Development of a qPCR Duplex Assay for simultaneous detection of Fascioloides magna and Galba truncatula in eDNA samples: Monitoring beyond boundaries

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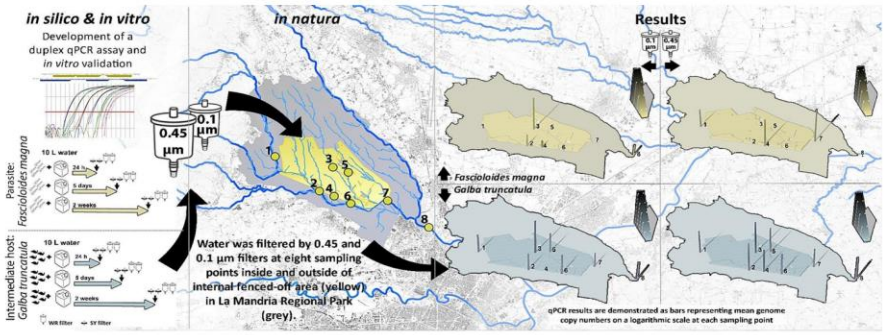
Abstract

Parasites constitute a significant economic burden and highly impact environmental, public, and animal health. The emergence of many parasitic diseases is environmentally mediated and they share the same biogeography with humans and both domestic and wild animals. American liver fluke, Fascioloides magna – a trematode parasite of domestic and wild ungulates – is an example of the anthropogenic introduction of an "invasive alien species" in Italy and Europe. Multiple introductions to Europe have led to the biogeographical expansion of the parasite across the Danube region mainly provided by the presence of suitable habitats for all hosts involved in the parasite's life cycle, human-assisted transport, and drastic environmental events such as flooding. In Italy, it was introduced and established in La Mandria Regional Park (LMRP) near Turin in 1865 along with imported wapitis (Cervus elaphus canadensis) from North America (Bassi, 1875), but with no reported expansion to the surrounding areas. LMRP isolated F. magna focus, poses an important threat of possible expansion since the enclosed area is vulnerable to occasional bidirectional passage of roe deer. Additionally, tributary rivers to the Po river system, traversing the enclosed area, could further bolster the possibility of such spread. In this study, we developed a duplex qPCR assay for F. magna and its principal intermediate host Galba truncatula optimized for testing eDNA samples to meet the needs for surveillance of the parasite. Moreover, we validated the developed assay in natura by testing samples derived from filtered water and sediments collected inside and outside LMRP's fenced-off area. Our findings for the first time demonstrate the presence of F. magna's eDNA outside the park's internal fenced-off area.



in vitro validation

Duplex qPCR assay design:

Table 1. Primers and Probes sequence.

Assay	Primer	Amplicon length	Sequence (5'-3')
F. magna	Forward	69bps	TTTATCGTCGGTTTGATGCTA
	Reverse		GAAGGATACCGTCTTTAAACAAC
	Probe		FAM – GGCTTGGTCATGTATCTGATGC – BHQ1
G. truncatula	Forward	82bps	ACTTTATTTATTATCGTGGCGTTC
	Reverse		CGTCTTAGAGCCCCTTGT
	Probe		CY5 – TCCATGGCATCGCAGCTC – BHQ3

Specificity of the *F. magna* assay was assessed by testing it with closely related or co-occurring parasites' DNA samples (Fig 1.). Sensitivity of designed assays were assessed before conducting water tank experiments (Fig 2.). Water tank experiments were conducted by sampling with four different filters at three different time points. (Fig 3.)

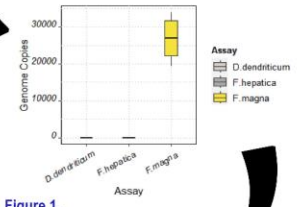
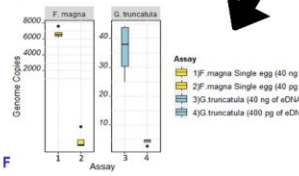


Figure 1.



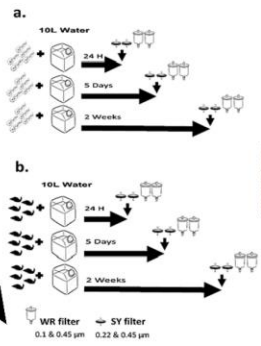
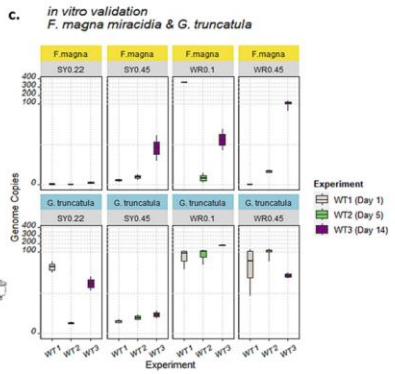


Figure 3.



in natura validation

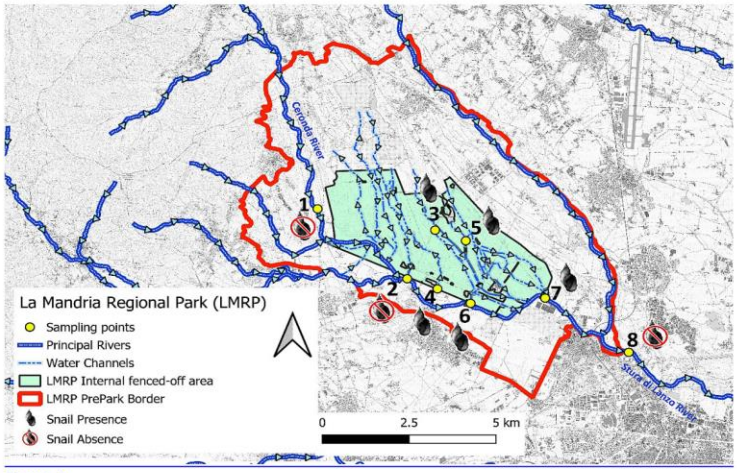


Figure 4.

Based on the results obtained from water tank experiments (Fig. 3), in natura filtration of water was carried out at water/sediment surface using WR filters with different pore-sizes (0.1 and 0.45 µm) and a prefilter with a 300 µm mesh plate. Eight sampling points inside and outside the LMRP's area were identified depending on the noticeable presence or absence of DL snails in the proximate surrounding area (Fig. 4). Sampling points were designated from 1 to 8 with point 1 being in the upstream and point 8 in the downstream of the study area. Moreover, sampling points 1, 7 and 8 were located outside the internal fenced-off area.

G. truncatula's eDNA was detected in all sampling points, irrespective of its visual presence/absence. Detected genome copy numbers for each assay are represented separately for WR 0.01 and WR 0.45 filters in Figs. 5(a) and 6(a) respectively. Figures b and c in Figs. 5 and 6 demonstrate mean genome copy number results from WR filters on logarithmic scale and exact coordination (dark color on the density maps highlights internal fenced-off area).

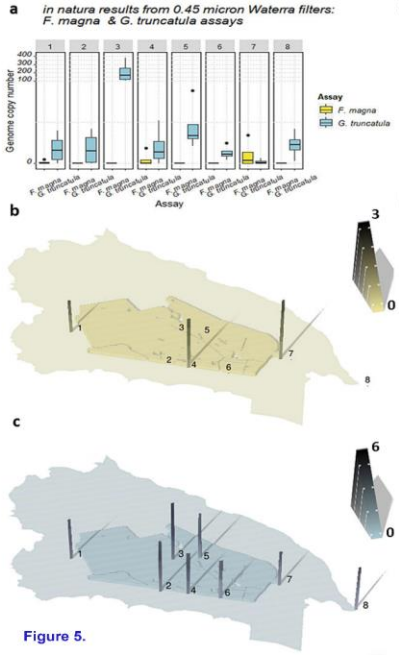


Figure 5.

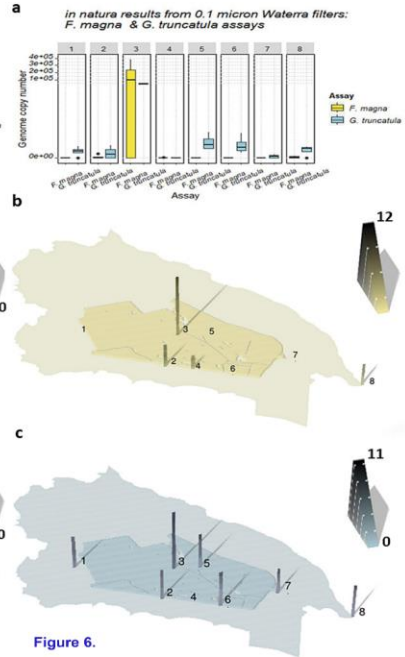


Figure 6.

Conclusion:

The developed duplex qPCR assay demonstrated high sensitivity and specificity towards both target organisms of interest, making it highly suitable for analyzing environmental DNA samples. When combined with sampling methods adapted for collecting samples from large volumes of water of varying levels of turbidity, this assay becomes a valuable tool facilitating environmental surveillance of F. magna simultaneously with G. truncatula in areas without prior information. Detection of F. magna in eDNA samples collected outside the LMRP's internal fenced-off area asserts applicability of this sampling method and assay for disease hotspot identification beyond the boundaries of LMRP, encompassing other riverine systems like the Po River.

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