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# A phylogenetic assessment of nematodes (Oxyuroidea: Pharyngodonidae) infecting Moroccan lizards

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**Summary**

Molecular tools can be used to estimate the phylogeny of species and to identify cryptic diversity, but their use for parasites has lagged behind that of free-ranging organisms. As an example, in North Africa there is minimal molecular data available for helminth parasites of lizards. In this work we used two molecular markers (the nuclear 18S rRNA and the mitochondrial Cytochrome *c* Oxidase subunit 1) to investigate the diversity of nematodes of the family Pharyngodonidae parasitizing three genera of lizards from Morocco (*Chalcides*, *Quedenfeldtia* and *Tarentola*) and to explore their co-evolutionary history. Morphological assessments indicated that members of three genera were present: *Spauligodon*, *Thelandros*, and *Parapharyngodon.* Phylogenetic analysis of 18S rRNA sequences indicated the monophyly of the genus *Spauligodon,* and that some lineages could be distinguished, including *Spauligodon auziensis* from the host species *Tarentola mauritanica*, and another unnamed lineage from hosts of the genus *Chalcides*. However, with this slow-evolving marker some species could not be distinguished. The genus *Thelandros* was not monophyletic, although relationships were not strongly supported. Analysis of the faster evolving mitochondrial marker clearly separated various species of *Spauligodon*, as well as distinct unnamed lineages identified in the host genus *Chalcides* and the host *Quedenfeldtia moerens*.

**Keywords:** *Spauligodon*; *Thelandros*; *Parapharyngodon*; Pharyngodonidae; parasites; cryptic species

## Introduction

Defining, cataloguing, mapping and preserving biodiversity is generally accepted to be one of the key challenges for the 21st Century. The Kingdom of Morocco possesses the richest and most varied herpetofauna of the Maghreb and the western Mediterranean, is characterized by high richness of reptiles, endemism and European relict species (del Mármol *et al*., 2019). Phylogenetic analyses performed over recent decades have identified notable evolutionary lineages in the Moroccan herpetofauna, several of which represent new (cryptic) species or species complexes (e.g. Barata *et al*., 2012; Salvi *et al*., 2018), demonstrating the value of incorporating molecular tools into diversity assessments. Still, despite the well-known biodiversity of reptiles, parasite diversity associated with these hosts remains poorly known. Part of the problem in estimating parasite diversity has historically been that,

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especially for endoparasites, identification based on morphological characters and life-cycle traits is often difficult. Parasites often present a simplified morphology (Jorge *et al*., 2011), and while DNA sequencing approaches should help overcome these problems, molecular studies have lagged behind those of free-ranging organisms (Criscione *et al*., 2005). However, application of molecular tools is starting to show that some parasites currently considered as a single species actually consist of genetically different lineages or cryptic species (e.g. Jorge *et al*., 2012, 2013a).

Reptiles are parasitized by various helminth species, typically occurring in depauperate communities (Aho, 1990), possibly due to characteristics of the hosts such as the simplicity of the alimentary canal, their low vagility, a nonspecialized diet, and characteristics of the parasites such as direct life cycles (Roca & Hornero, 1994). Various studies have suggested that the typical helminth infection pattern in reptiles is that few species occur frequently, while many species are rare (Birlik *et al*., 2015). In studies of reptiles from the Iberian Peninsula and the North of Africa, helminth fauna of assessed lizards was poor, and mainly composed by members of the family Pharyngodonidae that are often detected in insectivorous reptiles (Chabaud & Golvan, 1957; Ibrahim *et al*., 2005; Carretero *et al*., 2011; Roca *et al*., 2020). These are usually identified on the basis of male morphology, since females are generally similar among species (Jorge *et al*., 2014). Although this family includes 21 genera, only a few of these are typically found in insectivorous lizards, with *Spauligodon* the most commonly reported (Roca *et al*., 2020).

Recent nematode surveys of reptiles in the Mediterranean region resulted in several new host records and descriptions of new species (Jorge *et al*., 2011, 2013a, 2014). Despite the high diversity of reptiles in Morocco, parasites from these hosts remain poorly studied, with a few incidental assessments as part of surveys at the Canary Islands (e.g. Jorge *et al*., 2011, 2018). In this sense, no survey including molecular data has been performed for nematodes of reptiles from Morocco. Indeed, with one or two exceptions (e.g. Carretero *et al*., 2011), there have been few molecular studies in nematodes from reptiles across the whole of the Maghreb. The aim of this study was to fill this gap, by assessing diversity of Pharyngodonidae nematodes from six lizard species from Morocco. By sequencing exemplars for two molecular markers (fragments of the 18S rRNA and Cytochrome Oxidase 1 gene), we aim to place these in a phylogenetic framework, to potentially identify cryptic forms, and to examine patterns of host-specificity.

## Materials and Methods

*Parasitological procedures*

Helminths were collected from pellets, which were obtained through spontaneous defecation or by gentle abdominal massage of six lizard species: *Chalcides mionecton, Chalcides montanus, Chalcides polylepis, Quedenfeldtia moerens, Quedenfeldtia trachyblepharus*, and *Tarentola mauritanica* from Morocco (Table 1 and Fig. 1), between January 2019 to September 2021. These pellets were stored in 96 % ethanol. Faecal pellets were inspected for nematodes using a stereomicroscope. Specimens were mounted on temporary slides with a glycerol : water (1 : 1) solution, after which they were identified under a microscope, based on previous descriptions (Lucker, 1952; Skrjabin *et al*., 1967; Ashour *et al*.,

Table 1. Fecal samples from which parasitic nematodes were recovered and included in the genetic analysis.

| Code | Nematode species | Host species | Locality | 18S | Col |
|---|---|---|---|---|---|
| 26 | *Spauligodon auziensis* | *Tarentola mauritanica* | Ounagha, Essaouira | | |
| 32 | *Spauligodon auziensis* | *Tarentola mauritanica* | Dar Bouzza, Casa | OP548559 | OP558784 |
| 111 | *Spauligodon* sp. | *Quedenfeldtia trachyblepharus* | Oukaïmeden, Marrakech | OP548558 | |
| 112 | *Spauligodon* sp. | *Quedenfeldtia trachyblepharus* | Oukaïmeden, Marrakech | OP548557 | |
| 131 | *Spauligodon* sp. | *Chalcides montanus* | Oukaïmeden, Marrakech | OP548556 | |
| 164 | *Spauligodon* sp. | *Quedenfeldtia trachyblepharus* | Oukaïmeden, Marrakech | OP548555 | |
| 205 | *Spauligodon* sp. | *Quedenfeldtia moerens* | Fom Jrana, Chichaoua | OP548554 | |
| 219 | *Spauligodon* sp. | *Quedenfeldtia trachyblepharus* | Ait El Qaq, Marrakech | OP548553 | |
| 234 | *Spauligodon auziensis* | *Tarentola mauritanica* | Aït Aammour ou Ali, Azrou | OP548552 | OP558785 |
| 283 | *Spauligodon* sp. | *Quedenfeldtia trachyblepharus* | Ait El Qaq, Marrakech | OP548551 | |
| 377 | *Spauligodon* sp. | *Quedenfeldtia trachyblepharus* | Imlil, Marrakech | OP548550 | |
| 1149 | *Spauligodon auziensis* | *Tarentola mauritanica* | Admin Forest, Agadir | OP548549 | |
| 1213 | *Parapharyngodon micipsae* | *Chalcides mionecton* | El Ghazoua, Essaouira | OP548548 | |
| 1447 | *Spauligodon* sp. | *Quedenfeldtia moerens* | Bigoudine, Argana | OP548547 | OP558786 |
| 1476 | *Spauligodon* sp. | *Chalcides polylepis* | Oued Tensift, Marrakech | OP548546 | OP558787 |
| 1480 | *Thelandros alatus* | *Chalcides mionecton* | Oued Tensift, Marrakech | OP548545 | |
| 1550 | *Thelandros alatus* | *Chalcides polylepis* | Oued Tensift, Marrakech | OP548544 | |

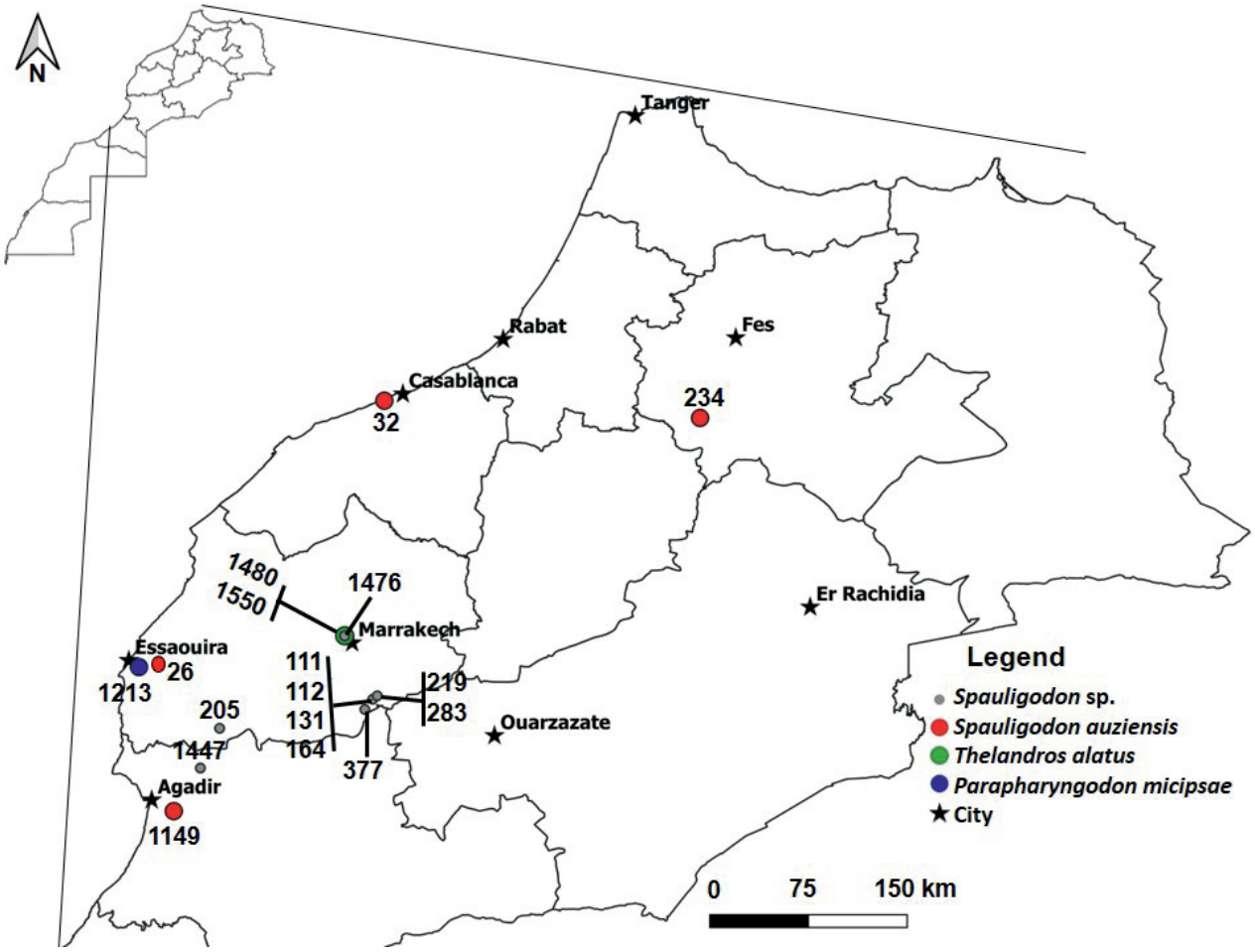


Fig. 1. Map of Northern Morocco with the geographic locations of sampled nematodes. Their respective hosts species are in Table 1.

1992; Amer & Bursey, 2008; Mašová *et al*., 2009; Pereira *et al*., 2017). All extracted specimens were photographed as a record. Further details regarding helminth collection and identification are given in Er-Rguibi *et al*. (2022).

*Genetic analysis*

Extractions of genomic DNA were performed using individual nematodes, according to the saline method (Maia *et al*., 2014). Two partial genes were amplified: the nuclear 18S rRNA (18S) gene and the mitochondrial Cytochrome c Oxidase subunit I (COI). The COI fragment was amplified using the primers LCO and HCO from Folmer *et al*. (1994), while the 18S was amplified using Nem 18SF and Nem 18SR from Floyd *et al*. (2005). Polymerase chain reactions were performed in a total volume of 15 µl, consisting of PCR buffer at 1 × concentration; $MgCl_2$ at 1.5 mM; dNTPs at a concentration of 0.2 mM for each nucleotide; primers at 0.5 µM each; BSA at 0.4 µg/µl (bovine serum albumin) (Roche Applied Science); and Taq DNA polymerase (Invitrogen Corporation) 0.025 units/µl and 1 µl of DNA template. PCR conditions were 35 cycles of: 30 sec at 94°C, 30 sec at 50 – 54°C and 1 min at 72°C, with an additional denaturation step of 3 min at 94°C and ending with a final extension at 72°C for 10 min. Successful amplified products, confirmed through electrophoresis, were cleaned and sequenced by a commercial facility (Gene Wiz, Germany).

*Phylogenetic analysis*

Sequences obtained were compared with those from GenBank using BLAST to confirm the taxonomic identity of the amplified products. Related sequences of *Spauligodon*, *Parapharyngodon*, and *Thelandros* published in previous studies and available in GenBank were included in the analyses. Sequences were aligned using MUSCLE alignment tool implemented in MEGA 10.1.8 with default parameters. The alignment lengths consisted of 595 bp (36 taxa) and 867 bp (47 taxa) for the CO1 and 18S respectively. Maximum likelihood was performed using PhyML 3.0 ( Guindon & Gascuel, 2003) executed online (http://www.atgc-montpellier.fr/phyml/), both for defining the most appropriate model of molecular evolution under the AIC criterium, and to estimate a phylogeny. Branch support was estimated using bootstrap (Felsenstein, 1985) with 1000 replicates. The model selected in both cases was

GTR+I+G. Phylogenies were also performed using Bayesian inference, implementing the most appropriate parameters according to the estimated models. Bayesian analyses were performed in MRBAYES 3.2.7a (Huelsenbeck & Ronquist, 2001) and run in duplicate for $10 \times 10^6$ generations with random starting trees, sampling every 1000 generations. The first 250 trees were discarded as 'burn-in', after verifying that stationarity was reached by plotting log-likelihood values against generation time. A 50 % majority-rule consensus tree was used to summarize the trees sampled from the post-burn-in trees. New sequences generated in this study were submitted to GenBank (Table 1).

**Ethical Approval and/or Informed Consent**

The research related to animal handling complied with all the relevant national regulations and institutional policies for the care and use of animals. The authorization for sampling of wild animals was granted by Cadi Ayyad University, Marrakech, Morocco. A field-work permit was issued by "Haut-Commissariat aux Eaux et Forêts et à la Lutte Contre la Désertification (HCEFLCD)".

**Results**

Three different genera were identified using microscopy. Identification of *Spauligodon* was based on 125 adult specimens (81 females and 44 males). These were identified as small cylindrical nematodes, sexually dimorphic, with males approximately one quarter the length of female. Lateral alae present in male, absent in female. Anterior extremity tapered, mouth surrounded by 3 small lips, each with shallow midline indentation; dorsal lip with 2 sessile papillae, ventrolateral lips with 2 sessile papillae and 1 prominent lateral amphid. Identification of *Paraphyngodon* was based on 6 adult specimens (4 females and 2 males). These were identified as small nematodes, whitish in colour, males smaller than females, sexual dimorphism moderate. Body fusiform, cuticle with distinct transverse striations beginning just behind the cephalic extremity and continuing to the anus. In males, lateral alae well developed, initiating anteriorly somewhere at the level of bulbus and extending posteriorly to last third of body. Oral opening subtriangular, surrounded by three lips, in female separated into six parts. Identification of *Thelandros* was based on 104 adult specimens (80 females and 24 males). These were identified as robust nematodes with prominent annulations beginning just behind cephalic extremity and continuing to anus. Cuticle with distinct longitudinal striations approximately 4 apart. Moderate sexual dimorphism. Triangular oral opening surrounded by 3 bilobed lips, 1 small pedunculate amphid on each ventrolateral lobe. Lateral alae absent. Males without caudal alae; caudal filament terminal, directed posteriorly. Females with vulva post equatorial. We followed Hering-Hagenbeck *et al*. (2002), considering *Parapharyngodon* distinct from *Thelandros* for the following reasons: Males of *Thelandros* have a genital cone, pendulant papillae outside the genital cone, lateral alae are absent, and the tail is terminal and directed posteriorly; males of *Parapharyngodon* lack a genital cone, mammiliform papillae surround a more-or-less terminal anus, lateral alae are present, and the tail is subterminal and directed dorsally.

Following previous descriptions (Lucker, 1952; Skrjabin *et al*., 1967; Ashour *et al*., 1992; Amer & Bursey, 2008; Mašová *et al*., 2009; Pereira *et al*., 2017; Er-Rguibi *et al*., 2022) samples from hosts of the genus *Chalcides* were identified as *Thelandros alatus* or *Parapharyngodon micipsae*. All other samples were identified as belonging to the genus *Spauligodon*. Of these, only *Spauligodon auziensis* from *Tarentola mauritanica* hosts could be identified to the species level. Sixteen of 17 nematode specimens had their 18S rRNA successfully sequenced. Based on the estimate of phylogeny derived from 18S rRNA sequences (Fig. 2), the species of *Spauligodon* formed a highly distinct clade. Within this, some forms could be distinguished, including *S. nicolauensis* from the Cape Verde islands, *S. auziensis* from the hosts *T. mauritanica*, and a group from hosts of the genus *Chalcides* (*C. montanus, C. ocellatus* and *C. polylepis*). However, several specimens from the geckos *Quedenfeldtia moerens* and *Quedenfedtia trachyblepharus* were identical for this region, and also identical to sequences from GenBank of both *Spauligodon saxicolae* and *Spauligodon carbonelli*. The sample identified as *P. micipsae* was sister taxa to *P. micipsae* from *Gallotia caesaris* from the Canary Islands. *Thelandros* was not a monophyletic group, although some estimates of relationships were poorly supported. Considering the CO1 marker, only 4 samples (all *Spauligodon*) were successfully amplified. Based on this more variable marker (Fig. 3) two samples from *Tarentola mauritania* hosts were apparently *S. auziensis*, related to other specimens from this host species. A specimen from *Quedenfeldtia* was distinct from all comparative sequences, but with poorly supported relationships. The remaining sample from a *Chalcides polylepis* host formed a distinct lineage with another *Spauligodon* sp. from a *Chalcides* sp. host, also from Morocco. These together were sister taxa to *S. nicolauensis*, from the Cape Verde islands.

**Discussion**

In principle, the geographic distribution of parasite diversity is predicted to match that of host diversity (Jorge & Poulin, 2018). Under this expectation, Morocco with its high diversity of reptile species should also harbor extensive nematode diversity within these hosts. However, this diversity remains essentially unassessed. In this study, we made a preliminary assessment of nematodes in pellets from six reptile host species. While it is known that there is a significantly lower detectability of nematodes from pellets compared to studies of intestines (Jorge *et al*., 2013b), this noninvasive approach can be used to give some baseline data on the parasites occurring in reptiles in this region.

As expected, the nematodes identified all belonged to three genera, *Parapharyngodon*, *Thelandros* and *Spauligodon*, which are typically identified within insectivorous reptiles (e.g. Mašová *et al*., 2009;

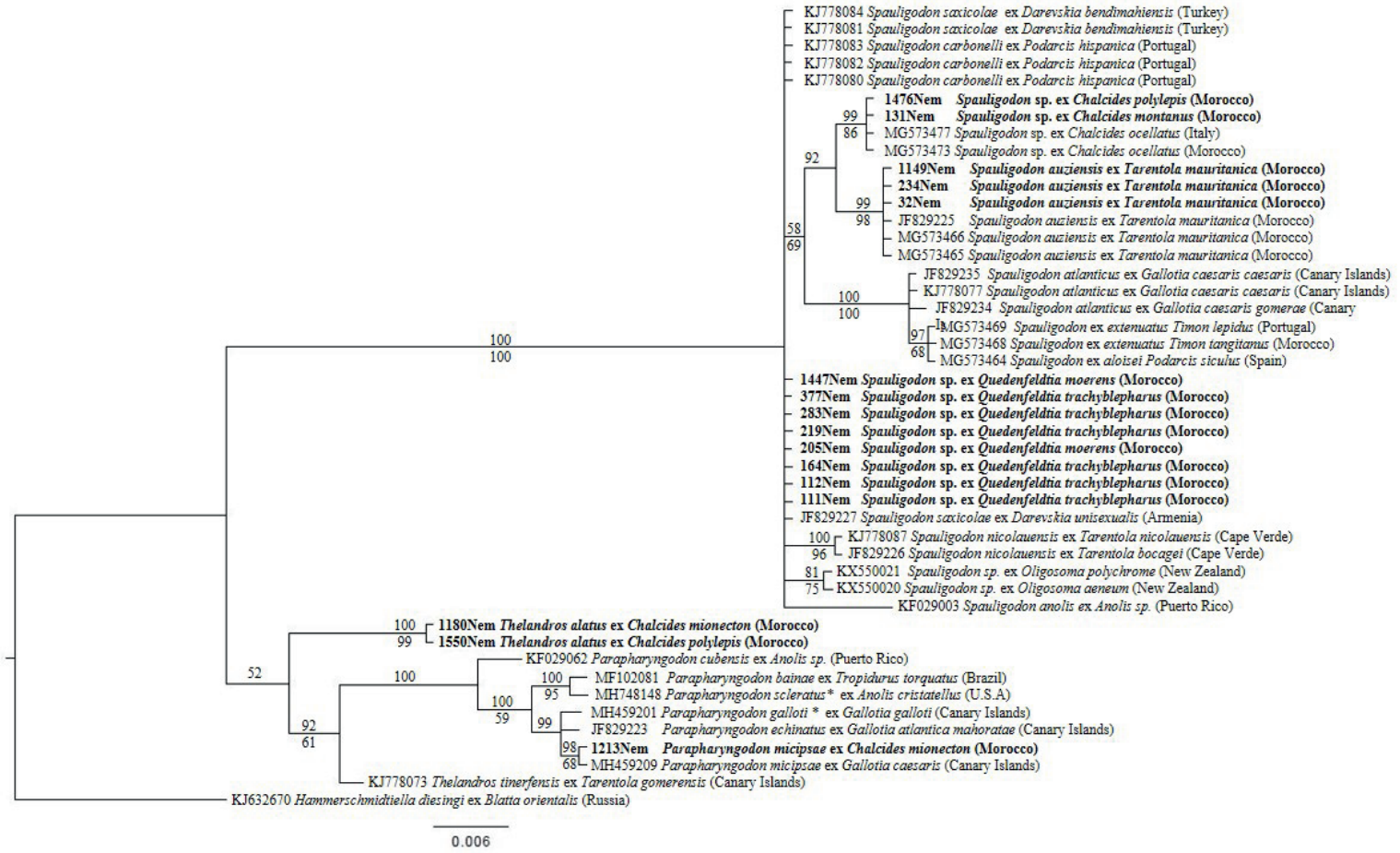


Fig. 2. Estimate of relationships derived from the 18S rRNA gene sequences using a Bayesian approach. Values above branches represent Bayesian posterior probabilities and those below represent ML bootstrap support values (both given as percentages). Specimen code descriptions are given in Table 1. * These samples are considered as belonging to *Parapharyngodon* following de Sousa et al. (2018), but are listed on GenBank as *Thelandros*.

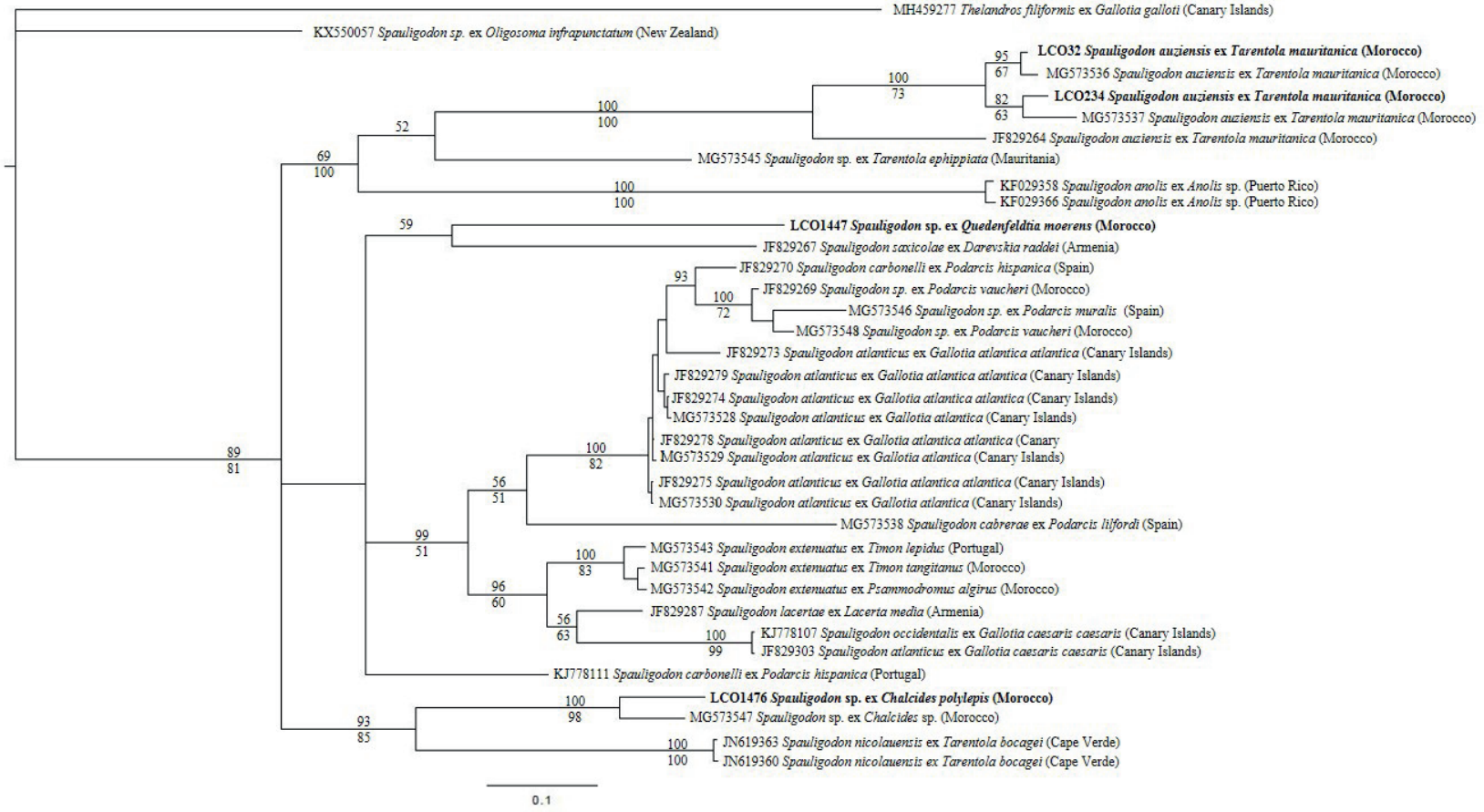


Fig. 3. Estimate of relationships derived from the CO1 mitochondrial gene sequences using a Bayesian approach. Values above branches represent Bayesian posterior probabilities and those below represent ML bootstrap support values (both given as percentages). Specimen code descriptions are given in Table 1.

Carretero *et al*., 2011; De Sousa *et al*., 2018). Exact relationships between these, and even the monophyly of these genera, has been widely debated (e.g. De Sousa *et al*., 2018; Pereira *et al*., 2018).

Using universal 18S rRNA primers, we were able to amplify and sequence a region of this gene for all but one of our samples. The specimens of *Spauligodon* from Morocco fell into three distinct lineages, reflecting the reptile hosts they were recovered from. Seven specimens of *Spauligodon* from the geckos *Quedenfeldtia moerens* and *Quedenfedtia trachyblepharus* were identical for the 18S rRNA region, and also identical to sequences from GenBank of *Spauligodon saxicolae* and *Spauligodon carbonelli*, that had been recovered from lacertid lizards in the Iberian Peninsula and Turkey (Jorge *et al*., 2011, 2014). Three samples of *Spauligodon* from the gecko *Tarentola mauritanica* were also identical, to *Spauligodon auziensis* previously recovered from *T. mauritanica* from central Morocco (Jorge *et al*., 2011, 2018). The two samples of *Spauligodon* from the skinks *Chalcides polylepis* and *Chalcides montanus* were related to *Spauligodon* sp., also from skinks *Chalcides ocellatus*, from Sardinia (Jorge *et al*., 2018).

While the distinction of *Spauligodon* was evident in our analysis of 18S rRNA, the separation of *Parapharyngodon* was more complex. Although molecular data does support distinction of this genus relative to *Thelandros*, many species have not been included in analyses. Pereira *et al*. (2018), using an integrative approach, proposed a clear separation between *Thelandros* and *Parapharyngodon*, but included only one representative species of *Thelandros* in the molecular phylogeny. De Sousa *et al*. (2018) also indicated that *Parapharyngodon* was distinct from *Thelandros* collected from the Canary Islands and Cape Verde islands, but that from *Gallotia* lizards previously considered *Thelandros galloti* should be reassigned to *Parapharyngodon*, as *P. galloti*. They further suggested that preliminary morphological assessments supported this reassignment, for example since this species lacks caudal alae and has long and wide lateral alae, both typical characteristics of *Parapharyngodon* (Astasio-Arbiza *et al*., 1988). On the other hand, *P. galloti* males had wider alae and longer oesophagus than representatives of *Parapharyngodon*, highlighting the difficulties of assigning these species to the appropriate genus without molecular data. Abdel-Ghaffar *et al*. (2020) recently described a new species as *Thelandros chalcidiae* from *Chalcides ocellatus* in Egypt. Based on 28S rRNA sequence data, they showed it was closely related to *P. galloti* (still considered as a member of *Thelandros*) and *Parapharyngodon micipsae*. In our analysis, we identified a *Parapharyngodon* sp. from *Chalcides mionecton*. Based on morphological aspects, it was considered to be *P. micipsae*, a species found in many different reptile hosts including lacertids (Martin & Roca, 2004), skinks (Ibrahim *et al*., 2005), geckos (Mašová *et al*., 2009) and agamas (Elmahy & Harras, 2019). Following the 18S rRNA analysis, this was closely related to *P. micipsae* from *Gallotia* lizards from the Canary Islands (De Sousa *et al*., 2018), and then *P. galloti* and *P. echinatus.* That *T. chalcidiae* is also closely related to *P. galloti* shows how difficult it is to assign species to the different genera based on morphological characters. Highlighting the diversity of nematodes found in *Chalcides* skinks, in another *C. mionecton* and *Chalcides polylepis*, representatives of *Thelandros* were identified, which we considered as *T. alatus* based on morphological characters, the type species of *Thelandros*. Based on the 18S rRNA analysis, these were distinct from *Parapharyngodon,* but also from *Thelandros tinerfensis* from *Tarentola gomerensis* hosts in the Canary Islands, thus making *Thelandros* paraphyletic. Overall, representatives of *Spauligodon*, *Thelandros* and *Parapharyngodon* were identified in *Chalcides* skinks in Morocco, but which species of *Spauligodon* is still unclear.

Although we only obtained 4 sequences from *Spauligodon* species using the CO1 primers, the estimate of phylogenetic relationships derived from this gene is still highly informative (Fig. 3). Unlike with the more conservative 18S rRNA sequences, all *Spauligodon* species could be distinguished with this marker, and intra-specific variation identified. Two samples from *T. mauritanica* again grouped with samples identified as *S. auziensis*. Intraspecific variation within *S. auziensis* was high, up to 12.6 % uncorrected sequence divergence, although all the samples were collected from the same host species. One specimen from *Quedenfeldtia moerens* was highly distinct from all other comparative sequences, but relationships of this from were poorly supported. Specimens of *Spauligodon* were recently identified in *Quedenfedtia trachyblepharus* (Er-Rguibi *et al*., 2021), and the results of this study demonstrate that they are genetically distinct from other known *Spauligodon* species. The fourth specimen, from *C. polylepis*, was found to be sister taxa to another *Spauligodon* sp., also from a *Chalcides* sp., and these in turn were related to *Spauligodon nicolauensis* from the Cape Verde islands.

To conclude, our new data clarifies several aspects regarding the presence of nematodes in reptiles from Morocco. Each lineage of parasite is quite specific to a related group of hosts, with *S. auziensis* in *Tarentola mauritanica*, another lineage in both species of *Quedenfeldtia*, another only in species of *Chalcides*, and another previously identified lineage from *Podarcis vaucheri*. While the universal 18S primers employed successfully amplified almost all samples, variation was too low to distinguish between some accepted species, while the CO1 region showed much greater variation. While classification of species to *Spauligodon* is relatively straightforward, some species considered as *Thelandros* may be better reassigned to *Parapharyngodon*, highlighting the difficulty of distinguishing between these genera using morphological characters. Other markers, such as 28S rRNA, and improved primers for amplifying CO1 across additional species, may help resolve some of these taxonomic issues. Several lineages of *Spauligodon*, particularly those from *Chalcides* and possibly also from *Quedenfeldtia* hosts form divergent lineages and probably warrant consideration as full species, pending detailed morphological assessments. This highlights the undescribed diversity within Morocco and the Maghreb region, and the need for additional surveys of this poorly known parasite fauna.

**Conflict of Interest.**

Authors state no conflict of interest.

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