The high resolution melting analysis (HRM) as a molecular tool for monitoring parasites of the wildlife

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SUMMARY

In an interconnected world, the international pet trade on wild animals is becoming increasingly important. As a consequence, non-native parasite species are introduced, which affect the health of wildlife and contribute to the loss of biodiversity. Because the investigation of parasite diversity within vulnerable host species implies the molecular identification of large samples of parasite eggs, the sequencing of DNA barcodes is time-consuming and costly. Thereby, the objectives of our study were to apply the high resolution melting (HRM) approach for species determination from pools of parasite eggs. Molecular assays were validated on flatworm parasites (polystomes) infecting the Mediterranean pond turtle *Mauremys leprosa* and the invasive red-eared slider *Trachemys scripta elegans* in French natural environments. HRM analysis results indicated that double or multiple parasitic infections could be detected from wild animal populations. They also showed that the cycle of parasite eggs production was not regular over time and may depend on several factors, among which the ecological niche and the target species. Thereby, monitoring parasites from wild endangered animals implies periodic parasitological surveys to avoid false negative diagnostics, based solely on eggs production.

Key words: HRM, 12S rRNA gene, polystome, *Mauremys leprosa*, *Trachemys scripta elegans*, endangered wildlife.

INTRODUCTION

Global biodiversity is increasingly threatened by anthropogenic activities that contribute to alter the structure and functioning of ecosystems (Vitousek *et al*. 1997; Sala *et al*. 2000). The causes of species extinction mainly involve habitat loss, land cover change, pollution, overexploitation and introduction of alien species (Groom, 2005). Regarding biological invasions, if direct competition between invaders and native species has been well documented as a source of species decline (see Simberloff, 2013), the introduction of exotic parasites, which can affect the health of wildlilfe, also contributes to the loss of biodiversity (Gozlan *et al*. 2005; Barry *et al*. 2014; Iglesias *et al*. 2015). Thereby, surveying parasite diversity for conservation practices should not be neglected (Gómez and Nichols, 2013).

Parasites, which are commonly organisms with small body size, are mostly poor in morphological discriminant characters (Besansky *et al*. 2003). As a consequence, the 'DNA barcoding' procedure (Hebert *et al*. 2003) is increasingly used for species identification (Eszterbauer *et al*. 2006; Berthier *et al*. 2014). In practice, a DNA sequence of the marker region, i.e. the DNA barcode, is obtained from unknown species and compared with sequences that are referenced in genomic databases. Because investigation of parasite biodiversity within vulnerable host species usually implies the molecular identification of large samples of parasite eggs, the sequencing of DNA barcodes can be time-consuming and costly. This has been the case when studying the parasite diversity of endangered freshwater turtle species in Southern Europe (Meyer *et al*. 2015; Héritier *et al*. 2016, submitted).

The molecular approach high resolution melting (HRM), which was introduced by Ririe *et al*. (1997) and Lay and Wittwer (1997), was originally used as a diagnostic tool for genotyping and mutation scanning in humans (Gundry *et al*. 2003; Wittwer *et al*. 2003). Variations between polymorphic sequences are detected through the melting properties of double-stranded amplified DNA (dsDNA). The melting profiles of each amplicon, which depend on the melting temperature (Tm), are monitored through the release of a saturating fluorescent dsDNA binding dye as temperature

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increases. This approach was subsequently applied in a wide range of surveys (see Tong and Giffard, 2012), such as the identification of pathogenic parasites for medical and veterinary purposes (Pangasa *et al*. 2009; Robertson *et al*. 2010; Ngui *et al*. 2012) and the prevention of zoonoses (Morick *et al*. 2009). It has to date never been used for the monitoring of parasites within native wild host populations in a framework of biological invasions.

The Mediterranean pond turtle *Mauremys leprosa* (Schweigger, 1812) is a freshwater chelonian encountered in Northern Africa and South-Western Europe (Van Dijk *et al*. 2014). Though this taxon has not yet been assessed for the IUCN red list, it has been classified as 'Vulnerable' in France (UICN France, MNHN and SHF, 2015), partly because of small and isolated endemic populations occurring solely in the province of Languedoc – Roussillon (see Palacios *et al*. 2015). In this area, this species co-occurs with the invasive red-eared slider *Trachemys scripta elegans* (Wied, 1839) that may compete for basking, territory and feeding resources (Polo-Cavia *et al*. 2009, 2010, 2011). Meyer *et al*. (2015) also showed that non-native parasite species (Platyhelminthes, Monogenea, Polystomatidae) carried by *T. s. elegans* were transmitted to *M. leprosa* in natural environments of Southern Europe. Polystome diversity was assessed from a non-invasive approach involving the collection of polystome eggs from both turtles and DNA barcoding. Because one turtle can accommodate up to three distinct polystome species, each of them being specific of distinct ecological niches within the host, i.e. the pharyngeal cavity, the bladder and the conjunctival sacs, the molecular tool HRM was applied here after for the analysis of parasite diversity from pools of eggs. Our objectives were first to obtain reference melting curves for all documented parasite species in *M. leprosa* (see Meyer *et al*. 2015) and to develop cross-DNA experiments from parasites of interest in order to illustrate cases of multiple infestations. Because *T. s. elegans* is considered as an invasive species in French natural environments, we also performed HRM analyses on polystome eggs and adults collected from this species in the field for validation *in situ*. In the context of conservation of threatened species, this approach should help stakeholders of environmental management for a more accurate development of parasitological monitoring.

MATERIALS AND METHODS

Polystome selection for melting curves references

Six of the polystome species that were documented within populations of *M. leprosa* in natural environments (Meyer *et al*. 2015) were selected to obtain reference melting curves: (i) *Polystomoides tunisiensis* Gonzales and Mishra (1977) and (ii) *Neopolystoma euzeti* Combes and Ktari (1976) were originally described from the pharyngeal cavity and the bladder of *M. leprosa*, respectively; (iii) *Polystomoides oris* (Paul, 1938) and (iv) *Neopolystoma orbiculare* (Stunkard, 1916) were originally described from the pharyngeal cavity and the bladder of the American painted turtle *Chrysemys picta marginata* (Agassiz, 1857), respectively; two undescribed species, namely (v) *Polystomoides* sp. from the pharyngeal cavity and (vi) *Neopolystoma* sp. from the bladder were reported from *T. s. elegans* and *M. leprosa* in French natural environments and considered as non-native species for the native freshwater turtle (see Meyer *et al*. 2015). All the DNAs used were from our DNA collection.

Sampling of polystome eggs and adults from wild T. s. elegans

Red-eared sliders were captured using catfish traps that were set from 2 to 3 days along the watercourse La Fosseille in Southern France (see Meyer *et al*. 2015 for GPS coordinates). Traps were baited with pork liver and firmly attached to the vegetation. They were checked every day. Captured turtles were kept in the laboratory and maintained separately in small containers equipped with UVB lights and filled partially with water heated to 26 °C. Polystome eggs were collected from four turtles following the procedure of Verneau *et al*. (2011) at two distinct periods, the first period extending from day 1 to day 7 after capture, the second period extending from day 37 to day 44 after capture. Eggs that were gathered were pooled and separated into eight samples, depending on the infected turtle and sampling period, and stored at −20 °C until molecular assays. Finally, the four specimens of *T. s. elegans* were euthanized by cardiac injection of 10% sodium pentabarbitone following French national rules for invasive species (Euthapent, Kryon Laboratories, South Africa) and dissected to collect parasites of the pharyngeal cavity and the bladder. Worms were stored at −20 °C until use.

DNA extraction

Adult worms and egg samples were crushed with the help of a pestle in 300 μL of ultra-pure water. Then, 300 μL of Lysis Buffer and 25 μL of 10 mg mL^{-1} proteinase K were added and mixtures were heated at 56 °C for 1 h. The DNAs were extracted using the DNA extraction Maxwell LEV Simply DNA Tissue Kit (Promega) following the manufacturer's instructions and eluted in 30 μL of Sigma water. Their quality was evaluated by electrophoresis migration on a 1% agarose gel and their concentration assessed by spectrophotometry at 260 nm (Nanodrop ND-1000, Thermo Fisher scientific Inc., MA, USA).

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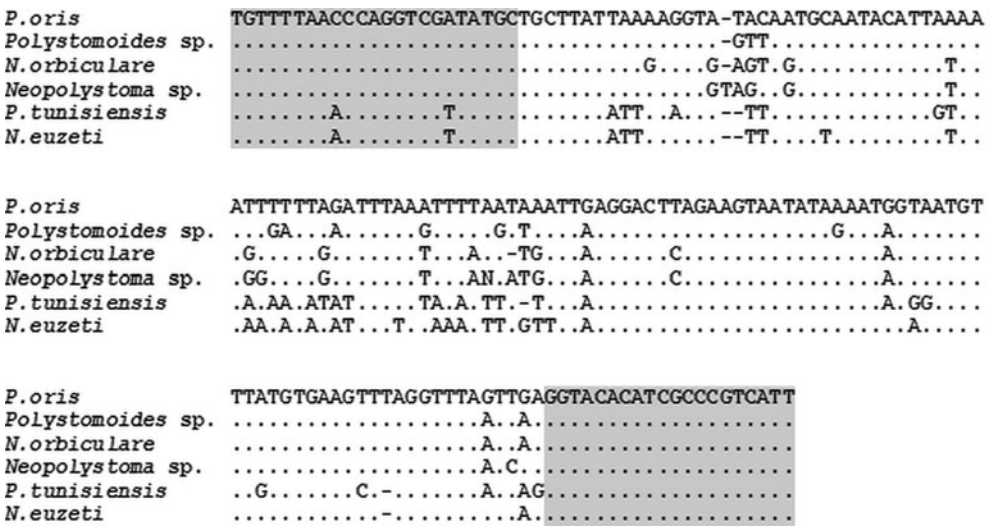

Fig. 1. Alignment 5′–3′ of 12S rRNA amplicons within polystomes. Identical nucleotides are represented by '.' and indels by '-'. Areas used for the design of primers are shaded.

Cross-DNA experiments

DNAs from species recovered from the pharyngeal cavity (*P. tunisiensis, P. oris* and *Polystomoides* sp.) were crossed by two in equal proportions with the DNAs of species recovered from the bladder (*N. euzeti, N. orbiculare* and *Neopolystoma* sp.) to simulate double infections. DNA from *Polystomoides* sp. was subsequently crossed with DNA from *N. orbiculare* in different proportions, respectively 1/10, 1/4, 1/2, 3/4 and 9/10, to simulate double infections with different parasite loads. Finally, DNAs from three polystome species, namely *Polystomoides* sp., *N. orbiculare* and *Neopolystoma* sp., were crossed to illustrate cases of multiple infestations.

Primers design and genetic divergences

Partial 12S rRNA sequences of *Polystomoides* sp. (KY0G2405) and *Neopolystoma* sp. (KY0J2406) were obtained following the procedure described in Héritier *et al.* (2015). Complete alignment was performed using MEGA6 (Tamura *et al.* 2013) with the two former sequences and the four others extracted from Genbank, namely *P. tunisiensis* (KR856116), *P. oris* (KR856115), *N. euzeti* (KR856101) and *N. orbiculare* (KR856103). The HRM primers were designed from the two most conserved regions flanking a variable portion of the 12S rRNA gene (Fig. 1), using Primer Express v2·0 software (Applied Biosystems). The resulting primers, 12SF (5′-TGTTTTAACCCAGGTCGATATGC-3′) and 12SR (5′-AATGACGGGCGATGTGTACC-3′) allowed the amplification of fragments of about 121 bp, depending on the species. Genetic divergences between each pair of sequences (Indels, transversions and transitions) were estimated with MEGA6 and reported in Table 1.

qPCR-HRM assays

Real time polymerase chain reaction (PCR) were run in a 384-well plate on a LightCycler® 480 Instrument (Roche), in a final volume of 10 μL with $MgCl_2$ at 3 mM, each primer at 0·5 μM, 1X Master Mix Resolight® dye and 15 ng of genomic DNA. Amplification consisted of 10 min of pre-incubation at 95 °C followed by 40 cycles consisting in denaturation at 95 °C for 15 s (ramping rate, 4·4 °C s^{-1}), touchdown annealing from 65 to 53 °C for 15 s (ramping rate, 2·2 °C s^{-1}) and extension at 72 °C for 20 s (ramping rate, 4·4 °C s^{-1}). After the amplification, the HRM conditions consisted of a denaturing step at 95 °C for 1 min followed by a hybridization step at 40 °C for 1 min. Melting curves were then generated by ramping from 65 to 95 °C at 0·02 °C s^{-1}, taking 25 acquisitions per each degree centigrade. A negative 'no-template' control was included in the qPCR-HRM assay to detect potential DNA contamination. Reactions were conducted in duplicate and experiments were repeated twice for validation of the results. HRM products obtained for reference species were subsequently sequenced using both HRM primers by the company Genoscreen (Lille, France) to confirm species identity.

Data analyses and interpretation

Data were analysed using the HRM analysis tools implemented in the LightCycler® 480 Software v.1.5.0 (Roche, USA). Only samples successfully amplified with a Ct lower than 30 were considered for further analysis. Curve shapes differences between samples were discriminated with the Gene Scanning module of the LightCycler 480 v.1.5.0 Software (Roche, USA). Melting curve data were adjusted manually and normalized fluorescence conditions were established and adjusted, with the threshold

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Table 1. Number of genetic changes between 12S rRNA amplicons for six polystome species.

	P. oris	*Polystomoides* sp.	*N. orbiculare*	*Neopolystoma* sp.	*P. tunisiensis*
Polystomoides sp.	Indels = 0 Tv = 5; Ts = 9				
N. orbiculare	Indels = 1 Tv = 6; Ts = 12	Indels = 1 Tv = 7; Ts = 10			
Neopolystoma sp.	Indels = 1 Tv = 8; Ts = 10	Indels = 1 Tv = 9; Ts = 12	Indels = 2 Tv = 1; Ts = 4		
P. tunisiensis	Indels = 3 Tv = 20; Ts = 12	Indels = 2 Tv = 17; Ts = 8	Indels = 4 Tv = 18; Ts = 12	Indels = 4 Tv = 19; Ts = 14	
N. euzeti	Indels = 2 Tv = 21; Ts = 6	Indels = 2 Tv = 21; Ts = 5	Indels = 3 Tv = 22; Ts = 10	Indels = 3 Tv = 21; Ts = 13	Indels = 1 Tv = 7; Ts = 10

Indels = Insertions/Deletions; Tv = Tranversions; Ts = Transitions.

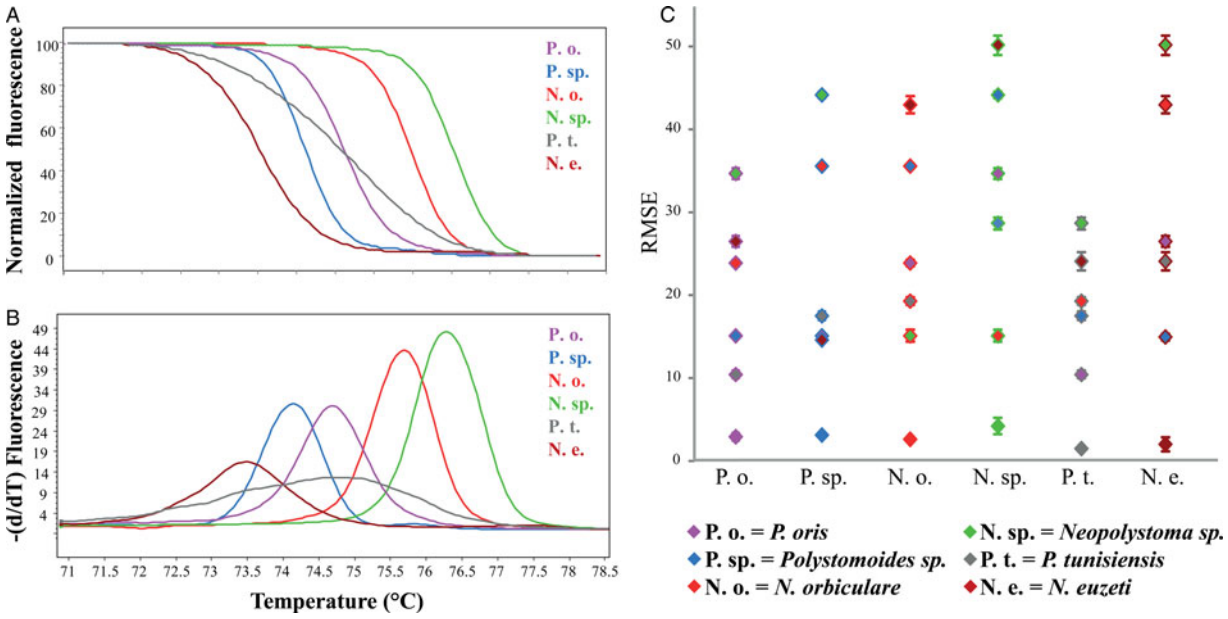

Fig. 2. (A) Normalized melting curves for the six polystome species. (B) Melting peaks derived from normalized melting curves shown in A. (C) RMSE values calculated from normalized fluorescence data of intraspecific and interspecific inter-runs duplicata.

set up at 0 and the sensitivity at 0·26. Two melting peaks differing by a Tm > 0·5 °C were considered as different. Similarities or differences in the melting curves and reproducibility of HRM results were assessed by comparing the root-mean square error (RMSE) values. RMSE values were calculated using the normalized fluorescence data from comparisons of intraspecific and interspecific inter-runs duplicata, as previously recommended by Naue *et al.* (2014).

RESULTS

Genetic divergences within polystomes and melting curve references

The number of genetic changes between 12S rRNA amplicons extended from 7 up to 37 (Table 1). The development of the HRM assay associated with this molecular marker allowed the discrimination of the six polystome species recovered from *M. leprosa*, Tm temperatures ranging from 73·5 to 76·5 °C (Fig. 2A and B). Regardless of the species investigated, replicate reactions and experiments gave Ct values within each species that never varied more than a factor of 0·5 cycle (results not shown). The highest Ct values were observed for the two native species, i.e. *P. tunisiensis* and *N. euzeti*, probably because of the occurrence of two nucleotide mutations within the forward primer. Overall these results indicated good amplification efficiencies for all species. To the exception of *P. tunisiensis*, whose shape of the melting curve may reflect the presence of several melting domains (not shown), the melting curves of all the other species had a

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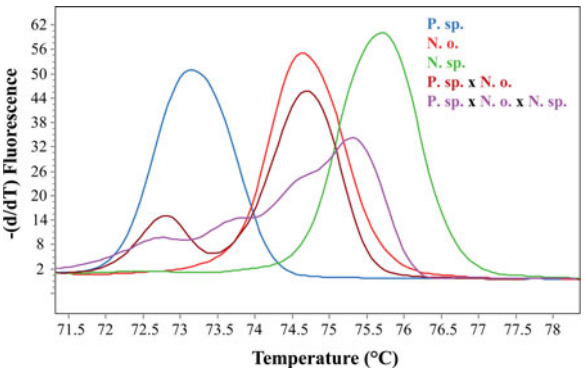

Fig. 3. Melting peaks for the reference species (*Polystomoides* sp., *N. orbiculare* and *Neopolystoma* sp.), for a mixture of two DNAs (*Polystomoides* sp. × *N. orbiculare*) and for a mixture of three DNAs (*Polystomoides* sp. × *N. orbiculare* × *Neopolystoma* sp.).

shape indicating the presence of a single melting domain. RMSE values inferred from intraspecific inter-runs were compared with RMSE values inferred from interspecific inter-runs and presented graphically (Fig. 2C). These values showed significant differences between melting curves of each species.

Cross-DNA experiments

When DNAs from the exotic species of the pharyngeal cavity were crossed by two, in equal proportions, with DNAs from the exotic species of the bladder, two melting peaks were revealed, indicating the presence of the two distinct DNAs (see Fig. 3). A similar result was obtained when crossing by two the DNAs from the two native species, i.e. *P. tunisiensis* of the pharyngeal cavity and *N. euzeti* of the bladder. On the opposite, when DNAs from the exotic species of the pharyngeal cavity were crossed with DNA from *N. euzeti* on the one hand, and when DNAs from the exotic species of the bladder were crossed with DNA from *P. tunisiensis* on the other, a single peak characterizing the exotic species was systematically revealed for each crossing experiment. Overall these results indicated less efficient amplification levels in both native species. Furthermore when DNA of *Polystomoides* sp. was crossed with that of *N. orbiculare* in the proportions 1/4, 1/2 and 3/4, two peaks were revealed whose fluorescence intensity corresponded to the relative amounts of each DNA used in the experiment. However, when DNAs of the same species were crossed together in the proportions 1/10 and 9/10, respectively, one single peak was revealed corresponding to the DNA present in the highest amount. Finally, when DNAs from three distinct species were crossed in the same proportions, several peaks were revealed indicating the presence of several DNAs (Fig. 3).

Table 2. Polystome eggs and worms collected from distinct specimens of *T. s. elegans* (Tse 1 to Tse 4). Eggs were sampled at two distinct periods of time and worms were subsequently recovered following turtles dissection

	Number of eggs		Number of worms	
	First sampling	Second sampling	Pharyngeal cavity	Bladder
Tse 1	32	50	5	3
Tse 2	50	50	9	6
Tse 3	48	50	5	5
Tse 4	3	30	9	9

Parasite sampling from wild T. s. elegans *and HRM assay on polystome eggs*

The number of polystome eggs collected from wild specimens of the red-eared slider during the two sampling periods and the number of parasite worms recovered after inspection of the bladder and pharyngeal cavity, are summarized in Table 2. For all the turtles examined, specimens of two polystome species were gathered and identified from DNA barcoding, namely *N. orbiculare* from the bladder and *Polystomoides* sp. from the pharyngeal cavity, which is in agreement with Meyer *et al.* (2015). HRM analyses showed contrasted results regarding surveyed turtles and polystome eggs samples. On *T. s. elegans* number 1 (Tse 1), regardless of the sampling period, one single species was revealed, i.e. *Polystomoides* sp. On Tse 2, the two species were revealed, however the fluorescence intensity was higher for *N. orbiculare* than *Polystomoides* sp. during the first sampling period. Inversely, it was lower for *N. orbiculare* than *Polystomoides* sp. during the second sampling period. On Tse 3, although the two species were revealed during the first sampling period, the fluorescence intensity was higher for *N. orbiculare* than *Polystomoides* sp. During the second sampling period, one single species was revealed, i.e. *Polystomoides* sp. On Tse 4, *N. orbiculare* was the single species revealed during the first sampling period, while *Polystomoides* sp. was the single species detected during the second sampling period (Table 3).

DISCUSSION

Experimental HRM assays for identification of parasites from endangered wildlife

The first steps in assessing global biodiversity embrace specimens' collection and species recognition. Whereas the sampling of free living organisms can be an increasingly difficult task regarding the conservation status of some species, surveying endoparasites

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Table 3. Parasite species detected by HRM analysis on polystome eggs collected from Tse 1 to Tse 4 during first and second sampling periods

	First sampling	Second sampling
Tse 1	*Polystomoides* sp.	*Polystomoides* sp.
Tse 2	*Polystomoides* sp. + *N. orbiculare*[a]	*Polystomoides* sp.[a] + *N. orbiculare*
Tse 3	*Polystomoides* sp. + *N. orbiculare*[a]	*Polystomoides* sp.
Tse 4	*N. orbiculare*	*Polystomoides* sp.

[a] Indicates the species for which the melting peak had the highest fluorescence intensity.

without host sacrifice can be even more difficult, or impossible. Therefore, the best way to assess internal parasites of threatened species involves parasite eggs detection. However, if the description of parasite species from the study of adult worms can be a difficult challenge regarding the paucity of good apomorphic morphological characters, species identification from the study of eggs morphology has never been completed. Consequently molecular techniques are still the best suitable approaches to carry out taxonomic research in parasitology. Nonetheless, analyzing a great number of parasite eggs using DNA barcodes, which entails the sequencing of large DNA samples, can be time-consuming and costly. For this reason, the HRM molecular method was applied for the study of parasite diversity from pools of eggs. This approach relies on the determination of reference melting curves that derive from target sequences. Those ones must be sufficiently variable to get species-specific Tm temperatures, but rather conserved for the design of amplification primers. The selected gene along this study, namely the mitochondrial 12S rRNA gene, was well appropriate for species recognition, since it allowed obtaining species-specific Tm temperatures for all six investigated polystome species.

Because co-infections of wildlife are the rule (Bordes and Morand, 2011), eggs usually reflect the production of several distinct parasite species. We therefore performed cross-DNA experiments in order to simulate double infections within hosts. When DNA from one exotic polystome species was crossed in equal proportion with DNA of another exotic species, the two DNAs were systematically revealed demonstrating the usefulness of the HRM tool for the detection of double infections. If a similar outcome was obtained when crossing the DNAs of both native parasite species, inversely, a single DNA was revealed when crossing the DNAs of native with exotic polystome species, suggesting the presence of a single parasite species, i.e. the exotic species. Because the consensus sequence used for the design of the forward HRM primer differed from the nucleotide sequence of the two native target parasite species by two mutations (Fig. 1), it could explain the decrease of the amplification efficiency of both species. This explanation holds as Ct values of the exotic species were lower than Ct values of the native ones. Similarly, when DNA from one exotic polystome species was crossed with DNA of another exotic species at a different concentration, the two DNAs were not systematically revealed. These results would indicate that double infections can be detected from HRM analysis only if parasite intensities of the less abundant species represent at least 1/4 of the total number of parasites. Finally, the HRM pattern resulting from the crossing of three distinct DNAs would indicate that multiple infections could be also detected, though the number of parasite species infecting host is difficult to evaluate. Overall, if the HRM molecular tool can be usefulness for characterizing double or multiple parasitic infections within host, some limitations were highlighted. HRM primers, first of all, must be 100% identical to the target gene sequence of all species if one may evidence all the species involved in the infection. Secondly, if one species is less abundant than another one, it may not be detected from an HRM analysis conducted on parasite eggs released by hosts. In conclusion, this approach may be well appropriate for preliminary studies based on parasite diversity among endangered wildlife as it gives a global overview of the parasite richness.

HRM assays for parasite identification in wild environments

Regarding the limitations of the HRM molecular approach discussed above, we explored the polystome diversity of the read-eared slider from French natural environments. This study was performed on pools of polystome eggs that were collected from four specimens of turtles co-infected with *Polystomoides* sp. and *N. orbiculare*. Though Tse 1 was infected with the two polystome species in the proportions of about 2/3 of *Polystomoides* sp. and 1/3 of *N. orbiculare*, only *Polystomoides* sp. was detected from HRM analysis, regardless of the sampling period. On the opposite, both polystome species were detected from Tse 2 during sampling periods 1 and 2, whereas it was also infected by the two parasite species in the same proportions as those reported for Tse 1. Tse 3 and Tse 4, which were infected by the two parasite species in

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the same proportions, i.e. 1/2 of *Polystomoides* sp. and 1/2 of *N. orbiculare*, also showed contrasted results. If the two polystome species were detected in Tse 3 during the first sampling period, a single polystome species, i.e. *Polystomoides* sp., was detected during the second sampling period. Within Tse 4, two polystome species were also detected, however only *N. orbiculare* was identified during the first sampling period, and only *Polystomoides* sp. was identified during the second sampling period. Overall, these results were unpredicted regarding infection levels of both parasite species (see Table 2). We expected to reveal the presence of both species within each turtle regardless of the sampling period, which was actually not the case. In fact, the most predominant parasite species that was revealed during the first sampling period was *N. orbiculare*, while it was *Polystomoides* sp. during the second sampling period. This suggests that the cycle of eggs production is not regular over time and may depend on several factors, among which the target parasite species and the ecological niche where parasites reproduce sexually. Badets *et al.* (2010) showed from field studies that eggs production of polystomes infecting the stripeless tree frog, *Hyla meridionalis* Boettger (1874), was intimately related to the frogs' ecology and physiology. Badets (unpublished data) also showed from the same host–parasite interaction that the patterns of polystome eggs releases were different regarding the preference of parasites towards their hosts, male or female, suggesting that host physiology was indeed an important factor influencing parasite eggs production. Thereby, monitoring parasites from wild endangered animal populations, but also from captive specimens used in breeding programs, must imply periodic parasitological surveys to avoid false negative diagnostics based solely on eggs production. This is also very important in a veterinary framework, especially because the international pet trade on wild animals can lead to the introduction of non-native parasite species and to the emergence of new infectious diseases.

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