Invasive species threat: parasite phylogenetics reveals patterns and processes of host-switching between non-native and native captive freshwater turtles

O. VERNEAU[1,*,†], C. PALACIOS[1]†, T. PLATT[2], M. ALDAY[1], E. BILLARD[1],
J.-F. ALLIENNE[1], C. BASSO[3] *and* L. H. DU PREEZ[4]

[1] *UMR 5244 CNRS-UPVD, Biologie et Ecologie Tropicale et Méditerranéenne, Parasitologie Fonctionnelle et Evolutive, Université de Perpignan Via Domitia, 52 Avenue Paul Alduy, 66860 Perpignan Cedex, France*
[2] *Department of Biology, Saint Mary's College, Notre Dame, IN 46556, USA*
[3] *Unidad de Entomología, Facultad de Agronomía, Universidad de la República, Av. Garzón 780, 12900 Montevideo, Uruguay*
[4] *School of Environmental Sciences and Development, North-West University, Potchefstroom Campus, Private Bag X6001, Potchefstroom 2520, South Africa*

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SUMMARY

One of the major threats to biodiversity involves biological invasions with direct consequences on the stability of ecosystems. In this context, the role of parasites is not negligible as it may enhance the success of invaders. The red-eared slider, *Trachemys scripta elegans*, has been globally considered among the worst invasive species. Since its introduction through the pet trade, *T. s. elegans* is now widespread and represents a threat for indigenous species. Because *T. s. elegans* coexists with *Emys orbicularis* and *Mauremys leprosa* in Europe, it has been suggested it may compete with the native turtle species and transmit pathogens. We examined parasite transfer from American captive to the two native species that co-exist in artificial pools of a Turtle Farm in France. As model parasite species we used platyhelminth worms of the family Polystomatidae (Monogenea) because polystomes have been described from American turtles in their native range. Phylogenetic relationships among polystomes parasitizing chelonian host species that are geographically widespread show patterns of diversification more complex than expected. Using DNA barcoding to identify species from adult and/or polystome eggs, several cases of host switching from exotic to indigenous individuals were illustrated, corroborating that parasite transmission is important when considering the pet trade and in reintroduction programmes to reinforce wild populations of indigenous species.

Key words: Parasitic invasions, phylogenetic systematics, *Trachemys scripta elegans*, *Emys orbicularis*, *Mauremys leprosa*, Platyhelminthes, Polystomatidae, cytochrome c oxidase I.

INTRODUCTION

Following both the exponential increase in global movement of people within the past few decades and the global trade or transport of many plants and animals, the introduction of non-native species into new biogeographic areas has been considerably accelerated. Alien species may disrupt the delicate balance of the ecosystem and therefore cause drastic or irremediable changes to environments (Vitousek *et al*. 1997; Mooney and Cleland, 2001). Accordingly, intentional or accidental biological invasions are considered a major threat to biodiversity second only to the destruction of natural environments (Vitousek *et al*. 1997). The success of introduced species depends on niche availability (Shea and Chesson, 2002), which may be driven by a combination of non-exclusive factors like competitive exclusion of resident species (Salo *et al*. 2007), lack of natural predators and/or enemies (Torchin *et al*. 2001, 2003; Keane and Crawley, 2002; Clay, 2003; Mitchell and Power, 2003), hybridization and introgression (Huxel, 1999; Ellstrand and Schierenbeck, 2000). In the context of biological invasions, parasitism must be also considered. Invaders may lose their parasites and subsequently outperform native species in their home range, or they may transmit their own parasites to naive host species (i.e. species not infected by exotic parasites), which in turn may have detrimental effects on survival rates of native species (Daszak *et al*. 2000; Anderson *et al*. 2004). This has been extensively documented from studies following translocation of vertebrate host-parasite complexes (e.g. Daszak *et al*. 1999; Tompkins *et al*. 2002; Taraschewski, 2006). Invasive parasites may,

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* Corresponding author: Olivier Verneau, UMR 5110 CNRS-UPVD, Centre de Formation et de Recherche sur les Environnements Méditerranéens, Université de Perpignan Via Domitia, 52 Avenue Paul Alduy, 66860 Perpignan Cedex, France. Tel.: 33 4 68 66 21 10. Fax: 33 6 07 47 86 07. E-mail: verneau@univ-perp.fr
† Present address: UMR 5110 CNRS-UPVD, Centre de Formation et de Recherche sur les Environnements Méditerranéens, Université de Perpignan Via Domitia, 52 Avenue Paul Alduy, 66860 Perpignan Cedex, France.

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therefore, play a key role in animal and plant invasions (Prenter *et al*. 2004).

The American red-eared slider, *Trachemys scripta elegans*, has been the most popular pet among turtles in the second half of the twentieth century. This and other American turtle species from the genera *Apalone, Graptemys, Pseudemys* and *Chrysemys* have been exported worldwide, especially to Asian and European markets. According to Telecky (2001), about 52 million sliders were exported between 1989 and 1997. Because sliders can grow rapidly and attain a large size as adults (carapace length up to 300 mm), they become less attractive as pets. As a result owners, not aware of the environmental risk, have released them into the wild. *T. s. elegans* is now widespread in natural wetlands all over Western Europe and Asia (France: Servan and Arvy, 1997; Arvy and Servan, 1998; Spain: Da Silva and Blasco, 1995; Alarcos Izquierdo *et al*. 2010; Valdeón *et al*. 2010; Italy: Ficetola *et al*. 2009; Asia: Chen, 2006; Ramsay *et al*. 2007) and is in fact considered as one of the worst invasive species (see the Global Invasive Species Database: http://www.issg.org/database/welcome/). Numerous studies have indeed reported that the invasive red-eared sliders are able to breed successfully in their new habitats (e.g. Arvy and Servan, 1998; Cadi *et al*. 2004; Ficetola *et al*. 2002, 2009; Perez-Santigosa *et al*. 2008; Kikillus *et al*. 2009) with the consequent threat of outcompeting indigenous species (Cadi *et al*. 2004).

Two European freshwater terrapins are potentially endangered by the American slider, namely the European pond turtle *Emys orbicularis* and the Mediterranean turtle *Mauremys leprosa*. Both are listed in the Annexes II and IV of the European Union habitats directive and as "near threatened" in the IUCN Red List of Threatened Species$^{\text{TM}}$ for *E. orbicularis* and "in danger" in the Liste Rouge de l'IUCN Français for *M. leprosa*. *E. orbicularis* is primarily distributed in the European and North African countries surrounding the Mediterranean Sea, while *M. leprosa* is mainly found in countries of North Africa, the Iberian Peninsula and Southern France (Bonin *et al*. 1998). Only recently have questions been addressed on the actual impact of American turtles on wild resident populations (Cadi and Joly, 2003, 2004; Polo-Cavia *et al*. 2008, 2009*a*,*b*, 2010*a*,*b*; 2011). Some studies have shown that the exotic turtles were more competitive than *E. orbicularis* and *M. leprosa* in the use of basking sites within experimental pools (Cadi and Joly, 2003; Polo-Cavia *et al*. 2010*b*). A negative impact on the weight variations and survival rates of the European pond turtles was also demonstrated under experimental conditions in the presence of *T. s. elegans* (Cadi and Joly, 2004). Similarly, experimental results suggested that chemical cues released from invasive species could modify adversely the behaviour of *M. leprosa* (see Polo-Cavia *et al*. 2009*a*). Polo-Cavia *et al*. (2009*a*) concluded that exotic sliders could ultimately contribute to the displacement of endemic turtles in natural environments. However, Cadi and Joly (2004) did not exclude the possibility that *E. orbicularis* could be more sensitive to exotic pathogens transmitted from *T. s. elegans*, which would explain its lower fitness. This has been documented in *Emys* (formerly *Clemmys*) *marmorata*, an endemic endangered North American freshwater turtle, in which the herpes-like virus transmitted from introduced captive exotic turtles may be responsible of the decline of some populations (Hays *et al*. 1999) since that virus was able to kill captive individuals of the endemic turtle (Frye *et al*. 1977).

Trachemys s. elegans may carry its own parasites when released into natural environments but, to the best of our knowledge, only a single study assessed parasite transmission from American invaders to native European turtles and found transmission from indigenous to exogenous species (Hidalgo-Vila *et al*. 2009). Among parasites reported from chelonians (Harper *et al*. 1982; Une *et al*. 1999; Du Preez and Lim, 2000; Pasmans *et al*. 2002; Eiras, 2005; Segade *et al*. 2006; Hidalgo-Vila *et al*. 2007, 2008, 2009; Mihalca *et al*. 2007, 2008), species of the Polystomatidae (Platyhelminthes, Monogenea) are widespread among amphibians and freshwater turtles (Verneau, 2004). Polystomatids arose early in the course of vertebrate evolution and dispersed to ancestral freshwater chelonians in the Upper Triassic (Verneau *et al*. 2002). Chelonian polystomes are divided into three genera, *Polystomoides, Polystomoidella* and *Neopolystoma*, based on the number of hamuli located between the posterior pair of suckers on the opisthaptor: two pairs, one pair and none, respectively. These parasites have a direct life cycle with free swimming infective larvae, i.e. oncomiracidia, and are mostly host and site specific (Verneau, 2004). Different polystome species have been recorded from the same chelonian host in three different microhabitats: the urinary bladder and cloaca, the conjunctival sacs under the eyelids, or the pharyngeal cavity, as it is the case, for instance, in the Southeast Asian box turtle *Cuora amboinensis* (Rohde, 1963, 1965; Richardson and Brooks, 1987; Du Preez and Lim, 2000). However Littlewood *et al*. (1997) showed no evidence for intra-host speciation from a phylogenetic analysis. Because polystomes have been described from American terrapins in their native range (e.g. Wright, 1879; Stunkard, 1916, 1924; Harwood, 1932; Platt, 2000) as well as from wild populations of *M. leprosa* and *E. orbicularis* in Maghreb and Europe (e.g. Rudolphi, 1819; Combes and Ktari, 1976; Gonzales and Mishra, 1977; Knoeppfler and Combes, 1977; Mishra and Gonzalez, 1978; Combes and Thierry, 1983), they are a good model to study parasitic transmission from the potentially invasive chelonian species. Our primary objective was to search for such a transfer within captive turtles of a

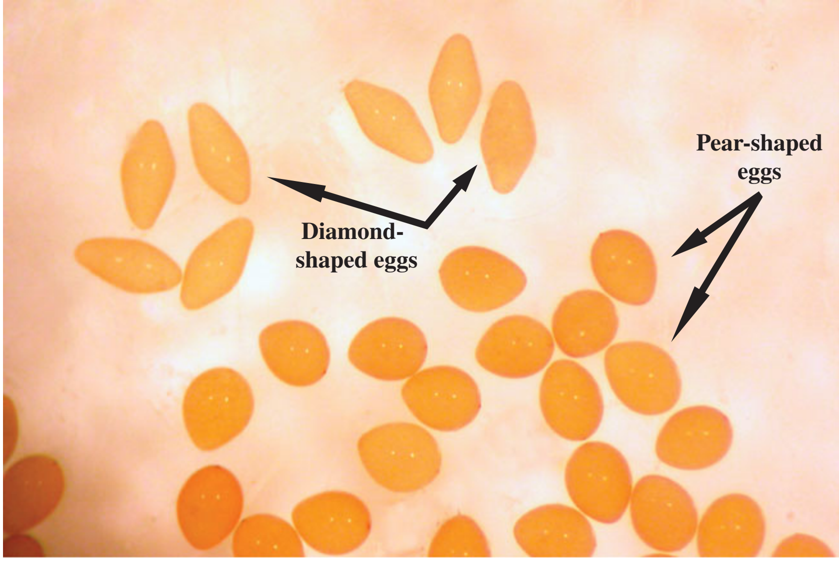

Fig. 1. Polystome eggs recovered from infected turtles. Pear-shaped eggs are from parasites located either in the urinary bladder or in the pharyngeal cavity of their host whereas diamond-shaped eggs are exclusively released by parasites located in the conjunctival sacs.

Turtle Farm in Southern France where introduced American species such as *Apalone spinifera, Chrysemys picta marginata, Graptemys pseudogeographica, T. s. elegans* and *T. s. scripta* occur with the two native species in artificial pools. Due to the endangered status of both indigenous terrapins, the search of polystomes was conducted mainly from a non-invasive approach that relies on the presence of parasite eggs collected from infected turtles, and to a lesser extent from dissecting turtles. Phylogenetic systematics of polystomes recovered from captive animals was inferred from DNA sequences of the fast evolving gene, the cytochrome c oxidase I (COI), and from subsequent genetic comparisons with polystome species sampled from wild animals in their home range.

MATERIALS AND METHODS

Host sampling

Wild populations of *E. orbicularis* and *M. leprosa* were sampled from the province Languedoc-Roussillon in the South of France. Field surveys were conducted yearly from 2006 to 2009 in a small pond in the village of Leucate (42°50′32.21″N; 3°02′13.31″E) for *E. orbicularis*, and during 2008 and 2009 in a small pond in Canet-en-Roussillon (42°42′03.83″N; 3°01′18.91″E) and in the Tech River close to Le Boulou (42°32′02.12″N; 2°50′54.43″E) respectively for *M. leprosa*. Turtles were trapped overnight using catfish traps baited with fish and pork liver. Captured individuals were individually marked by a combination of cuts in peripheral scuts of the carapace following the procedure of the “Conservatoire des Espaces Naturels du Languedoc-Roussillon” for Capture-Mark-Recapture studies. Specimens of *E. orbicularis, M. leprosa, T. s. elegans, T. s. scripta* and *A. spinifera* were collected with landing nets in the Turtle Farm of Sorède (42°30′56.83″N; 2°57′26.76″E), mainly in 2009 and 2010. Ministerial authorisation numbers 06/71/AUT, 07/168/AUT and 09/247/DEROG for capture and sacrifice of *E. orbicularis* and *M. leprosa*, from March 2006 to November 2010, were obtained.

Parasite sampling

After capture, turtles were transported to the laboratory in Perpignan and placed in individual plastic boxes with water to a depth of about 5 cm. Polystome eggs were collected over three consecutive days by pouring the water through a set of sieves of 500 μm and 100 μm, respectively. The coarse material was collected on the 500 μm sieve while fine debris and eggs were collected on the 100 μm sieve. The contents of this sieve were then washed into a Petri dish and observed using a dissecting microscope. Polystome eggs were separated according to their shape, pipetted out and preserved in 70% ethanol until DNA extraction. Polystomes located in the urinary bladder or pharyngeal cavity release pear-shaped eggs, while parasites from the conjunctival sacs release diamond-shaped eggs (Fig. 1). After screening, all turtles were released in the exact location from which they were trapped except for two infected individuals of *E. orbicularis* from the natural pond of Leucate, and also two infected *E. orbicularis* and one individual each of *M. leprosa* and *T. s. elegans* from the Turtle Farm of Sorède which were dissected to study adult worms. Prior to dissection, turtles were euthanized by cardiac injection of 1 mL sodium pentabarbitone (Euthapent) diluted in 9 mL of luke warm water. Animals were dissected and the urinary bladder,

Table 1. List of hosts and parasites investigated, geographical origin, source of polystomes, field and DNA samples and GenBank accession numbers

Host species	Haplotype – Parasite species	Origin	Source of polystomes	Field Accession numbers/ DNA samples	GenBank Accession numbers
Apalone spinifera	H1 – *Neopolystoma* sp1	USA: Indiana	Pharyngeal cavity	TRP-2003/4-1/722	FR822527
Cryptodira: Trionychidae	H2	France: TF	P-shaped egg	-/Mi-76	FR822528
Chelidra serpentine	H3 – *Neopolystoma* sp2	USA: Nebraska	Conjunctival sacs	MTS-00023F/223	FR822529
Cryptodira: Chelydridae					
Chelodina longicollis	H4 – *Neopolystoma chelodinae*	Australia	Urinary bladder		Z83005[1]
Pleurodira: Chelidae	H5 – *Neopolystoma spratti*	Australia	Conjunctival sacs		Z83007[1]
Cuora amboinensis	H6 – *Neopolystoma liewi*	Malaysia	Conjunctival sacs	-/6	FR822530
Cryptodira: Geomydidae	H7 – *Polystomoides asiaticus*	Malaysia	Pharyngeal cavity		Z83009[1]
	H8 – *Polystomoides malayi*	Malaysia	Urinary bladder		Z83011[1]
Chrysemys picta marginata	H9 – *Neopolystoma orbiculare*	USA: Indiana	Urinary bladder	P-00-4/4/221	FR822531
Cryptodira: Emydidae	H10 – *Neopolystoma orbiculare*	USA: Indiana	Urinary bladder	P-00-4/3/Mi188	FR822532
	″	″	″	P-00-4/4/Mi189	FR822538
	″	″	″	P-00-4/4/Mi190	FR822539
	″	″	″	P-00-4/4/Mi191	FR822540
	″	″	″	P-00-4/4/Mi192	FR822541
	″	″	″	P-00-4/4/Mi193	FR822542
	H11 – *Polystomoides oris*	USA: Indiana	Pharyngeal cavity	P-00-4/2/219	FR822533
	H12 – *Polystomoides oris*	USA: Indiana	Pharyngeal cavity	TRP-2002//7-1/719	FR822534
Emydura kreftii	H13 – *Polystomoides australiensis*	Australia	Urinary bladder		Z83013[1]
Pleurodira: Chelidae					
Emys orbicularis	H14	France: TF	P-shaped egg	Eos6/Mi692	FR822535
Cryptodira: Emydidae	″	″	″	Eos6/Mi693	FR828365
	″	″	″	Eos10/Mi709	FR828366
	H15	France: TF	P-shaped egg	Eos23/Mi932	FR846486
	H16 – *Polystomoides* sp1	France: TF	Pharyngeal cavity	PL060528E1/Mi4	FR822537
	H17 – *Neopolystoma* sp3	France: TF	Urinary bladder	PL060528D1/Mi3	FR846487
	H18 – *Neopolystoma* sp4	France: Leucate	P-shaped egg	Eol5/Mi96	FR822543
	″	″	″	Eol5/Mi97	FR822544
	″	″	″	Eol5/Mi98	FR822545
	″	″	″	Eol5/Mi119	FR822546
	″	″	″	Eol22/986	FR822547
	″	″	″	Eol22/987	FR822548
	″	″	″	Eol34/Mi95	FR822549
	″	″	″	Eol34/Mi102	FR822550
	″	″	″	Eol34/Mi103	FR822551
	″	″	″	Eol34/Mi118	FR822552
	″	″	″	Eol34/Mi145	FR828367
	″	″	″	Eol53/Mi121	FR828368
	″	″	″	Eol87/Mi122	FR828369
	″	″	″	Eol87/Mi123	FR828370
	H19	France: TF	P-shaped egg	Eos10/Mi707	FR846488
	H20	France: TF	P-shaped egg	-/Mi125	FR846489
	″	″	″	-/Mi126	FR828371

Table 1. (*Cont.*)

Host species	Haplotype – Parasite species	Origin	Source of polystomes	Field Accession numbers/ DNA samples	GenBank Accession numbers
	″	″	″	Eos9/Mi702	FR828372
	″	″	″	Eos9/Mi704	FR828373
	″	″	″	Eos9/Mi705	FR828374
	H21	France: Leucate	D-shaped egg	Eol92/Mi84	FR828375
	″	France: TF	″	Eos2/Mi687	FR828376
	H22 – *Neopolystoma* sp5	France: TF	Conjunctival sacs	PL060529A1/Mi2	FR828377
	″	″	″	PL060529A2/Mi162	FR828378
Graptemys pseudogeographica Cryptodira: Emydidae	H21 – *Neopolystoma* sp6	USA: Indiana	Conjunctival sacs	TRP-2003//5-1/723	FR822553
Kinosternon baurii Cryptodira: Kinosternidae	H23 – *Polystomoidella* sp1	USA: Florida	Urinary bladder	PL040601A2/800	FR822554
Kinosternon leucostomum Cryptodira: Kinosternidae	H24 – *Neopolystoma fentoni*	Costa Rica	Conjunctival sacs	02-PARA-483-4/717	FR822555
Mauremys leprosa	H14	France: TF	P-shaped egg	Mls6/Mi-725	FR822556
Cryptodira: Geomydidae	H15	France: TF	P-shaped egg	Mls1/Mi711	FR822536
	″	″	″	Mls1/Mi713	FR822557
	H19	France: TF	P-shaped egg	Mls10/Mi732	FR822559
	″	″	″	Mls10/Mi733	FR822560
	″	″	″	Mls10/Mi734	FR822561
	″	″	″	Mls21/Mi908	FR822562
	″	″	″	Mls24/Mi916	FR822563
	H20	France: TF	P-shaped egg	-/Mi135	FR822564
	″	″	″	Mls5/Mi721	FR822565
	″	″	″	Mls10/Mi735	FR822566
	″	″	″	Mls20/Mi905	FR822567
	H21	France: TF	D-shaped egg	Mls28/MiAB10	FR822568
	H22	France: TF	D-shaped egg	Mls6/Mi939	FR822569
	H25 – *Polystomoides tunisiensis*	Algeria	Pharyngeal cavity	Mla1/Mi-110	FR822570
	H25	″	P-shaped egg	Mla1/Mi-114	FR822571
	″	″	″	Mla1/Mi-116	FR822572
	″	″	″	Mla1/Mi-142	FR822573
	″	″	″	Mla1/Mi-143	FR822574
	″	″	″	Mla1/Mi-144	FR822575
	H26	France: Canet	P-shaped egg	Mlc1/Mi-225	FR822576
	″	″	″	Mlc1/Mi-226	FR822577
	″	″	″	Mlc1/Mi-227	FR822578
	″	″	″	Mlc1/Mi-228	FR822579
	H27	France: TF	P-shaped egg	Mls24/Mi-914	FR822580
	H28	France: TF	P-shaped egg	Mls24/Mi-915	FR822581
	H29	France: TF	P-shaped egg	Mls24/Mi-917	FR822582
	H30	France: Le Boulou	P-shaped egg	Mlb21/Mi-657	FR822583
	″	″	″	Mlb22/Mi-987	FR822584
	″	″	″	Mlb22/Mi-989	FR822585
	″	″	″	Mlb25/Mi-669	FR822586

	H31 – *Neopolystoma euzeti*	Algeria	Urinary bladder	Mla1/Mi-111	FR822587
	H32	France: TF	P-shaped-egg	Mls2/Mi-715	FR822588
	″	″	″	Mls2/Mi-716	FR822589
	H33	France: TF	P-shaped-egg	-/Mi-137	FR822590
	H34	France: TF	P-shaped egg	Mls1/Mi712	FR822591
	H35	France: TF	P-shaped egg	Mls5/Mi-720	FR822592
	″	″	″	Mls6/Mi-724	FR822593
	″	″	″	Mls10/Mi-740	FR822594
	H36	France: TF	P-shaped egg	Mls4/Mi-719	FR822595
	H37	France: TF	P-shaped egg	-/Mi-85	FR822596
	″	″	″	Mls6/Mi728	FR822597
	H38	France: TF	D-shaped egg	Mls6/MiAB9	FR822598
	H39	France: TF	D-shaped egg	Mls18/MiAB12	FR822599
	H40	France: TF	D-shaped egg	Mls18/MiAB13	FR822600
Pelodiscus sinensis Cryptodira: Trionychidae	H41 – *Neopolystoma palpebrae*	Vietnam	Conjunctival sacs	-/130	FR822601
Pelusios castaneus Pleurodira: Pelomedusidae	H42 – *Polystomoides bourgati*	Togo	Urinary bladder	-/Pdsp	FR822602
Pseudemys nelsoni Cryptodira: Emydidae	H43 – *Polystomoides* sp2	USA: Florida	Pharyngeal cavity	PL040625C1/Mi200	FR822603
Siebenrockiella crassicollis Cryptodira: Geomydidae	H44 – *Polystomoides siebenrockiellae*	Malaysia	Urinary bladder	-/4	FR822604
Trachemys scripta elegans Cryptodira: Emydidae	H15 – *Polystomoides* sp3	France: TF	Pharyngeal cavity	PL090522B1/Mi848	FR822558
	″	″	″	PL090522B2/Mi849	FR822605
	″	″	″	PL090522B3/Mi850	FR822606
	H17 – *Neopolystoma* sp3	France: TF	Urinary bladder	PL090522A1/Mi767	FR822607
	″	France: TF	P-shaped egg	Tses10/Mi762	FR822608
	″	″	″	Tses10/Mi765	FR822609
	H20	France: TF	P-shaped egg	Tses5/Mi747	FR822610
	″	″	″	Tses5/Mi748	FR822611
	″	″	″	Tses15/Mi890	FR822612
	″	″	″	Tses15/Mi891	FR822613
	″	″	″	Tses15/Mi892	FR822614
	H35	France: TF	P-shaped egg	Tses2/Mi744	FR828344
	″	″	″	Tses2/Mi745	FR828345
	″	″	″	Tses2/Mi746	FR828346
	″	″	″	Tses6/Mi751	FR828347
	″	″	″	Tses19/Mi894	FR828348
	″	″	″	Tses19/Mi895	FR828349
	″	″	″	Tses46/Mi898	FR828350
	″	″	″	Tses46/Mi899	FR828351
	″	″	″	Tses46/Mi901	FR828352
	H38	France: TF	D-shaped egg	Tses10/Mi950	FR828353
	H45 – *Neopolystoma* sp3	USA: Indiana	Urinary bladder	TRP-2002//7-5/721	FR828354
	H46 – *Neopolystoma* sp3	USA: Kansas	Urinary bladder	MTS-0007/222	FR828355
	H47 – *Neopolystoma* sp7	USA: Indiana	Pharyngeal cavity	TRP-2002//7-1/720	FR828356

Table 1. (*Cont*.)

Host species	Haplotype – Parasite species	Origin	Source of polystomes	Field Accession numbers/ DNA samples	GenBank Accession numbers
Trachemys scripta scripta	H15	France: TF	P-shaped egg	Tsss55/Mi925	FR828357
Cryptodira: Emydidae	H35	France: TF	P-shaped egg	Tsss55/Mi922	FR828358
	H48 – *Neopolystoma* sp8	USA: Florida	Urinary bladder	PL040602A1/Mi198	FR828359
	H49 – *Neopolystoma* sp9	USA: Maine	Pharyngeal cavity	-/724	FR828360
	H50 – *Polystomoides* sp4	USA: Florida	Pharyngeal cavity	PL040604A5/Mi196	FR828361
	H51 – *Polystomoides* sp4	USA: Florida	Pharyngeal cavity	PL040505A2/Mi195	FR828362
	″	″	″	PL040602D2/Mi199	FR828363
Trachemys dorbigni	H52 – *Polystomoides coronatum*	Uruguay	Pharyngeal cavity	-/ Pv2	FR828364
Cryptodira: Emydidae					
Ougroups					
Bufo margaritifer	*Wetapolystoma almae*	French Guiana			AM913867^2
Smilisca baudenii	*Polystoma naevius*	Costa Rica			AM913864^2
Sparus auratus	*Bivagina pagrosomi*				Z83003^1

1: From Littlewood *et al*. (1997); 2: From Du Preez *et al*. (2007). TF means Turtle Farm; P means pear; D means diamond.

accessory bladders and cloaca were removed intact. The head was severed well below the pharyngeal pouch and eyelids and nictitating membranes were lifted to enable examination of all crevices along the eye balls. The urinary bladder, the pharyngeal cavity, the conjunctival sacs and all potential sites were carefully examined using a dissecting microscope for the presence of polystomes. Adult worms were fixed under coverslip pressure in 10% neutral buffered formalin for morphological determination or in 70% ethanol for molecular analyses. All other adult polystomes used in this study were from our collections. Collaborators supplied some taxa while we collected others during field surveys conducted in many different countries and areas: e.g. Australia, Africa, Eurasia and South, Central and North America. Three other monogeneans infecting amphibian and fish species were used for outgroup comparison. GenBank sequences were already available for eight of these adult polystomes, the rest were sequenced as part of this study (see Table 1 for details).

DNA Extraction, PCR and sequencing

Eggs and adult parasites were first ground with a micro-pestle and DNA extractions were carried out for one hour at 55 °C in a final volume of 100 μL of Chelex 10% and proteinase K at 1 mg mL^{-1}. Reactions were conducted at 100 °C for 15 min and DNA samples were stored at 4 °C until use for PCR. Amplification and purification of partial COI were done according to Verneau *et al*. (2009) and Du Preez *et al*. (2010), using the forward L-CO1p (5′-TTTTTTGGGCATCCTGAGGTTTAT-3′) and reverse H-COX1p2 (5′-TAAAGAAAGAACATAATGAAAATG-3′) primers (Littlewood *et al*. 1997). PCR products of about 440 bp were run in a 1% agarose gel and stained with ethidium bromide. DNA was purified using the Wizard SV Gel and PCR Clean-up System of Promega and sent to GATC (Biotech, France) for sequencing with both PCR primers. Sequences were edited and corrected with Sequencher^{TM} software (Gene Codes Corporation, Ann Arbor, Michigan, USA).

Phylogenetic analyses and molecular divergence level within species

Nucleic acid sequences from adult worms and polystome eggs were aligned using Clustal W (Thompson *et al*. 1994), which is implemented in MEGA version 4 (Tamura *et al*. 2007). DNA sequences were also translated to their corresponding protein sequences with the EMBOSS Transeq online software, following the alternative flatworm mitochondrial code, and aligned as before. The complete nucleic and amino acid sequence alignments were each subdivided in two sets of sequences depending on their origin. The first dataset comprised 35 haplotypes recovered exclusively

Table 2. List of species investigated for polystome eggs and prevalence of parasite infection in wild and captive turtles

Surveyed species, localities and years of investigation	No. of specimens examined	No. of specimens infected with prevalence in brackets	No. of specimens releasing only pear-shaped eggs	No. of specimens releasing only diamond-shaped eggs	No. of specimens releasing both types of eggs
Emys orbicularis					
Leucate (2006)	32	13 (40,6%)	13	0	0
Leucate (2007)	98	59 (60,2%)	58	0	1
Leucate (2008)	64	46 (71,8%)	46	0	0
Leucate (2009)	79	53 (67%)	53	0	0
Turtle Farm (2009)	10	7 (70%)	1	1	5
Turtle Farm (2010)	25	7 (28%)	7	0	0
Mauremys leprosa					
Canet-en-Roussillon (2008)	1	1 (100%)	1	0	0
Le Boulou (2009)	15	8 (53,3%)	8	0	0
Turtle Farm (2009)	12	8 (66,6%)	6	0	2
Turtle Farm (2010)	39	20 (51,3%)	13	4	3
Trachemys scripta elegans					
Leucate (2007)	1	0	0	0	0
Turtle Farm (2009)	10	7 (70%)	6	1	0
Turtle Farm (2010)	32	15 (46,9%)	14	1	0
Trachemys scripa scripta					
Turtle Farm (2010)	17	6 (35,3%)	5	1	0
Apalone spinifera					
Turtle Farm (2007)	1	1 (100%)	1	0	0

from wild animals, while the second set included 55 haplotypes from both wild and captive turtles.

Maximum Likelihood (ML) analyses were performed on 345 nucleic acids characters without partitioning data-sets as follows. For the nucleic acids alignment including 35 haplotypes, a TVM + I + Γ model was selected by the Akaike Information Criterion (AIC) implemented in the program Modeltest 3.06 (Posada and Crandall, 1998), whereas a GTR + I + Γ was selected for the nucleic acids alignment including 55 haplotypes. Using these models, the search for the best ML trees was done following a heuristic procedure under the TBR branch swapping option with PAUP* 4.0b9 (Swofford, 2002). ML bootstrap support values were calculated with the same model of sequence evolution under the NNI branch swapping option after 500 replicates. Bayesian analyses were conducted using the software MrBayes 3.04b (Huelsenbeck and Ronquist, 2001), with four chains running for a million generations, sampling each 100 cycles. The Bayesian inferences were obtained using the selected models listed above for each data-set and Bayesian posterior probabilities were computed after removing the first 1,000 trees as the burn-in phase. Only ML analyses were conducted with the amino acid sequence alignment, which comprised 115 characters, using the PHYML online software (Guindon and Gascuel, 2003), under the amino acids substitution model LG and the NNI branch swapping option. Branch support values were performed after 500 replicates. Finally uncorrected pairwise genetic distances (p-distances) were estimated from the 55 nucleic acid haplotype sequences using MEGA in order to delineate the within species molecular divergence level.

RESULTS

Prevalence of infection in wild and captive turtles

Results obtained from polystome eggs investigation among specimens of *E. orbicularis, M. leprosa, T. s. elegans, T. s. scripta* and *A. spinifera* are summarized in Table 2. Among the wild 171 individuals of *E. orbicularis* captured between May 2006 and October 2009 in Leucate, all infected turtles produced pear-shaped eggs except one that presented diamond-shaped eggs. This specimen (Eol92) was dissected in 2007, and another (Eol48) in 2006. Three adult worms of *Neopolystoma* sp. were found in the urinary bladder of Eol48 and one in Eol92. No parasites were found in the pharyngeal cavity and, surprisingly, no parasites from the conjunctival sacs of Eol92 that released diamond-shaped eggs. Two infected *E. orbicularis* from the Turtle Farm were also dissected in 2006. The first specimen yielded three *Neopolystoma* sp. in the urinary bladder and 11 *Polystomoides* sp. in the pharyngeal cavity. The second specimen contained two *Neopolystoma* sp. from the conjunctival sacs. Similarly a single specimen of captive *M. leprosa* was dissected in 2006. It contained one *Polystomoides* sp. in the pharyngeal cavity and four *Neopolystoma* sp. in the conjunctival sacs. Finally one *T. s. elegans* dissected in 2009 in the Turtle Farm was infected with 46 specimens of *Neopolystoma* sp. in the urinary bladder and 46 individuals of *Polystomoides* sp. in the pharyngeal cavity.

Organization of the haplotype diversity within polystomes

Sequences obtained from adult worms and polystome eggs were classified as corresponding haplotypes (from H1 to H52, Table 1). When adult worms had been previously identified, the species name is reported besides the haplotype in Table 1 and Figures 2a and 2b. Conversely, when worms were a new species, only the generic name is reported according to the number of hamuli (see Introduction). Haplotypes only found in polystome eggs are not assigned to a genus or species.

Phylogenetic relationships among polystomes

Bayesian phylograms obtained from the analyses of COI nucleic acid sequences with 35 and 55 haplotypes are shown in Figs. 2a and 2b, respectively. Because both topologies did not differ significantly from topologies inferred from ML analyses on nucleic and amino acid sequences, ML bootstrap values are given directly in Figs. 2a and 2b. Phylogenetic relationships among polystomes collected exclusively in wild animals show numerous polytomies (Fig. 2a). If we consider the basal placement of *Polystomoidella* sp1 (H23) as unresolved, thus six taxa and three sub-lineages (A, B and C) fall in a basal polytomy (Fig. 2a). Those six taxa, which infect turtles from disparate geographic localities (USA, Africa, Asia and Australia), and from distinct families of the suborders Pleurodira and Cryptodira, are found in the urinary bladder (in red, Fig. 2a), and in the conjunctival sacs (in blue) of their host. Clade A associates exclusively bladder parasites, but from geographically distant turtles (Asia and Australia) of distinct suborders and families. Similarly, clade B links only parasites of the conjunctival sacs, but from turtles of closely related families geographically less distant (Costa Rica and USA). Finally, clade C associates both parasites from the urinary bladder and pharyngeal cavity (in green). Whereas those parasites are from geographically distant turtles (Africa, Eurasia, South America and USA), all of them are from related families of the suborder Cryptodira. Within clade C, all North American bladder parasites form a monophyletic group (clade D). Concerning phylogenetic relationships among polystomes collected from wild and captive animals (Fig. 2b), additional haplotypes fall only in sub-lineages B and C and are tightly related to haplotypes previously recognized within polystomes of wild turtles.

Molecular species delineation within polystomes

Uncorrected pairwise divergences between haplotype sequences from eggs and adult worms collected in wild and captive turtles are reported in Appendix S1 (see Appendix S1, can be viewed at http://journals.cambridge.org/par). Adults of *Neopolystoma orbiculare* (see squared haplotypes H9 and H10, in Fig. 2a) diverge from each other by no more than 0·3%. The same level of divergence is also estimated between parasite worms belonging to the species *Polystomoides oris* (H11 and H12), and between specimens of *Polystomoides* sp4 (H50 and H51). A level of 0·9% is found between two adults belonging to *Neopolystoma* sp3 (H45 and H46). Similarly, sequences from worms or polystome eggs from *M. leprosa* in allopatry (Algeria and France), show divergences ranging from 0·9% to 1·7% (H25, H26 and H30). Conversely, a polystome collected from the urinary bladder of *T. s. scripta* in Florida (H48) differs by 1·7% to 2·0% from *N. orbiculare* individuals (H9 and H10) that were both sampled from the urinary bladder of *C. p. marginata* from Indiana. Polystomes collected from the pharyngeal cavity of *T. s. elegans* and *T. s. scripta* living in allopatry (H47 and H49) also differ by 2·6%. Summarizing, the genetic divergence within polystomes collected from the same body site across turtles of the same species, even in allopatry, ranges from 0 to 1·7%, whereas it ranges from 1·7% to more than 2·0% within polystomes collected from the same body site across turtles of different species or subspecies. These results suggest that the molecular species delineation can be fixed approximately to about 1·5%–2·0% divergence level in the COI, which is in accordance with the level found in amphibian polystomes by Du Preez *et al.* (2007).

DISCUSSION

Modes of polystome diversification over geological times

From a sample of six chelonian polystome species, Littlewood *et al.* (1997) showed that congeneric polystome species infecting the same body site from different host species were more related to each other than polystome species infecting different body sites of the same host species. Using a larger sample of polystome species recovered from geographically widespread chelonian host species belonging to different suborders and families, we demonstrate that patterns of polystome diversification are more complex (Fig. 2a). Our results show unambiguously that the two genera *Neopolystoma* and *Polystomoides*, based on the number of haptoral hamuli, are polyphyletic. Second, though our molecular marker could be highly saturated for tracking the phylogenetic route of chelonian polystomes since their origin

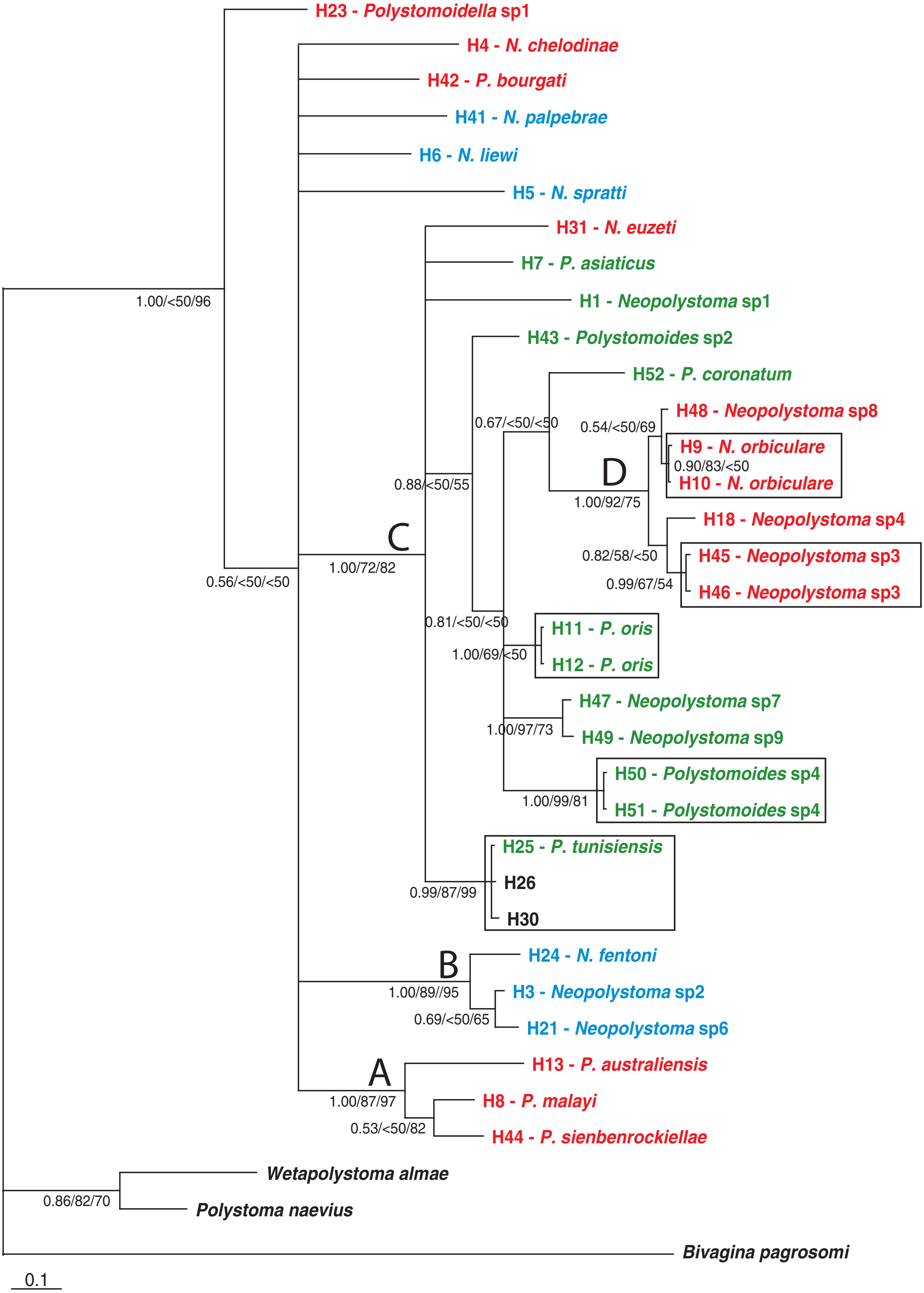
H23 - Polystomoidella sp1
H4 - N. chelodinae
H42 - P. bourgati
H41 - N. palpebrae
H6 - N. liewi
H5 - N. spratti
H31 - N. euzeti
H7 - P. asiaticus
H1 - Neopolystoma sp1
H43 - Polystomoides sp2
H52 - P. coronatum
H48 - Neopolystoma sp8
H9 - N. orbiculare
H10 - N. orbiculare
H18 - Neopolystoma sp4
H45 - Neopolystoma sp3
H46 - Neopolystoma sp3
H11 - P. oris
H12 - P. oris
H47 - Neopolystoma sp7
H49 - Neopolystoma sp9
H50 - Polystomoides sp4
H51 - Polystomoides sp4
H25 - P. tunisiensis
H26
H30
H24 - N. fentoni
H3 - Neopolystoma sp2
H21 - Neopolystoma sp6
H13 - P. australiensis
H8 - P. malayi
H44 - P. sienbenrockiellae
Wetapolystoma almae
Polystoma naevius
Bivagina pagrosomi
1.00/<50/96
0.56/<50/<50
C
1.00/72/82
0.88/<50/55
0.67/<50/<50
0.54/<50/69
D
1.00/92/75
0.90/83/<50
0.82/58/<50
0.99/67/54
0.81/<50/<50
1.00/69/<50
1.00/97/73
1.00/99/81
0.99/87/99
B
1.00/89//95
0.69/<50/65
A
1.00/87/97
0.53/<50/82
0.86/82/70
0.1

Fig. 2a.

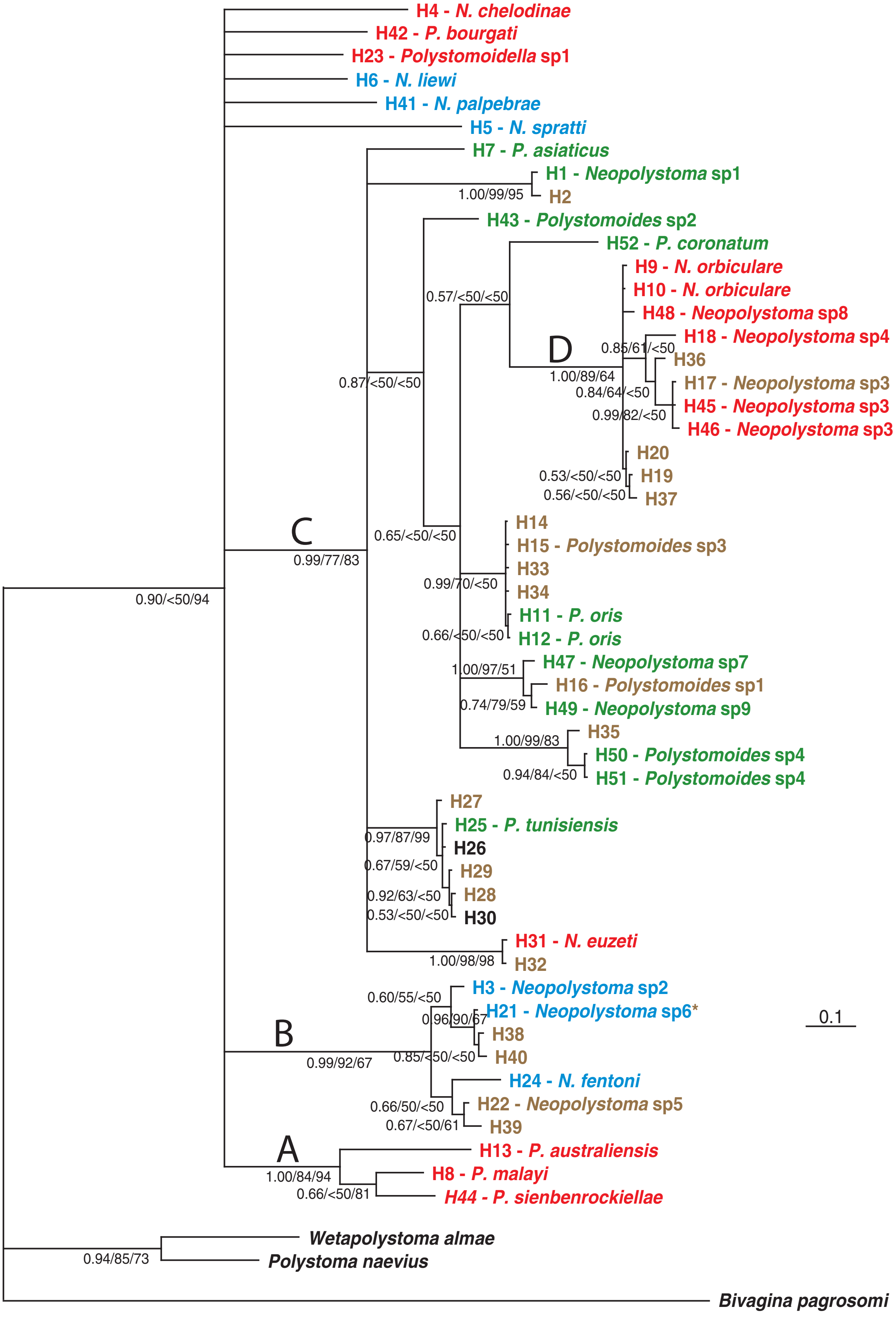
H4 - N. chelodinae
H42 - P. bourgati
H23 - Polystomoidella sp1
H6 - N. liewi
H41 - N. palpebrae
H5 - N. spratti
H7 - P. asiaticus
H1 - Neopolystoma sp1
1.00/99/95
H2
H43 - Polystomoides sp2
H52 - P. coronatum
H9 - N. orbiculare
0.57/<50/<50
H10 - N. orbiculare
H48 - Neopolystoma sp8
H18 - Neopolystoma sp4
0.85/61/<50
D
H36
0.87/<50/<50
1.00/89/64
H17 - Neopolystoma sp3
0.84/64/<50
H45 - Neopolystoma sp3
0.99/82/<50
H46 - Neopolystoma sp3
H20
0.53/<50/<50
H19
0.56/<50/<50
H37
H14
C
0.65/<50/<50
H15 - Polystomoides sp3
0.99/77/83
H33
0.99/70/<50
0.90/<50/94
H34
H11 - P. oris
0.66/<50/<50
H12 - P. oris
H47 - Neopolystoma sp7
1.00/97/51
H16 - Polystomoides sp1
0.74/79/59
H49 - Neopolystoma sp9
H35
1.00/99/83
H50 - Polystomoides sp4
0.94/84/<50
H51 - Polystomoides sp4
H27
H25 - P. tunisiensis
0.97/87/99
H26
0.67/59/<50
H29
0.92/63/<50
H28
0.53/<50/<50
H30
H31 - N. euzeti
1.00/98/98
H32
0.60/55/<50
H3 - Neopolystoma sp2
0.96/90/67
H21 - Neopolystoma sp6*
0.1
B
H38
0.85/<50/<50
0.99/92/67
H40
H24 - N. fentoni
0.66/50/<50
H22 - Neopolystoma sp5
0.67/<50/61
H39
A
H13 - P. australiensis
1.00/84/94
H8 - P. malayi
0.66/<50/81
H44 - P. sienbenrockiellae
Wetapolystoma almae
0.94/85/73
Polystoma naevius
Bivagina pagrosomi

Fig. 2b.

in the Upper Triassic (Verneau *et al*. 2002), the basal polytomy of parasites infecting either the urinary bladder or the conjunctival sacs of their host (Fig. 2a) confirms that polystomes could have originated and diverged very early. In fact, those parasites infect geographically distant turtles (Table 1) whose long isolation prevented host switching and speciation over recent geological time. The clustering of some parasites into clades that associate, respectively, polystomes of the urinary bladder (sub-lineage A, Fig. 2a) and polystomes of the conjunctival sacs (sub-lineage B, Fig. 2a) corroborates this hypothesis. Both clades, but mainly clade A, contains parasites from geographically and phylogenetically distant turtles. Third, the sub-lineage arising from the basal polytomy associates parasites infecting exclusively the urinary bladder and the pharyngeal cavity of their host (sub-lineage C, Fig. 2a). With the exception of *Neopolystoma euzeti* (H31), which is among the most basal taxa within that clade, all the remaining polystomes from the urinary bladder form a monophyletic group, suggesting a switch from the pharyngeal cavity to the urinary bladder. We can hypothesize that polystomes may have originated very early in ancestral chelonian hosts and specialized in particular body sites. During host evolution, they would have remained in their respective microhabitats and diversified following host speciation, but we demonstrate that host switching may also have occurred from one ecological niche to another, as exemplified for *N. euzeti* and the terminal clade of American bladder parasites (sub-lineage D, Fig. 2a).

Polystome species diversity in wild populations of M. leprosa *and* E. orbicularis

Polystomoides tunisiensis haplotypes (H25, H26 and H30) are reported in wild populations of *M. leprosa* in Algeria and France (Figs. 2a and 2b). Three other haplotypes (H27, H28 and H29) found among polystomes infecting captive *M. leprosa* are closely related to previous haplotypes (Fig. 2b). Genetic divergences estimated between each of these haplotypes range from 0·6% to 2·3%. In fact a single haplotype, H27, shows 2·3% divergence with H28, H29 and H30, but only 1·2% with H25 and H26. Though a threshold up to 2·0% was defined previously to delineate species using polystomes recovered from wild populations (see Results), it is obvious that H25 to H30 haplotypes belong to the same species. Therefore, *P. tunisiensis* would occur in wild populations and captive individuals of *M. leprosa*. This is also the case of *N. euzeti* (H31), which differs from H32 by only 0·6% divergence.

Haplotype H18, which infests *E. orbicularis* in the wild population of Leucate, has a genetic divergence with the most phylogenetically related haplotypes (H17, H36, H45 and H46, Fig. 2b) that range from 5·2% to 6·7%. The bladder polystome of *E. orbicularis* in Leucate (H18) can therefore be considered a new species. On the other hand, H21, which was recovered from a single individual of *E. orbicularis* in Leucate, corresponds to *Neopolystoma* sp6 (Fig. 2a) which occurs in the conjunctival sacs of *G. pseudogeographica* (Table 1). Only a single uninfected specimen of *T. s. elegans* has been recorded in the pond of Leucate in 2007, and *G. pseudogeographica* has never been found in natural environments during our field survey in Languedoc-Roussillon. This result questions the origin of that parasite from wild animals. It is very unlikely that after four years studying Leucate's population (20 different sessions of capture-mark-recapture), we missed specimens of *G. pseudogeographica*. Two explanations are possible for the presence of this exotic parasite in a wild population of *E. orbicularis*. Either *G. pseudogeographica* was introduced and disappeared from the pond after its parasite switched to *E. orbicularis*, or *G. pseudogeographica* was never there, and at least one individual of *E. orbicularis* has been introduced in that pond with an exotic parasite. Because only one turtle was found infected with an exotic parasite in Leucate, the second hypothesis seems the most plausible at the moment.

Polystome species diversity in captive turtles

Our results illustrate four cases of parasite transmission from exotic turtles to indigenous *E. orbicularis* and *M. leprosa* in the Turtle Farm. Among the four closely related haplotypes H14, H15, H33 and H34 in Fig. 2b, H14 was found from both endemic

Fig. 2. a. Bayesian polystome phylogram resulting from analysis of 35 nucleic acid sequences (only polystomes from wild turtles). Species in red are from the urinary bladder, in blue from the conjunctival sacs and in green from the pharyngeal cavity. Boxes indicate species groups that were used to determine the molecular level of polystome species delineation. Values along branches indicate, from left to right, the Bayesian posterior probabilities and the ML bootstrap proportions resulting from analysis of nucleic and amino acids sequences, respectively. Letters A, B, C and D indicate polystome sub-lineages. N refers to *Neopolystoma* and P to *Polystomoides*. b. Bayesian polystome phylogram resulting from analysis of 55 nucleic acid sequences (polystomes collected from wild and captive turtles). Species in red are from the urinary bladder, in blue from the conjunctival sacs and in green from the pharyngeal cavity. Brown indicates polystome haplotypes recorded from captive animals. Values along branches indicate, from left to right, the Bayesian posterior probabilities and the ML bootstrap proportions resulting from analysis of nucleic and amino acids sequences, respectively. Letters A, B, C and D indicate polystome sub-lineages. N refers to *Neopolystoma* and P to *Polystomoides*. * indicates haplotype that is found from captive and wild turtles.

captive species, H15 from captive *E. orbicularis, M. leprosa, T. s. elegans* (pharyngeal cavity) and *T. s. scripta* whereas H33 and H34 were recorded only from captive *M. leprosa*. Genetic divergences for these four haplotypes range from 0·3% to 0·9% when compared to haplotypes H11 and H12 (*P. oris*) recorded from the pharyngeal cavity of wild *C. p. marginata*, suggesting the transfer of an American polystome species to captive European and American turtles. Similarly, among the three related haplotypes H19, H20 and H37 in Fig. 2b, H19 was found from captive *E. orbicularis* and *M. leprosa*, H20 from captive *E. orbicularis, M. leprosa* and *T. s. elegans* whereas H37 was only recovered from captive *M. leprosa*. Genetic divergences among these three haplotypes range from 0·3% to 1·7% when compared to haplotypes H9 and H10 (*N. orbiculare*) recorded from the urinary bladder of wild *C. p. marginata*, implying the transfer of a second American polystome species to captive European and American turtles. Because H17, which is found only in captive *E. orbicularis* and *T. s. elegans*, differs by 0·3% to 1·2% to H45 and H46 (*Neopolystoma* sp3) found in wild *T. s. elegans*, this is surely a third American polystome species that has switched to captive indigenous turtles. Finally, genetic divergences within three other closely related haplotypes, H21, H38 and H40, range from 0·9% to 1·5%. H21 (*Neopolystoma* sp6) was recovered from both captive *E. orbicularis* and *M. leprosa*, but also from wild *E. orbicularis* and American *G. pseudogeographica*, H38 was recorded from captive *M. leprosa* and *T. s. elegans*, whereas H40 was only found from captive *M. leprosa*. Therefore, this suggests the transfer of *Neopolystoma* sp6 to wild *E. orbicularis* and captive indigenous European and exotic American species. Because all of those American parasites have never been described from *E. orbicularis* and *M. leprosa* in natural environments, parasitic transmission from European to American turtles is unlikely. According to these results, polystomes appear less host specific in confined conditions than in natural environments. This was also documented by MacCallum (1918) who reported *N. orbiculare* among different host species in an aquarium in New York. In summary, host switching would have occurred from host to host within the same body site, within the urinary bladder for *N. orbiculare* and *Neopolystoma* sp3, within the pharyngeal cavity for *P. oris*, and within the conjunctival sacs for *Neopolystoma* sp6. These results shed light on the evolutionary history of chelonian polystomes that would have mainly diversified within the same microhabitat, following cospeciation and host-switching events.

Even if our study comprises the most thorough revision of chelonian polystomes so far we have found some haplotypes that do not match any known polystome species: H16, only found in *E. orbicularis*; H22 found in *E. orbicularis* and *M. leprosa*; H35 recorded from *M. leprosa, T. s. elegans* and *T. s. scripta*; and, H36 plus H39 found in *M. leprosa*. While H16 might correspond to the native polystome species infecting the pharyngeal cavity of *E. orbicularis* (i.e. *Polystomoides ocellatum*) which was originally described from wild turtles by Rudolphi (1819), but not recovered in the wild population of *E. orbicularis* in Leucate, and H39 could be an undescribed polystome species of the conjunctival sacs of *M. leprosa*, H22, H35 and H36 cannot be yet ascribed to any known polystome species. Therefore the remaining three haplotypes probably reflect also undescribed species and may well represent instances of host switching.

Conclusions

Parasite transmission has been previously documented from indigenous wild populations of *M. leprosa* and *E. orbicularis* to red-eared sliders in Spain (Hidalgo-Vila *et al*. 2009). Our study is the first to illustrate multiple cases of host switching between American turtles to *E. orbicularis* and *M. leprosa* in captivity. In addition, host switching is also illustrated here between captive American turtles in the Turtle Farm. Together these results demonstrate that American polystomes exhibit low host specificity, particularly when potential host species share the same habitats in confined areas, as demonstrated at least by *P. oris, N. orbiculare, Neopolystoma* sp3 and *Neopolystoma* sp6 that infest wild American turtles in their home range and captive American and indigenous European species. Though American and indigenous turtles in the Turtle Farm are found in distinct pools being located in small areas surrounded by fences of about 60 cm height, turtles can escape occasionally and transmit their own parasites. The small size of the pools (about 20 square meters each) and the relatively high densities of turtles (about 30 turtles per pool) would increase probabilities of host-parasite encounter, which in turn may facilitate parasite transmission and host switching from American to indigenous turtles. Nothing is known about polystome pathogenicity but other parasites (e.g. viruses) may have disastrous consequences when introduced to native host turtles (Hays *et al*. 1999). Because captive turtles of both species are currently used in numerous reintroduction programmes to reinforce wild populations (Bertolero, 1999; Miquet and Cadi, 2002; Cadi and Miquet, 2004; Mosimann and Cadi, 2004; Bertolero and Oro, 2009) it is important to search for the presence of exogenous parasite species in animals used in restocking programmes. Parasite surveys of wild populations of *E. orbicularis* and *M. leprosa* throughout their natural home range are now necessary to assess the risks of pathogens transmitted by red-eared sliders and other American turtles that are widespread in natural environments and occur syntopically with the indigenous species.

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REFERENCES

Alarcos Izquierdo, G., Flechoso del Cuerto, F., Rodríguez-Pereira, A. and Lizana Avia, M. (2010). Distribution records of non-native terrapins in Castilla and León region (Central Spain). *Aquatic Invasions* **5**, 303–308. doi:10.3391/ai.2010.5.3.08

Anderson, P. K., Cunningham, A. A., Patel, N. G., Morales, F. J., Epstein, P. R. and Daszak, P. (2004). Emerging infectious diseases of plants: pathogen pollution, climate change and agrotechnology drivers. *Trends in Ecology & Evolution* **19**, 535–544. doi:10.1016/j.tree.2004.07.021

Arvy, C. and Servan, J. (1998). Imminent competition between *Trachemys scripta* and *Emys orbicularis* in France. *Mertensiella* **10**, 33–40.

Bertolero, A. (1999). Suivi de la population de cistude *Emys orbicularis* dans le delta de l'Ebre (Ne Espagne). *Chelonii* **2**, 63–68.

Bertolero, A. and Oro, D. (2009). Conservation diagnosis of reintroducing Mediterranean pond turtles: what is wrong? *Animal Conservation* **12**, 581–591. doi:10.1111/j.1469-1795.2009.00284.x

Bonin, F., Devaux, B. and Dupré, A. (1998). *Toutes les Tortues du Monde*. Delachaux et Niestlé, Lausanne. ISBN-13: 978-2603010242

Cadi, A., Delmas, V., Prévot-Julliard, A.-C., Joly, P., Pieau, C. and Girondot, M. (2004). Successful reproduction of the introduced slider turtle (*Trachemys scripta elegans*) in the South of France. *Aquatic Conservation: Marine and Freshwater Ecosystems* **14**, 237–246. doi:10.1002/aqc.607

Cadi, A. and Joly, P. (2003). Competition for basking places between the endangered European pond turtle (*Emys orbicularis galloitalica*) and the introduced red-eared slider (*Trachemys scripta elegans*). *Canadian Journal of Zoology* **81**, 1392–1398. doi:10.1139/z03-108

Cadi, A. and Joly, P. (2004). Impact of the introduction of the read-eared slider (*Trachemys scripta elegans*) on survival rates of the European pond turtle (*Emys orbicularis*). *Biodiversity and Conservation* **13**, 2511–2518. doi:10.1023/B:BIOC.0000048451.07820.9c

Cadi, A. and Miquet, A. (2004). A reintroduction programme for the European pond turtle (*Emys orbicularis*) in Lake Bourget (Savoie, France): first results after two years. *Biologia* **59**, 155–159.

Chen, T.-H. (2006). Distribution and status of the introduced red-eared slider (*Trachemys scripta elegans*) in Taiwan. In *Assessment and Control of Biological Invasion Risks* (eds. Koike, F., Clout, M. N., Kawamichi, M., De Poorter, M. and Iwatsuki, K.), pp. 187–195. Shoukadoh Book sellers, Kyoto, Japan and IUCN, Gland, Switzerland. ISBN-13: 978-4879746047

Clay, K. (2003). Parasites lost. *Nature* **421**, 585–586. doi:10.1038/421585a

Combes, C. and Ktari, M. H. (1976). *Neopolystoma euzeti* n. sp. (Monogenea, Polystomatidae) premier représentant du genre *Neopolystoma* Price, 1939 en Afrique. *Annales de Parasitologie Humaine et Comparée* **51**, 221–225.

Combes, C. and Thiery, A. (1983). Sur un *Polystomoides* (Monogenea), parasite du chélonien *Clemys caspica* au Maroc. *Bulletin de l'Institut Scientifique (Rabat)* **7**, 165–170.

Da Silva, E. and Blasco, M. (1995). *Trachemys scripta elegans* in Southwestern Spain. *Herpetological Review* **26**, 133–134.

Daszak, P., Berger, L., Cunningham, A. A., Hyatt, A. D., Green, D. E. and Speare, R. (1999). Emerging infectious diseases and amphibian population declines. *Emerging Infectious Diseases* **5**, 735–748.

Daszak, P., Cunningham, A. A. and Hyatt, A. D. (2000). Emerging infectious diseases of wildlife – Threats to biodiversity and human health. *Science* **287**, 443–449. doi:10.1126/science.287.5452.443

Du Preez, L. H. and Lim, L. H. S. (2000). *Neopolystoma liewi* sp. n. (Monogenea: Polystomatidae) from the eye of the Malayan box turtle (*Cuora amboinensis*). *Folia Parasitologica* **47**, 11–16.

Du Preez, L. H., Raharivololoniaina, L., Verneau, O. and Vences, M. (2010). A new genus of polystomatid parasitic flatworm (Monogenea: Polystomatidae) without free-swimming life stage from the Malagasy poison frogs. *Zootaxa* **2722**, 54–68.

Du Preez, L. H., Verneau, O. and Gross, D. (2007). *Polystoma floridana* n. sp. (Monogenea: Polystomatidae) a parasite in the green tree frog, *Hyla cinerea* (Schneider), of North America. *Zootaxa* **1663**, 33–45.

Eiras, J. C. (2005). An overview on the myxosporean parasites in amphibians and reptiles. *Acta Parasitologica* **50**, 267–275.

Ellstrand, N. C. and Schierenbeck, K. A. (2000). Hybridization as a stimulus for the evolution of invasiveness in plants? *Proceedings of the National Academy of Sciences, USA* **97**, 7043–7050. doi:10.1007/s10681-006-5939-3

Ficetola, G. F., Monti, A. and Padoa-Schioppa, E. (2002). First record of reproduction of *Trachemys scripta* in the Po Delta. *Annali del Museo Civico di Storia Naturale di Ferra* **5**, 125–128.

Ficetola, G. F., Thuiller, W. and Padoa-Schioppa, E. (2009). From introduction to the establishment of alien species: bioclimatic differences between presence and reproduction localities in the slider turtle. *Diversity and Distributions* **15**, 108–116. doi:10.1111/j.1472-4642.2008.00516.x

Frye, F. L., Oshiro, L. S., Dutra, F. R. and Carney, J. D. (1977). Herpesvirus-like infection in two pacific pond turtles. *Journal of the American Veterinary Medical Association* **17**, 882–884.

Gonzales, J. P. and Mishra, G. S. (1977). *Polystomoides tunisiensis* n. sp. (Monogenea, Polystomatidae) et *Telorchis temimi* n. sp. (Digenea, Telorchiidae). Deux nouvelles espèces de Trématodes de tortues paludines de Tunisie. *Archives de l'Institut Pasteur de Tunis* **54**, 29–38.

Guindon, S. and Gascuel, O. (2003). A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic Biology* **52**, 696–704. doi:10.1080/10635150390235520

Harper, P. A. W., Hammond, D. C. and Heuschele, W. P. (1982). A herpesvirus-like agent associated with a pharyngeal abscess in a desert tortoise. *Journal of Wildlife Diseases* **18**, 491–494.

Harwood, P. D. (1932). The helminths parasitic in the Amphibia and Reptilia of Houston, Texas and vicinity. *Proceedings of the United States National Museum* **81**, 1–71.

Hays, D. W., McAllister, K. R., Richardson, S. A. and Stinson, D. W. (1999). *Washington State Recovery Plan for the Western Pond Turtle.* Washington Department of Fish and Wildlife, Olympia, Washington.

Hidalgo-Vila, J., Díaz-Paniagua, C., de Frutos-Escobar, C., Jiménez-Martínez, C. and Pérez-Santigosa, N. (2007). *Salmonella* in free living terrestrial and aquatic turtles. *Veterinary Microbiology* **119**, 311–315. doi:10.1016/j.rvsc.2008.01.011

Hidalgo-Vila, J., Díaz-Paniagua, C., Pérez-Santigosa, N., de Frutos-Escobar, C. and Herrero-Herrero, A. (2008). *Salmonella* in free living exotic and native turtles in pet exotic turtles from SW Spain. *Research in Veterinary Science* **85**, 449–452. doi:10.1016/j.rvsc.2008.01.011

Hidalgo-Vila, J., Díaz-Paniagua, C., Ribas, A., Florencio, M., Pérez-Santigosa, N. and Casanova, J. C. (2009). Helminth communities of the exotic introduced turtle, *Trachemys scripta elegans* in southwestern Spain: transmission from native turtles. *Research in Veterinary Science* **86**, 463–465. doi:10.1016/j.rvsc.2008.08.003

Huelsenbeck, J. P. and Ronquist, F. (2001). MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* **17**, 754–755. doi:10.1093/bioinformatics/17.8.754

Huxel, G. R. (1999). Rapid displacement of native species by invasive species: effects of hybridization. *Biological Conservation* **89**, 143–152. doi:10.1016/S0006-3207(98)00153-0

Keane, R. M. and Crawley, M. J. (2002). Exotic plant invasions and the enemy release hypothesis. *Trends in Ecology & Evolution* **17**, 164–170. doi:10.1016/S0169-5347(02)02499-0

Kikillus, K. H., Hare, K. M. and Hartley, S. (2009). Minimizing false-negatives when predicting the potential distribution of an invasive species: a bioclimatic envelope for the red-eared slider at global and regional scales. *Animal Conservation* **13**, 5–15. doi:10.1111/j.1469-1795.2008.00299.x

Knoepffler, L.-P. and Combes, C. (1977). Présence en Corse de *Polystomoides ocellatum* (Rudolphi, 1819) chez *Emys orbicularis* (L., 1758) (Chelonia, Emydidae). Considérations sur la répartition mondiale du genre *Polystomoides*. *Vie Milieu, Série C* **27**, 221–230.

Littlewood, D. T. J., Rohde, K. and Clough, K. A. (1997). Parasite speciation within or between host species? Phylogenetic evidence from site-specific polystome monogeneans. *International Journal for Parasitology* **27**, 1289–1297. doi:10.1016/S0020-7519(97)00086-6

MacCallum, G. A. (1918). Studies on the Polystomatidae. *Zoopathologica* **1**, 103–120.

Mihalca, A. D., Gherman, C., Ghira, I. and Cozma, V. (2007). Helminth parasites of reptiles (Reptilia) in Romania. *Parasitology Research* **101**, 491–492. doi:10.1007/s00436-007-0486-y

Mihalca, A. D., Racka, K., Gherman, C. and Lonescu, D. T. (2008). Prevalence and intensity of blood apicomplexan infections in reptiles from Romania. *Parasitology Research* **102**, 1081–1083. doi:10.1007/s00436-008-0912-9

Miquet, A. and Cadi, A. (2002). Réintroduction de la cistude d'Europe en Savoie. Premier bilan (2000/2001). *Bulletin de la Société Herpétologique de France* **104**, 54–61.

Mishra, G. S. and Gonzalez, J. P. (1978). Les parasites des tortues d'eau douce en Tunisie. *Les archives de l'Institut Pasteur de Tunis* **55**, 303–326.

Mitchell, C. E. and Power, A. G. (2003). Release of invasive plants from fungal and viral pathogens. *Nature* **421**, 625–627. doi:10.1038/nature01317

Mooney, H. A. and Cleland, E. E. (2001). The evolutionary impact of invasive species. *Proceedings of the National Academy of Sciences, USA* **98** (Suppl.) S446–S451. doi:10.1073/pnas.091093398

Mosimann, D. and Cadi, A. (2004). On the occurrence and viability of the European pond turtle (*Emys orbicularis*) in Moulin-de-Vert (Geneva, Switzerland): 50 years after first introduction. *Biologia* **59**, 109–112.

Pasmans, F., De Herdt, P., Dewulf, J. and Haesebrouck, F. (2002). Pathogenesis of infections with *Salmonella enterica* subsp. *enterica* serovar Muenchen in the turtle *Trachemys scripta scripta*. *Veterinary Microbiology* **87**, 315–325. doi:10.1016/S0378-1135(02)00081-0

Perez-Santigosa, N., Diaz-Paniagua, C. and Hidalgo-Vila, J. (2008). The reproductive ecology of exotic *Trachemys scripta elegans* in an invaded area of southern Europe. *Aquatic Conservation Marine and Freshwater Ecosystems* **18**, 1302–1310. doi:10.1002/aqc.974

Platt, T. R. (2000). Helminth parasites of the western painted turtle, *Chrysemys picta belli* (Gray), including *Neopolystoma elizabethae* n. sp. (Monogenea: Polystomatidae), a parasite of the conjunctival sac. *Journal of Parasitology* **86**, 815–818. doi:10.1645/0022-3395(2000)086[0815:HPOTWP]2.0.CO;2

Polo-Cavia, N., López, P. and Martín, J. (2008). Interspecific differences in responses to predation risk may confer competitive advantages to invasive freshwater turtle species. *Ethology* **114**, 115–123. doi:10.1111/j.1439-0310.2007.01441.x

Polo-Cavia, N., López, P. and Martín, J. (2009*a*). Interspecific differences in chemosensory responses of freshwater turtles: consequences for competition between native and invasive species. *Biological Invasions* **11**, 431–440. doi:10.1007/s10530-008-9260-z

Polo-Cavia, N., López, P. and Martín, J. (2009*b*). Interspecific differences in heat exchange rates may affect competition between introduced and native freshwater turtles. *Biological Invasions* **11**, 1755–1765. doi:10.1007/s10530-008-9355-6

Polo-Cavia, N., Gonzalo, A., López, P. and Martín, J. (2010*a*). Predator recognition of native but not invasive turtle predators by naïve anuran tadpoles. *Animal Behaviour* **80**, 461–466. doi:10.1016/j.anbehav.2010.06.004

Polo-Cavia, N., López, P. and Martín, J. (2010*b*). Competitive interactions during basking between native and invasive freshwater turtle species. *Biological Invasions* **12**, 2141–2152. doi:10.1007/s10530-009-9615-0

Polo-Cavia, N., López, P. and Martín, J. (2011). Aggressive interactions during feeding between native and invasive freshwater turtles. *Biological Invasions* (in press). doi:10.1007/s10530-010-9897-2

Posada, D. and Crandall, K. A. (1998). MODELTEST: testing the model of DNA substitution. *Bioinformatics* **14**, 817–818. doi:10.1093/bioinformatics/14.9.817

Prenter, J., MacNeil, C., Dick, J. T. A. and Dunn, A. M. (2004). Roles of parasites in animal invasions. *Trends in Ecology & Evolution* **19**, 385–390. doi:10.1016/j.tree.2004.05.002

Ramsay, N. F., Ng, P. K. A., O'riordan, R. M. and Chou, L. M. (2007). The red-eared slider (*Trachemys scripta elegans*) in Asia: a review. In *Biological Invaders in Inland Waters: Profiles, Distribution, and Threat* (ed. Gherardi, F.), pp. 161–174. Springer series in Invasion Ecology. ISBN-13: 978-1402060281

Richarson, J. P. M. and Brooks, D. R. (1987). *Polystomoidella mayesi* n. sp. (Monogenea: Polystomatidae) from the urinary bladder of a Malaysian box turtle, *Cuora amboinensis*. *Canadian Journal of Zoology* **65**, 1567–1569.

Rohde, K. (1963). *Polystomoides malayi* n. sp. (Monogenea, Polystomatidae) aus der Harnblase von *Cyclemys amboinensis* in Malaya. *Zeitschrift für Parasitenkunde* **22**, 278–282.

Rohde, K. (1965). Studies on the genus *Polystomoides* Ward, 1917 (Monogenea). I. Description of 4 Malayan species, a key to the known species, and a comparison of the subcuticular layers in *Polystomoides* and some digenetic trematodes. *Zoologische Jahbuecher Abteilung fuer Systematik Oekologie und Geographie der Tiere* **92**, 345–368.

Rudolphi, C. A. (1819). *Entozoorum synopsis cui accedunt mantissa duplex et indices locupletissimi*. Berolini.

Salo, P., Korpimäki, E., Banks, P. B., Nordström, M. and Dickman, C. R. (2007). Alien predators are more dangerous than native predators to prey populations. *Proceedings of the Royal Society of London. Series B* **274**, 1237–1243. doi:10.1098/rspb.2006.0444

Segade, P., Crespo, C., Ayres, C., Cordero, A., Arias, M. C., García-Estévez, J. M. and Iglesias Blanco, R. I. (2006). *Eimeria* species from the european pond turtle, *Emys orbicularis* (Reptilia: Testudines), in Galicia (NW Spain), with description of two new species. *Journal of Parasitology* **92**, 69–72. doi:10.1645/GE-3491.1

Servan, J. and Arvy, C. (1997). Introduction de la tortue de Floride *Trachemys scripta* en France. Un nouveau compétiteur pour les espèces de tortues d'eau douce européennes. *Bulletin Français de la Pêche et de la Pisciculture* **344/345**, 173–177.

Shea, K. and Chesson, P. (2002). Community ecology theory as a framework for biological invasions. *Trends in Ecology & Evolution* **17**, 170–176. doi:10.1016/S0169-5347(02)02495-3

Stunkard, H. W. (1916). On the anatomy and relationships of some North American trematodes. *Journal of Parasitology* **3**, 21–27.

Stunkard, H. W. (1924). On some trematodes from Florida turtles. *Transactions of the American Microscopical Society* **43**, 97–117.

Swofford, D. L. (2002). PAUP*: Phylogenetic Analysis Using Parsimony (* and Other Methods), version 4.0b9 Sinauer Associates, Sunderland, Massachusetts.

Tamura, K., Dudley, J., Nei, M. and Kumar, S. (2007). *MEGA4*: Molecular evolutionary genetics analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* **24**, 1596–1599. doi:10.1093/molbev/msm092

Taraschewski, H. (2006). Hosts and parasites as aliens. *Journal of Helminthology* **80**, 99–128. doi:10.1079/JOH2006364

Telecky, T. M. (2001). United States import and export of live turtles and tortoises. *Turtle and Tortoise Newsletter. The Newsletter of Chelonian Conservationists and Biologists* **4**, 8–13.

Thompson, J. D., Higgins, D. G. and Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignments through sequence weighting, position specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22**, 4673–4680. doi:10.1093/nar/22.22.4673

Tompkins, D. M., Sainsbury, A. W., Nettleton, P., Buxton, D. and Gurnell, J. (2002). *Parapoxvirus* causes a deleterious disease in red squirrels associated with UK population declines. *Proceedings of the Royal Society of London. Series B* **269**, 529–533. doi:10.1098/rspb.2001.1897

Torchin, M. E., Lafferty, K. D., Dobson, A. P., McKenzie, V. J. and Kuris, A. M. (2003). Introduced species and their missing parasites. *Nature* **421**, 628–630. doi:10.1038/nature01346

Torchin, M. E., Lafferty, K. D. and Kuris, A. M. (2001). Release from parasites as natural enemies: increased performance of a globally introduced marine crab. *Biological Invasions* **3**, 333–345. doi:10.1023/A:1015855019360

Une, Y., Uemura, K., Nakano, Y., Kamiie, J., Ishibashi, T. and Nomura, Y. (1999). Herpesvirus infection in tortoises (*Malacochersus tornieri* and *Testudo horsfieldii*). *Veterinary Pathology* **36**, 624–627. doi:10.1354/vp.36-6-624

Valdeón, A., Crespo-Diaz, A., Egaña-Callejo, A. and Gosá, A. (2010). Update of the pond slider *Trachemys scripta* (Schoepff, 1792) records in Navarre (Northern Spain), and presentation of the Aranzadi turtle trap for its population control. *Aquatic Invasions* **5**, 297–302. doi:10.3391/ai.2010.5.3.07

Verneau, O. (2004). *HDR – Origine et évolution des monogènes Polystomatidae, parasites d'amphibiens et de chéloniens d'eau douce*. Université de Perpignan, France.

Verneau, O., Bentz, S., Sinnappah, N. D., Du Preez, L., Whittington, I. and Combes, C. (2002). A view of early vertebrate evolution inferred from the phylogeny of polystome parasites (Monogenea : Polystomatidae). *Proceedings of the Royal Society of London. Series B* **269**, 535–543. doi:10.1098/rspb.2001.1899

Verneau, O., Du Preez, L. H., Laurent, V., Raharivololoniaina, L., Glaw, F. and Vences, M. (2009). The double odyssey of Madagascan polystome flatworms leads to new insights on the origins of their amphibian hosts. *Proceedings of the Royal Society* **276**, 1575–1583. doi: 10.1098/rspb.2008.1530

Vitousek, P. M., Mooney, H. A., Lubchenco, J. and Metillo, J. M. (1997). Human domination of hearth's ecosystems. *Science* **277**, 494–499. doi:10.1126/science.277.5325.494

Wright, R. R. (1879). Contributions to American helminthology. *Proceedings of the Canadian Institute* **1**, 54–75.