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Supplementary material

Improving reliability in environmental DNA detection surveys through enhanced quality control

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Assay multiplex

To ensure the specificity of the *Cyprinus carpio* assay was maintained, we again tested for amplification of DNA in multiple fish species when used in combination with the endogenous control assay.

To test PCR amplification efficiency of the assay, calibration curves were generated from a series of five 10-fold dilutions of *C. carpio* DNA and run in triplicate. DNA was amplified using 20 µL qPCR reactions as detailed in the main text.

To ensure the endogenous control assay did not interfere with the efficiency of the species-specific assay, *C. carpio* DNA dilutions were amplified in a multiplex with the species-specific assay and the endogenous control assay. DNA was amplified using qPCR in 20-µL reactions consisting of 10 µL of TaqMan Environmental Master Mix 2.0 (Applied Biosystems), 1× qPCR species-specific assay mix, 0.75× qPCR endogenous control assay mix, 2 µL of DNA and made up to 20 µL with DNase/RNase free water (Bioline, Taunton, MA, USA). Note that the endogenous control assay is used in limiting amounts to avoid compromising the efficiency of the species-specific assay. Results were compared with efficiencies achieved when the species-specific assay was used alone.

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Table S1. Amplification results for freshwater fishes tested with the 16S rRNA generic fish endogenous control assay

Species	Common name	Native or introduced	Amplification
Gambusia holbrooki	Eastern mosquitofish	Introduced	Yes
Misgurnus anguillicaudatus	Oriental weatherloach	Introduced	Yes
Cyprinus carpio	European carp	Introduced	Yes
Perca fluvatis	Redfin perch	Introduced	Yes
Tinca tinca	Tench	Introduced	Yes
Carassius auratus	Goldfish	Introduced	Yes
Salmo trutta	Brown trout	Introduced	Yes
Rutilus rutilus	Roach	Introduced	Yes
Oreochromis mossambicus	Mozambique tilapia	Introduced	Yes
Archocentrus nigrofasciatus	Convict cichlid	Introduced	Yes
Pelmatolapia mariae	Spotted tilapia	Introduced	Yes
Geophagus brasiliensis	Pearl cichlid	Introduced	Yes
Macquaria australasica	Macquaire perch	Native	Yes
Gadopsis bispinosus	Two-spined blackfish	Native	Yes
Maccullochella peelii	Murray cod	Native	Yes
Nematalosa erebi	Bony bream	Native	Yes
Melanotaenia fluviatilis	Murray–Darling rainbow fish	Native	Yes
Melanotaenia splendida	Eastern rainbow fish	Native	Yes
Leiopotherapon unicolor	Spangled perch	Native	Yes
Bidyanus bidyanus	Silver perch	Native	Yes
Tandanus tandanas	Freshwater catfish	Native	Yes
Neosilurus hyrtlii	Hyrtyl's catfish	Native	Yes
Porochilus rendahli	Rendahl's catfish	Native	Yes
Ambassis agassizii	Olive cerchlet	Native	Yes
Pseudogobius olorum	Bluespot goby	Native	Yes
Afurcagobius tamarensis	Tamar goby	Native	Yes
Nannoperca australis	Southern pygmy perch	Native	Yes
Pseudaphritis urvillii	Tupong	Native	Yes
Atherinosoma microstoma	Small-mouth hardyhead	Native	Yes
Retropinna semoni	Australian smelt	Native	Yes
Galaxius auratus	Golden galaxias	Native	Yes
Hypseleotris klunzingeri	Western carp gudgeon	Native	Yes
Hypseleotris sp.	Midgley's carp gudgeon	Native	Yes
Aldrichetta forsteri	Yellow-eye mullet	Native	Yes[A]
Mugil cephalus	Flathead grey mullet	Native	Yes[A]
Mogurnda adspersa	Southern purple spotted gudgeon	Native	No[B]
Philypnodon grandiceps	Flathead gudgeon	Native	No[C]

[A]Known sequence mismatch – SNP in reverse primer region.

[B]Known sequence mismatch – SNP (insertion) in reverse primer region.

[C]Known sequence mismatch – SNP in probe region.

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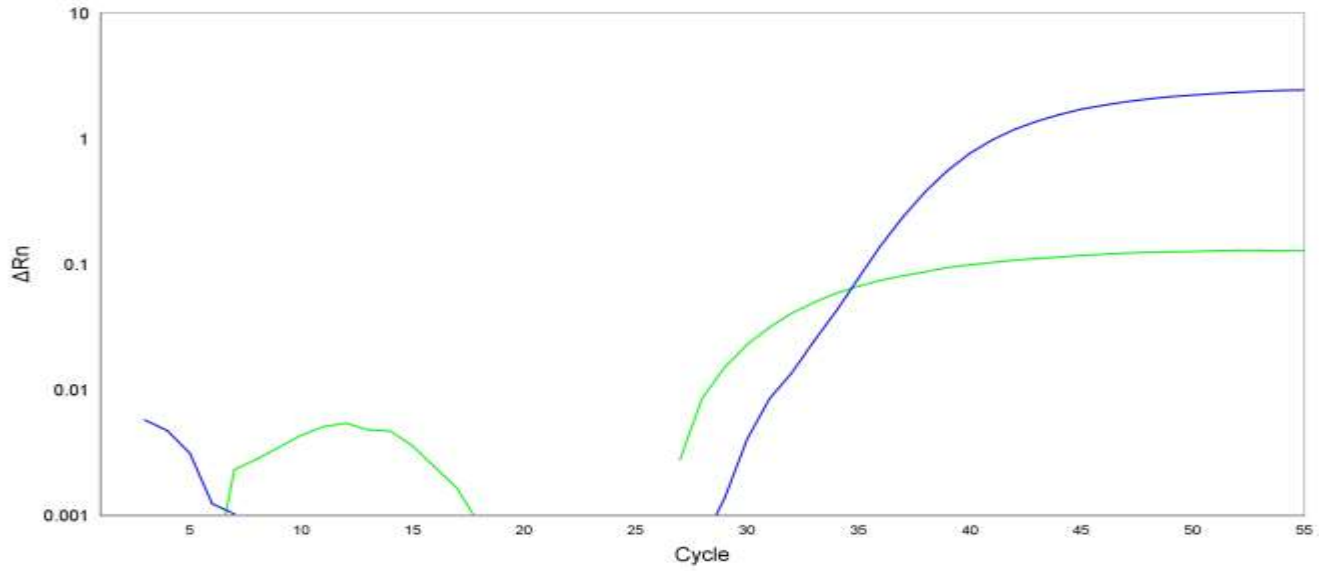

Fig. S1. Amplification plot for an environmental DNA sample showing multiplexed amplification of FAM dye-labelled species-specific assay (blue) and VIC dye-labelled endogenous control assay (green). The fluorescent dyes allow for clear differentiation of amplification curves to indicate the presence of species-specific DNA and generic, endogenous DNA in a sample.