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Research Article

Parasite host-switching from the invasive American red-eared slider, *Trachemys scripta elegans*, to the native Mediterranean pond turtle, *Mauremys leprosa*, in natural environments

Leon Meyer^{1,2,3}, Louis Du Preez¹, Elodie Bonneau^{2,3}, Laurent Héritier^{2,3}, Marc Franch Quintana⁴, Aitor Valdeón^{5,6}, Amel Sadaoui⁷, Nadia Kechemir-Issad⁷, Carmen Palacios^{2,3} and Olivier Verneau^{1,2,3*}

¹Unit for Environmental Sciences and Management, North-West University, Potchefstroom, 2520, South Africa

²Univ. Perpignan Via Domitia, Centre de Formation et de Recherche sur les Environnements Méditerranéens, UMR 5110, F-66860, Perpignan, France

³CNRS, Centre de Formation et de Recherche sur les Environnements Méditerranéens, UMR5110, F-66860, Perpignan, France

⁴Univ. Barcelona, Dept. Biol. Animal. Av. Diagonal 645, 08028 Barcelona, Spain

⁵Department of Geography and Regional Planning, University of Zaragoza, Pedro Cerbuna, 12 50009 Zaragoza, Spain

⁶Department of Herpetology, Aranzadi Society of Sciences, Zorroagaina, 11 20014 Donostia-San Sebastián, Gipuzkoa, Spain

⁷Laboratoire de Biodiversité et Environnement: Interactions, Génomes, Faculté des Sciences Biologiques, Université des Sciences et de la Technologie Houari Boumediene, El Alia, 16111 Bab-Ezzouar, Algeria

E-mail: leonmeyer8@gmail.com (LM), Louis.DuPreez@nwu.ac.za (LDP), bonneau.elodie@gmail.com (EB), laurent.heritier@univ-perp.fr (LH), apoarmatu@gmail.com (MFQ), emys@galapagosdenavarra.com (AV), sadaoui-amel@outlook.fr (AS), ndkechemir@gmail.com (NKI), carmen.palacios@univ-perp.fr (CP), verneau@univ-perp.fr (OV)

*Corresponding author

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Abstract

The red-eared slider turtle, *Trachemys scripta elegans*, is among the most over-exploited animals and is still exported annually from the USA all over the world. Once introduced into its new environment, feral populations may arise and pose threats to local biodiversity and ecosystem functioning. In France, it is in fact considered as a risk for the Mediterranean pond turtle, *Mauremys leprosa*, and the European pond turtle, *Emys orbicularis*, as they may compete for resources and habitat. Freshwater turtles are also host to a variety of parasites including protozoans and helminths. When introduced turtles escape, parasites may spread to native species. The objective of this study was to document the extent of platyhelminth invasions from *T. s. elegans* to natural *M. leprosa* populations in northern Spain and southern France and to evaluate the risks that parasite host-switching may pose on indigenous freshwater turtle species. From DNA barcoding analysis based on the sequencing of the Cytochrome c Oxidase I gene, the Bayesian tree and p-distance comparisons of closely related haplotypes revealed a greater polystome richness within *M. leprosa* than expected, suggesting that host switching may take place in natural environments. Because these parasites most typically infest American turtles like *Chrysemys picta marginata* and *Graptemys pseudogeographica* in their natural home range and because parasites were also found within *T. s. elegans* feral populations, it is suggested that the red-eared slider would serve as a carrier for a variety of not strictly host-specific polystomes that are transmitted to *M. leprosa* throughout the south of France. The global trade in freshwater turtles thus provides opportunity for parasites to be transported to new destinations which could impact the physiology, behavior and survival of native turtle species.

Key words: host switching, parasite invasion, turtle trade, Platyhelminthes, Monogenea

Introduction

With the increase in maritime and air traffic, more goods, including animals are being traded between countries and thus the introduction of non-native species into new biogeographic areas has been considerably accelerated over the past decades (Vitousek et al. 1997; Lowe et al. 2000;

Arena et al. 2012). Because introduced species do not share a common evolutionary pathway with native species in their new environment, behavior, ecology and demographic characteristics of native species may be impacted upon (Huxel 1999; Mooney and Cleland 2001; Shea and Chesson 2002). The organization and the functioning of local communities through assorted processes such as predation, competitive exclusion, parasite

loss or parasite transfer may also be altered (Lodge 1993; Williamson 1996; Vitousek et al. 1996; Hudson and Greenman 1998; Holway and Suarez 1999; Tompkins et al. 2002; Clay 2003; MacNeil et al. 2003; Torchin et al. 2003; Torchin and Mitchell 2004; Smith et al. 2006; Crowl et al. 2008). In turn this may cause drastic and irreversible changes in ecosystems (Williamson 1996; Vitousek et al. 1997; Chapin III et al. 2000; McNeely 2001; Mooney and Cleland 2001; Daszak et al. 2000; Ehrenfeld 2010).

One such animal group that is globally very popular in the pet trade is freshwater turtles. This is especially true for the red-eared slider turtle, *Trachemys scripta elegans* (Wied, 1839), because of its striking color, small body size of hatchlings and minimal husbandry requirements (Telecky 2001). As a result various turtle farms from the United States of America (USA) were involved in selling young red-eared sliders to mainly Europe (Warwick 1991; Lutz 2000; Telecky 2001) and Asia (Goh and O'Riordan 2007; Ramsey et al. 2007) in the 80s and 90s. However, because sliders grow rapidly and soon lose their striking juvenile color pattern, they become less attractive and are often released in the wild by owners without considering potential environmental implications. As a consequence, feral populations established in various natural freshwater ecosystems and the red-eared slider has become the most widely invasive reptile species in the world (Kraus 2009).

While habitat destruction and human pressure are the main factors in the decline of some turtle species, competition between native and introduced turtles could worsen the state of native turtle populations (Da-Silva and Blasco 1995; Pleguezuelos 2002; Gibbons et al. 2000; Cadi and Joly 2003, 2004; Ficetola et al. 2008; Polo-Cavia et al. 2008, 2009b, 2010, 2011, 2012). It has been shown for instance that *T. s. elegans* may outcompete the native European pond turtle, *Emys orbicularis* (Linnaeus, 1758), for food, basking sites or breeding habitats (Cadi and Joly 2003, 2004). Similar recent studies suggested that competition is also very likely to occur between sliders and the Mediterranean pond turtle, *Mauremys leprosa* (Schweigger, 1812) (Polo-Cavia et al. 2008, 2009b, 2010, 2011, 2012). Semiochemicals that are released from the various glands of the invasive species that facilitate species and sex recognition, could also adversely alter the behavior of *M. leprosa* leading to its displacement in natural environments (Polo-Cavia et al. 2009a). Furthermore sliders are

carriers of various parasites that could be released into new environments and potentially become established in unusual host species. Hays et al. (1999) hypothesized that *Actinemys marmorata* (Baird and Girard, 1852), which is an endangered endemic turtle to North America, could be vulnerable to contract the herpes-like virus from introduced captive *T. s. elegans* individuals. This might explain the decline observed in some *Actinemys* populations (Hays et al. 1999). Whereas studies have shown that the spread of parasites can occur from native European to introduced American turtles in wild populations (Hidalgo-Vila et al. 2009), Verneau et al. (2011) showed horizontal parasite transfers from exotic American to native European turtles in captivity.

Examples of such parasites include the polystomatid flatworms (Platyhelminthes, Monogenea, Polystomatidae), which are endoparasites in aquatic tetrapods but mainly infest anurans and freshwater chelonians (see Verneau 2004). These parasites are found as adults in the urinary bladder, cloaca, pharyngeal cavity and conjunctival sacs of freshwater turtles (see Morrison and Du Preez 2011). They have a reproductive strategy that is well adapted to the ecology of their hosts that are primarily aquatic and they have a direct life cycle with free swimming larvae (oncomiracidia). They were assumed to be mostly host and site specific (see Verneau 2004) until Verneau et al. (2011) reported host-switching in confined environments from introduced American turtles to native ones, namely *E. orbicularis* and *M. leprosa*. The Mediterranean pond turtle, which originated in the western region of North Africa according to Fritz et al. (2006), is mainly distributed in countries surrounding the Mediterranean Sea, namely Tunisia, Algeria and Morocco in North Africa, as well as Spain, Portugal and France in southern Europe (Bonin et al. 1998). While *M. leprosa* is considered as "Least Concern" in North Africa according to the IUCN criteria (Cox et al. 2006), it is classified as "Vulnerable" in the European Red List of Reptiles and in the Spanish Red List (Da-Silva 2002; Cox and Temple 2009) and as "Endangered" in France (IUCN France, MNHN and SHF, 2009) where it occurs only in the Languedoc-Roussillon province, more specifically in the Pyrénées Orientales region. Two polystome species were recorded and described from *M. leprosa* in Tunisia, i.e., *Neopolystoma euzeti* Combes and Ktari, 1976 of the urinary bladder and *Polystomoides tunisiensis* Gonzales and Mishra, 1977 of the pharyngeal cavity. Understanding how *T. s. elegans*

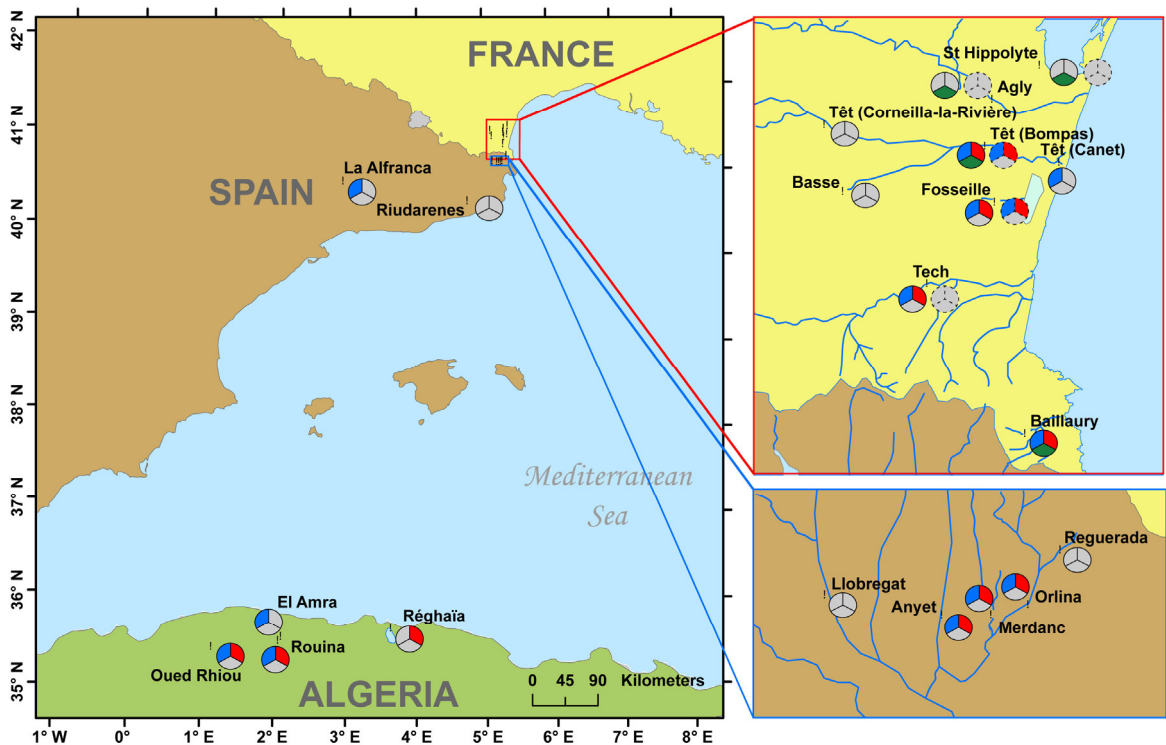


Figure 1. Map showing the sample sites in Algeria, northern Spain and southern France where *M. leprosa* was monitored for polystomes. The circles with continuous and dashed lines represent various infection sites within *M. leprosa* and *T. s. elegans*, respectively. Blue corresponds to polystomes from the pharyngeal cavity, red to polystomes from the urinary bladder and green to polystomes of the conjunctival sacs. No color means no infection detected.

may impact native turtle species is fundamentally important when considering conservation issues (Luiselli et al. 1997; Cadi and July 2004). Therefore our objectives were to determine the diversity of polystome species within *M. leprosa* and to what extent host-switching may take place between the introduced invasive freshwater turtle species and the native Mediterranean pond turtle in wild habitats of northern Spain and southern France.

Materials and methods

Host sampling (*M. leprosa* and *T. s. elegans*)

Traps were set in rivers, streams and ponds where turtles were observed or within habitats that might be suitable for turtles. Nine sites were investigated in natural water bodies of the Pyrénées Orientales region in southern France that correspond to the Agly, Baillaury, Basse, Fosseille, Têt (three distinct sites next to the villages of Canet, Bompas and Corneilla-la-

Rivière) and Tech rivers, and to a small canal that flows to the brackish lagoon of Salses-Leucate next to the village of St. Hippolyte (Figure 1). Six sites were explored along small rivers in the Catalonia province (Northeastern Spain), namely Anyet, Orlina, Merdanc, Llobregat, Reguerada and Riudarenes (Figure 1). Another locality in the Aragon province (Northern Spain) was sampled in an oxbow of the Ebro River, namely La Alfranca. Finally, four other localities were also investigated in Algeria, namely Rouina, Réghaïa, El Amra and Oued Rhiou (Figure 1). Crayfish traps were baited with pork liver, left overnight and removed the following day. Captured individuals of *M. leprosa* were then marked in the field by making marginal cuts on the carapace scutes following an international procedure for Capture-Mark-Recapture of turtles. Marked turtles were taken back to the experimental place for parasite screening over a period of usually three - four days. They were released at the exact locality where collected after the

screening process. Invasive *T. s. elegans* were not released but euthanized by cardiac injection of 10% sodium pentobarbitone (Euthapent, Kryon Laboratories, South Africa) following French national rules for invasive species.

Parasite sampling (polystomatids)

Because *M. leprosa* is a protected species in Europe, we followed a non-invasive method for parasite screening (Verneau et al. 2011). Turtles were kept individually at room temperature for three consecutive days in plastic boxes containing water to a depth of about 30 mm. When infected with mature polystomes, parasite eggs are released and washed out from the infection sites, i.e., the pharyngeal cavity and/or conjunctival sacs, or excreted with urine from the bladder. Water was then filtered daily through a set of sieves of 500 μm and 100 μm , respectively. Polystome eggs are retained on the 100 μm sieve while the 500 μm sieve retains most of the debris that foul the water. The content of the 100 μm sieve was then rinsed into a Petri dish using a wash bottle and eggs were searched for and collected using a dissecting microscope. They were identified by their orange-brown colour and pear (for parasites found either in the urinary bladder or the pharyngeal cavity) or fusiform shape (for parasites found in the conjunctival sacs) and preserved in 70% molecular grade ethanol pending DNA extraction. A few individuals of *M. leprosa* collected in Algeria were euthanized following the same procedure described above for *T. s. elegans* and dissected. Adult parasites were also preserved in 70% ethanol.

Molecular experiments

Polystome eggs and adult parasites were removed from ethanol and lyophilized by using a centrifugal evaporator (Universal Vacuum System Plus UVS400A). DNA extractions were carried out for 30 minutes at 55°C with 100 μL of 10% Chelex and 20 μL of proteinase K 10 mg mL⁻¹. Eggs were then grounded with a micro-pestle and DNA extractions were completed for 30 minutes at the same temperature. Enzymatic reaction was then stopped at 100°C for 15 minutes and DNA samples were stored at 4°C until used for PCR.

Amplification of the partial Cytochrome c Oxidase I (COI) gene and purification of PCR products were done according to the procedure developed in Verneau et al. (2009) and Du Preez et al. (2010), with the forward LCO1p

(5'-TTTTTTGGGCATCCTGAGGTTTAT-3') (Littlewood et al. 1997) and reverse HCOX1R (5'-AACAAACAAACCAAGAATCATG-3') primers. PCR followed one initial step of 5 minutes at 95°C for long denaturation, 30 cycles of 1 minute at 94°C for denaturation, 1 minute at 48°C for annealing, 1 minute at 72°C for elongation and one final step of 10 minutes at 72°C for terminal elongation, yielding a product of approximately 360 bp that was checked on a 1% agarose gel. Each DNA sample was amplified three times independently in 25 μL final volumes and PCR products were sent to the Genoscreen Company (Lille, France) for purification and sequencing that was performed with either HCOX1R or both PCR primers.

Sequence analysis

Sequence chromatograms were first checked with the program Genious (Biomatters Ltd) and then edited using the MEGA version 5 software (Tamura et al. 2011). New sequences were aligned with published sequences retrieved in Verneau et al. (2011) using Clustal W (Thompson et al. 1994). Phylogenetic analyses were conducted on the whole data set plus two other polystome species of amphibians (*Wetapolystoma almae* and *Polystoma naevius*) used as outgroups following three kinds of procedures. For the Bayesian analysis, the complete alignment was partitioned according to codon positions 1, 2 and 3. A GTR + I + Γ model was selected by the Akaike Information Criterion (AIC) implemented in the program Modeltest 3.06 (Posada and Crandall 1998) allowing rate variation across sites. The Bayesian Inference was obtained using the software MrBayes 3.04b (Huelsenbeck and Ronquist 2001), with four chains running for million generations, sampling each 100 cycles. Bayesian posterior probabilities were then computed after removing the first 1000 trees as the burn-in phase. The Maximum Likelihood (ML) analysis was conducted with the same model of evolution without partitioning. ML bootstrap support values were calculated under the Nearest Neighbor Interchange (NNI) branch swapping option using 500 replicates. Finally a Minimum Evolution (ME) tree based on the Kimura-2 parameters distance was constructed with the MEGA software and a bootstrap test (1000 replications) was also applied. Absolute differences (p-distance) between each pair of sequences were also measured for species identification, regarding the specific COI divergence threshold determined by Verneau et al. (2011) on chelonian polystomes.

Table 1. Sampling localities for *M. leprosa* (*M. l.*) and *T. s. elegans* (*T. s. e.*) in southern France (Fr), northern Spain (Sp) and Algeria (Alg), with GPS coordinates, number of individuals sampled for polystomes, number of infected hosts and prevalence.

Sampling localities	GPS coordinates	No. of individuals sampled	No. of individuals infected with prevalence (%)
St Hippolyte (Fr)	42°48'17.030"N / 2°58'15.09"E	12 (M. l.) 3 (T. s. e.)	10 (83.3%) 1 (33%)
Basse (Fr)	42°38'04.150"N / 2°46'30.28"E	12 (M. l.)	0
Agly (Fr)	42°45'38.020"N / 2°55'29.51"E	14 (M. l.) 1 (T. s. e.)	7 (50%) 0
Têt (Bompas) (Fr)	42°42'54.430"N / 2°56'02.48"E	16 (M. l.) 8 (T. s. e.)	7 (43,75%) 6 (75%)
Têt (Canet) (Fr)	42°42'34.690"N / 3°00'08.18"E	1 (M. l.)	1 (100%)
Têt (Corneilla-la-Rivière) (Fr)	42°41'25.400"N / 2°43'36.88"E	10 (M. l.)	0 (0%)
Tech (Fr)	42°35'07.490"N / 2°59'04.76"E	35 (M. l.) 6 (T. s. e.)	14 (40%) 0
Baillaury (Fr)	42°27'45.850"N / 3°05'27.16"E	154 (M. l.)	42 (27,3%)
Fosseille (Fr)	42°40'00.920"N / 2°58'00.80"E	45 (M. l.) 10 (T. s. e.)	28 (62,2%) 7 (70%)
Anyet (Sp)	42°21'39.261"N / 2°58'39.45"E	31 (M. l.)	12 (38,7%)
Orlina (Sp)	42°22'37.203"N / 3°01'50.56"E	23 (M. l.)	16 (69,6%)
Llobregat (Sp)	42°21'14.287"N / 2°54'08.70"E	10 (M. l.)	1 (10%)
Merdanc (Sp)	42°22'01.908"N / 3°00'26.68"E	13 (M. l.)	4 (30,7%)
Reguerada (Sp)	42°23'18.466"N / 3°03'08.98"E	7 (M. l.)	0 (0%)
Riudarenes (Sp)	41°49'18.600"N / 2°42'20.06"E	4 (M. l.)	0 (0%)
La Alfranca (Sp)	41°36'17.568"N / 0°45'43.72"E	2 (M. l.) 1 (T. s. e.)	2 (100%) 0
Rouina (Alg)	36°15'02.930"N / 1°49'00.45"E	16 (M. l.)	15 (93,75%)
Lake of Réghaïa (Alg)	36°46'16.700"N / 3°20'10.85"E	19 (M. l.)	3 (15,78%)
El Amra (Alg)	36°18'18.720"N / 1°50'31.81"E	10 (M. l.)	3 (30%)
Oued Rhiou (Alg)	35°58'24.010"N / 0°55'22.73"E	1 (M. l.)	1 (100%)

Naming of the various sequences

Chelonian polystomes can be identified to the genus level based on the number of hamuli, namely *Neopolystoma* with no hamuli, *Polystomoidella* with one pair and *Polystomoides* with two pairs. When sequences were obtained from previously described adult parasites, they were named according to the systematics of the species followed by its haplotype number (for example *Polystomoides tunisiensis* H25). For undescribed species, only the genus name was given followed by the corresponding haplotype number (for example *Neopolystoma* sp6H21). On the opposite, when sequences were obtained from polystome eggs, the corresponding haplotype number was just assigned (for example X. spH39). However if those sequences were identical to sequences characterizing adult polystomes, they were named following the nomenclature given to adult parasites.

Estimate of species diversity

Verneau et al. (2011) showed that polystomes occupying the same microhabitat within turtles of the same species always had genetic

divergence levels (p-distance) lower than 2% in the COI. On the other hand, genetic divergence levels between polystomes collected from the same biological niche within turtles of distinct species were always higher than 2% in the COI, although turtles occurred in sympatry. It was thus hypothesized that the divergence threshold for chelonian polystome delineation was about 2% (Verneau et al. 2011). The diversity of polystome species infecting *M. leprosa* and *T. s. elegans* was therefore evaluated from the Bayesian tree following p-distance comparisons of the most closely related haplotypes.

Results

Prevalence of infected hosts

Field investigation results are listed in Table 1. Among the 16 sampling sites where *M. leprosa* was surveyed in France and Spain, *T. s. elegans* was collected only at six of them. *M. leprosa* was found to be infected by polystomes almost everywhere except at the Basse and Têt (Corneilla-la-Rivière) rivers in France and at Reguerada and Riudarenes in Spain. Of the 389

M. leprosa specimens screened for parasite eggs in both countries, 144 (37%) were found to be infected as they released either pear or fusiform shaped eggs. Of the 28 *T. s. elegans* specimens screened, 14 (50%) were found to be infected, releasing only pear shaped eggs at three distinct sites, a canal next to St. Hippolyte, the Têt (Bompas) and Fosseille rivers.

Haplotype diversity within polystomes of M. leprosa and T. s. elegans

A total of 253 new sequences were obtained from parasite eggs or adults, among which 218 from polystomes of *M. leprosa* and 35 from polystomes of *T. s. elegans*. They were subsequently aligned with COI nucleic acid sequences depicting chelonian polystome species. A ME tree was first constructed from the analysis of all COI sequences (not shown). It helped to sort out groups of identical sequences and to collapse them into unique haplotypes (see Table S1). A total of 13 new haplotypes not present in the database of Verneau et al. (2011) were found (GenBank Accession numbers KM258884 to KM258896), among which 11 illustrated polystomes of *M. leprosa* (H57, H59, H69, H70, H78, H80, H82, H83, H85, H86 and H87) and two illustrated polystomes of *T. s. elegans* (H77 and H81). Two other haplotypes (H55 and H88) were also identified from polystome eggs and adults collected from *T. s. elegans* in the Turtle farm of Sorède (France) and from *Kinosternon leucostomum* (Duméril and Bibron, 1851) of Costa Rica, respectively (GenBank Accession numbers KM258897 and KM258898). At the end, 66 haplotypes were retained to analyze their phylogenetic relationships and to identify clades of interest (Figure 2).

Polystome species diversity within M. leprosa

Haplotypes depicting polystomes infesting wild *M. leprosa* were subdivided into seven clades (A to E and G to H) according to the Bayesian tree (Figure 2) and branch support values obtained from ML and ME analyses. Because p-distance estimates between haplotypes within each clade (A to E and H) were less than 2%, but higher when comparing haplotypes inside and outside of the clades of interest, all these clades were considered as distinct polystome species. This was the same for clade G that includes a single haplotype (H18) recovered from several polystomes of distinct host specimens and species. Finally,

group F was also considered as a separate species as ME branch support value was higher than 50% and p-distance estimates between haplotypes of group F less than 2%.

Clade A: Polystomoides tunisiensis

It comprises eleven distinct haplotypes (H25, H26, H27, H28, H29, H30, H59, H69, H78, H82 and H85) and is considered as *P. tunisiensis*, i.e., a natural polystome species of *M. leprosa*, which was collected for the first time from the pharyngeal cavity of Mediterranean pond turtles at Oued Rhiou in Algeria (Verneau et al. 2011). H25 characterizes polystome eggs and adults collected from a single turtle in Oued Rhiou, H26 characterizes polystome eggs collected from a single specimen of *M. leprosa* near to Canet on the Têt River, H27, H28 and H29 characterize polystome eggs collected from a single captive specimen of *M. leprosa* and H30 characterizes polystome eggs collected from nine distinct specimens of *M. leprosa* sampled in the Tech River (see Verneau et al. 2011 and Table S1). H59 characterizes polystome eggs collected from nine distinct specimens of *M. leprosa* in Spain, namely in Anyet, Merdanc, La Alfranca and Orlina, H69 characterizes two polystomes collected from a single specimen of *M. leprosa* in Rouina, H78 characterizes polystome eggs collected from eight distinct specimens of *M. leprosa* in Orlina and Anyet, H82 characterizes polystome eggs collected from nine distinct specimens of *M. leprosa* in Anyet, Merdanc and Orlina, and finally H85 characterizes a polystome collected from a single specimen of *M. leprosa* in El Amra.

Clade B: Neopolystoma euzeti

It comprises four distinct haplotypes (H31, H32, H70 and H87) and is considered as *N. euzeti*, i.e., a natural polystome species of *M. leprosa*, which was collected for the first time from the urinary bladder of Mediterranean pond turtles at Oued Rhiou in Algeria (Verneau et al. 2011). H31 characterizes one polystome adult collected from a single turtle in Oued Rhiou (Verneau et al. 2011), but also another polystome adult sampled from the urinary bladder of a single specimen of *M. leprosa* in Rouina and polystome eggs collected from five distinct specimens of *M. leprosa* in Spain, namely in Merdanc and Orlina. H32 characterizes polystome eggs collected from a single captive specimen of *M. leprosa* (Verneau et al. 2011), but also polystome eggs collected

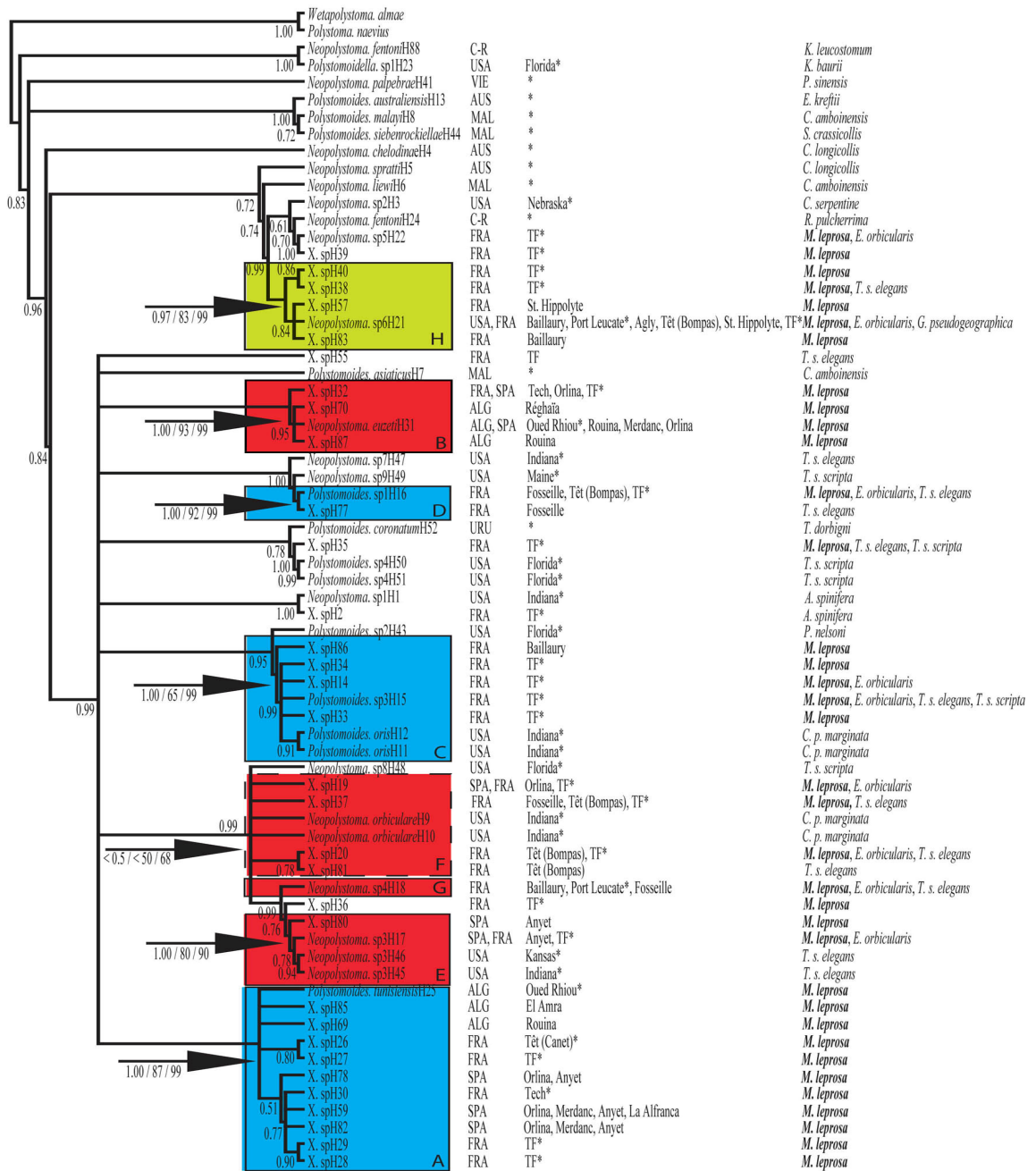


Figure 2. Bayesian tree resulting from the analysis of 66 nucleic acid sequences obtained from polystomes collected in captive and wild turtle populations. Values along branches indicate Bayesian posterior probabilities. Bootstrap proportions resulting from 500 and 1,000 resampling in ML and ME, respectively, are also reported for clades of interest depicted with arrows. * refers to results reported in Verneau et al. (2011). Polystome species boxed in blue (A, C and D) are from the pharyngeal cavity, in red (B, E, F and G) from the urinary bladder and in green (H) from the conjunctival sacs. Abbreviations used for countries are: ALG = Algeria; AUS = Australia; C-R = Costa-Rica; FRA = France; MAL = Malaysia; SPA = Spain; USA = United States of America; VIE = Vietnam; URU = Uruguay. Abbreviations used for turtle species names are from top to bottom: *K. baurii* = *Kinosternon baurii*; *K. leucostomum* = *Kinosternon leucostomum*; *C. p. marginata* = *Chrysemys picta marginata*; *M. leprosa* = *Mauremys leprosa*; *E. orbicularis* = *Emys orbicularis*; *T. s. elegans* = *Trachemys scripta elegans*; *T. s. scripta* = *Trachemys scripta scripta*; *P. nelsoni* = *Pseudemys nelsoni*; *C. amboinensis* = *Cuora amboinensis*; *T. dorbigni* = *Trachemys dorbigni*; *A. spinifera* = *Apalone spinifera*; *R. pulcherrima* = *Rhinoclemmys pulcherrima*; *C. serpentine* = *Chelidra serpentine*; *G. pseudogeographica* = *Graptemys pseudogeographica*; *C. longicollis* = *Chelodina longicollis*; *E. kreftii* = *Emydura kreftii*; *P. sinensis* = *Pelodiscus sinensis*; *S. crassicollis* = *Siebenrockiella crassicollis*.

from five distinct specimens of *M. leprosa* in Orlina and in the Tech River. Finally H70 and H87 characterize two polystome adults collected from two specimens of *M. leprosa* in Réghaïa and Rouina, respectively.

Clade C: *Polystomoides oris* Paul, 1938

It comprises seven distinct haplotypes (H11, H12, H14, H15, H33, H34 and H86) and is considered as *P. oris* which was collected for the first time from the pharyngeal cavity of wild American painted turtles *Chysemys picta marginata* Agassiz, 1857 (see Verneau et al. 2011). H11 and H12 characterize polystome adults collected from *C. p. marginata* of the USA (Verneau et al. 2011). H14 characterizes polystome eggs of captive *M. leprosa* and *E. orbicularis*, H15 characterizes polystome eggs of captive *M. leprosa*, *E. orbicularis* and *T. s. elegans* while H33 and H34 characterize only polystome eggs of captive *M. leprosa* (Verneau et al. 2011). Finally H86 characterizes polystome eggs collected from six distinct specimens of *M. leprosa* in the Baillaury River.

Clade D: *Polystomoides* sp1 (see Verneau et al. 2011)

It comprises two distinct haplotypes, namely H16 and H77. H16 was assigned to *Polystomoides* sp1 which was collected for the first time from the pharyngeal cavity of a single captive specimen of *E. orbicularis* (Verneau et al. 2011). It also characterizes polystome eggs collected from three individuals of *M. leprosa* and five of *T. s. elegans* sampled at the Fosseille River and from one specimen of *M. leprosa* and seven of *T. s. elegans* sampled at the Têt River, next to Bompas. H77 characterizes a polystome egg collected from a single individual of *T. s. elegans* at the Fosseille River.

Clade E: *Neopolystoma* sp3 (see Verneau et al. 2011)

It comprises four distinct haplotypes (H17, H45, H46 and H80). H45 and H46 were assigned to *Neopolystoma* sp3 which was collected for the first time from the urinary bladder of two specimens of American *T. s. elegans* (Verneau et al. 2011). H17 characterizes two polystome adults collected from the same ecological niche of captive *E. orbicularis* and *T. s. elegans*, but also polystome eggs collected from four specimens of *M. leprosa* in Anyet. Finally H80 characterizes polystome egg collected from a single specimen of *M. leprosa* in Anyet.

Group F: *Neopolystoma orbiculare* (Stunkard, 1916)

It comprises six distinct haplotypes (H9, H10, H19, H20, H37 and H81) and is considered as *Neopolystoma orbiculare* which was collected for the first time from the urinary bladder of American *C. p. marginata* (Verneau et al. 2011). H9 and H10 characterize seven polystome adults collected from two turtles in the USA (Verneau et al. 2011). H19 characterizes polystome eggs collected from three captive specimens of *M. leprosa* and from one captive specimen of *E. orbicularis*, H20 characterizes polystome eggs collected from four captive specimens of *M. leprosa*, but also from three captive specimens of *E. orbicularis* and from two captive specimens of *T. s. elegans* and H37 characterizes polystome eggs collected from two captive specimens of *M. leprosa* (Verneau et al. 2011). H19, H20 and H37 also characterize polystome eggs collected from wild specimens of *M. leprosa* in Orlina (H19), in the Têt (H20 and H37) and Fosseille (H37) rivers, but also polystome eggs and adults collected from wild specimens of *T. s. elegans* in the Têt (H20) and Fosseille (H37) rivers. Finally H81 characterizes polystome eggs collected from a single specimen of *T. s. elegans* in the Têt River.

Clade G: *Neopolystoma* sp4 (see Verneau et al. 2011)

It comprises a single haplotype, namely H18 that was previously assigned to *Neopolystoma* sp4 collected for the first time from the urinary bladder of wild *E. orbicularis* in France (Verneau et al. 2011). This haplotype also characterizes polystome eggs that were collected from 16 specimens of *M. leprosa* and from one specimen of *T. s. elegans* in the Baillaury and Fosseille rivers, respectively.

Clade H: *Neopolystoma* sp6 (see Verneau et al. 2011)

It comprises five distinct haplotypes (H21, H38, H40, H57 and H83). H21 was assigned to *Neopolystoma* sp6 collected for the first time from the conjunctival sacs of one specimen of the American Mississippi map turtle *Graptemys pseudogeographica* (Gray, 1831) (see Verneau et al. 2011). H21 also characterizes polystome eggs collected from two French specimens of wild *E. orbicularis* and captive *M. leprosa*, respectively (Verneau et al. 2011), but also polystome eggs and adult parasites collected from four specimens of *M. leprosa* in the Baillaury River and polystome eggs collected from two specimens

of *M. leprosa* in the Agly River, from one specimen of *M. leprosa* in the Têt River and from five specimens of *M. leprosa* at St. Hippolyte. H38 characterizes polystome eggs of captive *M. leprosa* and *T. s. elegans* turtles while H40 only characterizes polystome eggs of captive specimens of *M. leprosa* (Verneau et al. 2011). Finally H57 and H83 characterize polystome eggs collected from two specimens of *M. leprosa* at St. Hippolyte and in the Baillaury River, respectively.

Discussion

Polystome diversity within M. leprosa

Due to limited interspecific differences in morphological traits used in identifying polystome species (Tinsley 1973), it is often very complicated or even impossible to identify a polystome based only on morphological characteristics. Thus, emphasis has been placed on host-specificity although Pichelin (1995) stated that host identity cannot be used as a reliable taxonomic character among turtle polystomes. Whereas amphibian polystomes are only found in the urinary bladder of post-metamorphic frogs, turtle polystomes are known from three distinct biological niches, namely the urinary bladder, pharyngeal cavity and conjunctival sacs. The Southeast Asian box turtle *Cuora amboinensis* (Daudin, 1801), for example, harbours *Neopolystoma liewi* Du Preez and Lim, 2000 within conjunctival sacs, *Polystomoides asiaticus* Rohde, 1965 in the pharyngeal cavity, *Polystomoidella mayesi* Richardson and Brooks, 1987 and *Polystomoides malayi* Rohde, 1963 in the urinary bladder. This explains the relatively high diversity of chelonian polystome species, namely to about 50 species (see Verneau 2004; Morrison and Du Preez 2011), in comparison to the low diversity of freshwater chelonians, i.e. about 200 species (Bonin et al. 1998). Because DNA barcoding has proven to be an excellent tool for exploring biodiversity the last two decades (Darling and Blum 2007; Meusnier et al. 2008; Valentini et al. 2009), considering up to eight distinct polystome species within allopatric *M. leprosa* populations along its natural range, is not unrealistic. Among those parasites, four of them were recorded from the urinary bladder, i.e., *N. euzeti*, *N. orbiculare* and two undescribed species named for convenience *Neopolystoma* sp3 and *Neopolystoma* sp4 in Verneau et al. (2011). Three other species were reported from the pharyngeal cavity, i.e. *P. tunisiensis*, *P. oris*

and an undescribed species named *Polystomoides* sp1 by Verneau et al. (2011). The last species was located in the conjunctival sacs and named *Neopolystoma* sp6 by Verneau et al. (2011).

Although we cannot rule out the possibility that some polystomes are not specific to their hosts in natural environments on one hand and that some host species may be infected by distinct polystome species within the same microhabitat on the other hand, a correct description of host and/or parasite species is an important prerequisite to unravel evolutionary processes of biodiversity. *Polystomoides ocellatum* (Rudolphi, 1819), for instance, was described from the pharyngeal cavity of the European pond turtle *E. orbicularis* and later reported from the same ecological niche within *M. leprosa* in Morocco (Combes and Thiery 1983). We showed from a DNA barcoding survey (data not shown) that *P. ocellatum* is in fact specific to its host and has never been found on *M. leprosa*. Similarly *Neopolystoma fentoni* Platt, 2000, which was recovered from the conjunctival sacs of two distinct host species, namely *Rhinoclemmys pulcherrima* (Gray, 1855) and *K. leucostomum* of Costa Rica, could be also considered as non-host specific. Regarding the phylogenetic position of parasite specimens (H24 and H88) collected from the two host species in their natural environment (see the Bayesian tree in Figure 2), and according to the high level of genetic divergence between these two parasites (20,5% of nucleotide substitutions), *N. fentoni* should be definitely split into two distinct species, each of them being specific to its respective host (Figure 2). Conversely, distinct polystome species were reported from the same biological niche within the same host species, as is the case for instance for the two species *Polystomoides nabedei* Kulo, 1980 and *Polystomoides chabaudi* Euzet and Combes, 1965 that were described from the urinary bladder of their chelonian host species *Pelomedusa subrufa* (Lacépède, 1788), in Togo and Madagascar, respectively. A recent phylogeographic survey that was conducted on the widely distributed helmeted terrapin *P. subrufa* in Africa and Madagascar has shown this turtle could be a complex of up to nine non-overlapping species (Vargas-Ramírez et al. 2010). Therefore, we might also consider that the two polystomes *P. nabedei* and *P. chabaudi* infect the same ecological niche of two distinct host species. Concerning *M. leprosa*, it has been shown that this species originated in North Africa and dispersed to the Iberian Peninsula afterwards (Fritz et al. 2006). Although two genetic lineages were identified

from analysis of the cytochrome b suggesting the existence of two subspecies, i.e., *Mauremys leprosa leprosa* (Schweigger, 1812) which is confined to the Iberian Peninsula, France and northern Morocco, and *Mauremys leprosa saharica* Schleich, 1996 which is confined in southern Morocco, eastern Algeria and Tunisia (Fritz et al. 2006), misinterpretations in systematics of *M. leprosa* and their parasites cannot explain the occurrence of eight distinct polystome species within host populations (Figure 2). Thus our results suggest that *M. leprosa* may be infected by various polystome species in natural environments and that polystome diversity may be greater than expected, at least within the Mediterranean pond turtle.

Patterns and processes of polystome evolution within M. leprosa populations

In Algeria, *T. s. elegans* has never been recorded in natural environments, probably because the pet trade in North Africa has been less than in Europe and Asian countries. At Spanish sites American turtle species, particularly *T. s. elegans*, were rarely observed. They were also not very common in the Tech river of France. All these findings would explain why only natural *M. leprosa* polystome species, namely *P. tunisiensis* and *N. euzeti*, were collected from wild Algerian turtles (Figures 1 and 2). This would also explain the occurrence of both polystome species from the *M. leprosa* population of the Tech River, the occurrence of *P. tunisiensis* in all Spanish investigated sites and the occurrence of *N. euzeti* in Merdanc and Orlina *M. leprosa* populations. One may therefore question the lack of both natural polystome species from all other French *M. leprosa* populations. Parasite exclusion following competition with exotic parasites may be an explanation. However additional experimental data after cross infections are needed to conclude.

Although both natural polystome species were found within Spanish *M. leprosa* populations, two other species, namely *Neopolystoma* sp3 and *N. orbiculare* were found in Anyet and Orlina, respectively (Figures 1 and 2). *Neopolystoma* sp3 is an undescribed species that infests the urinary bladder of wild American *T. s. elegans*, while *N. orbiculare* is known as the natural polystome species that infests the urinary bladder of *C. p. marginata* (Stunkard 1916). Because no American turtle was reported from both localities, one may question the occurrence of these two non-native parasite species in *M. leprosa*. One possibility is that each parasite was introduced in both sites by

T. s. elegans and *C. p. marginata*, respectively, before they disappeared from the natural environments. If this hypothesis can not be ruled out, it seems very unlikely because both turtle species have never been documented at these sites. Furthermore *C. p. marginata* was also not exported as much as *T. s. elegans* that can be found in various natural environments across the world. The other possibility that may be considered is that *M. leprosa* was infected at another site, or in captivity, before it was introduced into the wild. This hypothesis seems more likely due to the fact that non-native polystomes are able to infect a broad range of turtles in captivity (Verneau et al. 2011), and since many turtle farms and people keep a variety of endemic and exotic turtles such as *T. s. elegans* and *C. p. marginata* whereby native turtles could be introduced at a later stage in natural environments.

The occurrence of the three polystome species, namely *P. oris*, *Neopolystoma* sp6 and *Neopolystoma* sp4, which are reported from the *M. leprosa* population of the Baillaury in France (Figures 1 and 2), may be explained in the same way as above. Regarding the two former species, *P. oris* is a parasite that infests the pharyngeal cavity of wild American *C. p. marginata* (Paul 1938) whereas *Neopolystoma* sp6 is an undescribed species that infests the conjunctival sacs of wild American *G. pseudogeographica* (Verneau et al. 2011). Because these two host species have never been observed in this river system, in spite of a very intensive sampling at this site since 2006, and since no program of reintroduction has taken place for *M. leprosa* in France, some native already infected turtles may probably have been released into this natural environment. This hypothesis is supported by recent genetic analyses that show from the analysis of the cytochrome b mitochondrial marker that some *M. leprosa* turtles of the subspecies *M. l. saharica* in the Baillaury would have been illegally translocated (Palacios et al. submitted). The same hypothesis may be applied to interpret the occurrence of *Neopolystoma* sp6 within *M. leprosa* turtles in the small canal next to the village of St. Hippolyte and in the Agly and Têt rivers. At last, regarding *Neopolystoma* sp4, it was previously reported from wild specimens of *E. orbicularis* in the pond of Port Leucate in France (Verneau et al. 2011). The occurrence of that species within specimens of *M. leprosa* in the Baillaury River, but also within one individual of *T. s. elegans* in the Fosseille River, raises the same questions about that parasite species. However

because *Neopolystoma* sp4 has not yet been documented from wild American turtles in their native range, we cannot conclude.

In the Têt and Fosseille rivers, the two polystome species, i.e., *N. orbiculare* and *Polystomoides* sp1, were reported from both *M. leprosa* and *T. s. elegans* turtles (Figures 1 and 2). Because *N. orbiculare* is a natural parasite of *C. p. marginata*, another American turtle, and because *M. leprosa* and *T. s. elegans* turtles share the same polystome at the Têt (H20) and Fosseille (H37) rivers, the most likely hypothesis is to consider parasite transfer between host species in the wild. Therefore, we may hypothesize that *T. s. elegans* serves as a carrier for *N. orbiculare* parasites and transmit them to native turtles in natural environments. Regarding the undescribed species, *Polystomoides* sp1, although that parasite has never been reported from the pharyngeal cavity of wild American turtles in their home range, it is likely a non-native polystome species for *M. leprosa*. Therefore, the same scenario can be highlighted to explain the occurrence of H16 within *M. leprosa* and *T. s. elegans* populations in both rivers. Considering *T. s. elegans* as a carrier for exotic polystome species in the wild is the most plausible hypothesis at this stage as this species is very common in confined environments and generally in contact with some other American turtles. Once released into the natural environments, this species could transmit some parasites to native turtles. However we cannot reject the alternative hypothesis which considers that some native turtles may also be released in the field after being kept as pets with some American turtles in closed environments.

Conclusion

We have shown that parasite host-switching is of big concern with reference to native *M. leprosa* turtles in natural environments of Southern France and Northern Spain. As a result, invasion of *T. s. elegans*, together with its associated parasitic load, could be a key stressor to endemic turtle species. In the context of ecological risk assessment frameworks (Kolar and Lodge 2002; Sergeant 2002; Andersen et al. 2004; Keller and Lodge 2007; Keller and Perrings 2011; Ricciardi et al. 2011), we therefore recommend that potential adverse effects of new pathogens on indigenous *M. leprosa* populations be evaluated, to get a better idea of how these parasites spread and establish in natural environments and to identify the threats these parasite introductions may pose.

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The following supplementary material is available for this article:

Table S1. Haplotype diversity within polystomes of *Mauremys leprosa* and *Trachemys scripta elegans* sampled in natural environments.

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