (Table 1).

ITS1-PCR and RFLP analysis

In ITS1-PCR amplification, all samples produced an amplicon of approximately 680 bp, indicating that they belong to the *Fasciola* genus. The negative control did not produce bands in any of the PCR reactions. The RFLP results generated a single profile consisting of three bands of approximately 370, 100, and 60 bp indicating that the amplicons belong to *F. hepatica* (Fig. 3). Neither *F. gigantica* nor an intermediate RFLP pattern was detected in all digested amplicons. Therefore, all specimens collected from sheep livers in the current study belonged to *F. hepatica.*

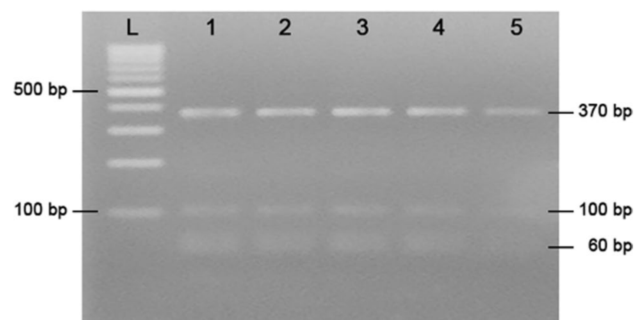

Fig. 3 RFLP pattern of ITS1-PCR products after digestion with RsaI enzyme. Lane L: 100 bp DNA ladder; lanes 1, 2, 3, 4, and 5: *Fasciola hepatica*

ITS2 amplification and phylogenetic analysis

The sequencing of 66 ITS2 PCR amplicon from Tunisian *Fasciola* spp. confirmed the presence of *F. hepatica* in sheep livers in the Sejnane region (Northwest Tunisia). Sequence alignment of the amplicons (approximately 500 bp in length) revealed two distinct variants (GenBank accession numbers OQ457027 and OQ457028). Both variants have two nucleotide differences at positions 15 (first variant) and 16 (second variant) (T/G and G/A, respectively), with 99.79% similarity between them. Of the 66 aligned sequences, 63.64% (42/66) belongs to the first variant and 36.36% (24/66) to the second variant. These results show that the first variant is more abundant than the second in the Tunisian *F. hepatica* specimens.

Phylogenetic trees of *F. hepatica* were constructed using the ITS2 rDNA gene sequence of our amplicons and those published in GenBank. Our *F. hepatica* amplicons showed 99.58–100% identity with the amplicons published in GenBank. The sequences of the two amplicons obtained in the present study fall into the same clade as amplicons from North Africa (Egypt and Libya), Asia (Japan, Vietnam, India, and Iran), Australia, and slightly distantly from amplicons from Switzerland and Iran. Our variants are clearly distinct from other *Fasciola* species such as *F. gigantica* (GenBank DQ385828) in sheep in Egypt (Fig. 4).

Discussion

The present study provides new insights into the liver fluke (*Fasciola* spp.) population infecting sheep in the Sejnane region (Northwest Tunisia). To our knowledge, this is the first morphometric study of adult liver flukes isolated from sheep in Tunisia, complemented by molecular characterization of the ITS1 gene (PCR-RFLP) and phylogenetic analysis based on the ITS2 gene.

The morphometric measurements showed that the mean body length of Tunisian *Fasciola* spp. was 21.1 ± 2.7 mm, with minimum and maximum lengths of 13 and 31 mm, respectively. The mean abdomen diameter of the flukes was 10.14 ± 3.04 mm, ranging from 2 to 18 mm. These results are consistent with those obtained in other studies conducted on *F. hepatica* from sheep in Bolivia and Iran. The mean

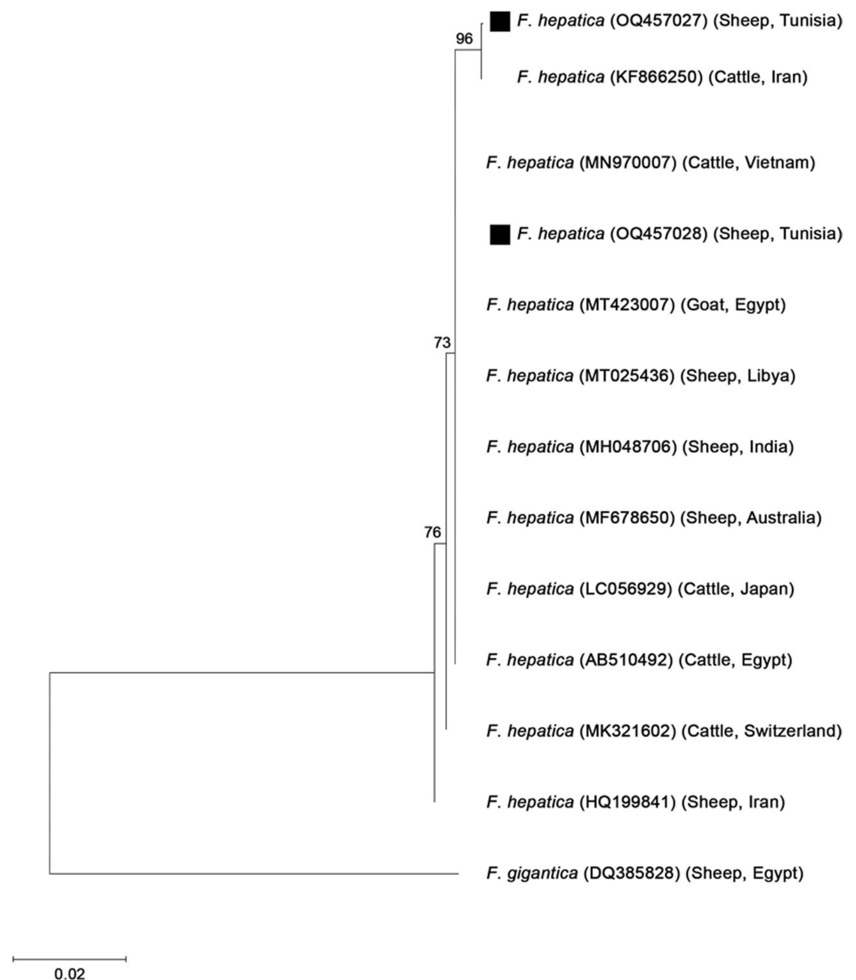

Fig. 4 Partial sequence ITS2 gene phylogenetic tree of *Fasciola hepatica* isolated from sheep liver (GenBank accession number: OQ457027 and OQ457028) and *Fasciola hepatica* amplicons deposited in GenBank. Amplicons described in this study are indicated with a black square, and numbers at the branches refer to bootstrap (1000 replications)

length of *F. hepatica* was 16.10 ± 4.80 mm, and its mean width was 7.11 ± 2.27 mm in Bolivia (Valero et al., 2001), whereas *F. hepatica* measured approximately 21.1 ± 2 mm in length and 12 ± 1.5 mm in width in Iran (Yakhchali et al., 2015). Moreover, *F. gigantica* isolated from the same host species in Iran measured 34.1 ± 4 mm in length and 9 ± 0.1 mm in width, respectively (Yakhchali et al., 2015). Other studies throughout the world described the morphology of *F. hepatica* isolated from other different species such as cattle, buffaloes, and pigs (Valero et al., 2001; Diyana et al., 2020; Sumruayphol et al., 2020; Haridwal et al., 2021; Abdel-Fatah et al., 2022). On the other hand, they compared the morphology of *F. hepatica* and *F. gigantica* isolated from the same host species and found that the latter was longer and narrower than *F. hepatica* (Valero et al., 2001; El-Rahimy et al., 2012; Amer et al., 2016; Akhlaghi et al., 2017; Diyana et al., 2020; Sumruayphol et al., 2020; Shykat et al., 2022). Similarly, previous studies based on morphometric differences between the two *Fasciola* species have shown that the average distance between the posterior testis and the posterior border of the body was shorter in *F. hepatica* than in *F. gigantica* (Bergeon and Laurent 1970; Sahba et al., 1972). Similarly, Mas-Coma and Bargues (1997) have shown that the shoulders of *F. hepatica* are more developed, the cephalic cone is longer, and the caeca are less branched than in *F. gigantica*. Moreover, Dar et al. (2003) showed that the rediae of *F. gigantica* developing in *Radix natalensis* and *Galba truncatula* are morphologically different from those of *F. hepatica*. Morphological measurements are a simple tool for discriminating *F. hepatica* and *F. gigantica* in areas with low occurrence and without intermediate forms. In contrast, morphometric assessments are unable to discriminate between the two liver fluke species in areas (e.g., Egypt, Japan, Korea, Vietnam, China) where the presence of co-infection with both *Fasciola* species and their intermediate forms has already been confirmed (Itagaki et al., 2005a, 2005b; Ichikawa and Itagaki, 2012; Anh et al., 2018; Itagaki et al., 2022). Furthermore, the morphometric characteristics of adult *Fasciola* spp. and eggs have been shown to be critically influenced by the definitive host species (Valero et al., 2001), thus confirming the limitations of morphometric analyses in discriminating *Fasciola* species.

The PCR-RFLP analysis of Tunisian *Fasciola* spp. of the ITS1 gene using the restriction enzyme RsaI revealed a unique profile consisting of three bands of approximately 370, 100, and 60 bp, thus indicating that the fluke samples belonged to the *F. hepatica* species. Moreover, neither *F. gigantica* nor intermediate RFLP patterns were detected in all digested amplicons The same profile of *F. hepatica* that we obtained (360, 100, and 60 bp) in the present study was also found in other studies in different countries around the world (Ichikawa and Itagaki 2010; Itagaki et al. 2011; Diyana et al. 2020; Hasanpour et al. 2020). On the other hand, *F. gigantica* yielded RFLP patterns of approximately 370, 170, and 60 bp using the same technique and restriction enzyme (Ichikawa and Itagaki, 2010; Itagaki et al., 2011; Ichikawa and Itagaki, 2012; Anh et al., 2018; Diyana et al., 2020; Hasanpour et al., 2020). It is noteworthy that an intermediate form has been detected by this technique in Japan, Korea, and Vietnam giving a profile consisting of four bands of approximately 370, 170, 100, and 60 bp (Itagaki et al., 2005b; Ichikawa and Itagaki, 2012; Anh et al., 2018). Other studies used a PCR-RFLP assay based on the partial rDNA of ITS1 and different restriction enzymes from RsaI to differentiate and identify *Fasciola* species such as AvaII and DraII, while the latter has no restriction sites in *F. gigantica* amplicons (Marcilla et al., 2002; El-Rahimy et al., 2012; Yakhchali et al., 2015). Molecular approaches based on DNA analyses are useful tools for the identification and genetic characterization of parasites with similar morphology. Therefore, various molecular targets, primarily deoxyribonucleic acid (DNA) sequences of the ITS1, ITS2, and 28S rRNA genes, have been used to differentiate *F. hepatica* from *F. gigantica* (Adlard et al., 1993; Marcilla et al., 2002; Itagaki et al., 2005a; Anh et al., 2018; Evack et al., 2020; Omar et al., 2021). In addition, RFLP of amplified DNA is a suitable method for differentiating *Fasciola* ITS1, using various restriction enzymes such as RsaI, AvaII, and DraII, while the PCR-RFLP method relies on patterns produced by the effects of endonucleases on ITS genes to identify *Fasciola* species (Ichikawa and Itagaki, 2010; Dar et al., 2012; Yakhchali et al., 2015; Anh et al., 2018; Diyana et al., 2020; Hasanpour et al., 2020).

The presence of *F. hepatica* in the Sejnane region of northwestern Tunisia was confirmed by sequencing of the ITS2 amplicon. The analysis revealed two distinct variants (GenBank accession numbers OQ457027 and OQ457028) with a similarity of 99.79%. This difference between the two variants could not be related to the host species or the geographical origin of the samples, as they are the same (Alasaad et al., 2007). However, this variation could be explained by the movements of *Fasciola*-infected animals in different regions of Tunisia or even by the introduction of infected animals imported from abroad (Schwantes et al., 2020). These results would require the sequencing of samples isolated from other host species and/or other regions in order to determine whether there is *Fasciola* species diversity in Tunisia.

The two sequences obtained in the present study clustered to those from Africa, Asia, and Australia, sharing high homology (99.58–100%) with amplicons from other regions and hosts deposited in GenBank. Our amplicons showed 99.6–100% identity with others isolated from sheep in Libya, India, and Australia (GenBank accession numbers: MT025436, MH048706, MF678650, respectively), from goats in Egypt (GenBank accession number:

MT423007), and from cattle in Japan, Egypt, Vietnam, and Iran (GenBank accession numbers: LC056929, AB510492, MN970007, KF866250, respectively).

The two variants differed slightly (99.58% identity) in cattle isolates from Switzerland and in sheep isolate from Iran (GenBank accession numbers: MK321602 and HQ199841, respectively). However, this study confirms that the amplicons were distinct from other *Fasciola* species such as *F. gigantica* in sheep from Egypt (GenBank accession number: DQ385828).

The molecular identity of Tunisian *F. hepatica* with other *F. hepatica* isolates from other countries bordering Tunisia could be explained by a genetic mixing between *Fasciola* ecotypes due to intensive international livestock trade and passive displacement of intermediate hosts.

Conclusion

The results from PCR-RFLP showed a unique profile consistent with that of *F. hepatica*, indicating that this technique is effective in differentiating the two species, *F. hepatica* and *F. gigantica*. Molecular analyses were in line with morphometric and phylogenetic analyses, indicating the presence of a single species of *F. hepatica* in the Sejnane region of northwestern Tunisia. Our results will be useful to support molecular profiling and comparison with other *Fasciola* specimens from different regions of Tunisia and other North Africa countries.

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Author contributions Ines Hammami: investigation, methodology, data analyses, writing—original draft. Lavina Ciuca: methodology, writing—review and editing. Maria Paola Maurelli: methodology, writing—review and editing. Rihab Romdhane: investigation. Limam Sassi: investigation. Mohamed Ridha Rjeibi: methodology. Nadia Farhat: investigation. Alain Kouam Simo: investigation, methodology. Laura Rinaldi: funding acquisition, writing—review and editing. Mourad Rekik: funding acquisition. Mohamed Gharbi: conceptualization, supervision, funding acquisition, writing—review and editing.

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Data availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Ethical approval The present study was performed in a certified slaughterhouse under the supervision of an officially certified veterinarian by the Tunisian state.

Consent to participate Informed consent was obtained from the slaughterhouse staff prior to sampling the *Fasciola* flukes.

Consent for publication All authors read and consent to the publication of the manuscript.

Conflict of interest The authors declare no competing interests.

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