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Development of novel real-time TaqMan® PCR assays for the species and sex identification of otter (*Lutra lutra*) and their application to noninvasive genetic monitoring

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Abstract

Developing strategies to maintain biodiversity requires baseline information on the current status of each individual species. The development of genetic techniques and their application to noninvasively collected samples have the potential to yield information on the structure of elusive animal populations and so are important tools in conservation management. Using DNA isolated from faecal samples can be challenging owing to low quantity and quality. This study, however, presents the development of novel real-time polymerase chain reaction assays using fluorescently labelled TaqMan® MGB probes enabling species and sex identification of Eurasian otter (*Lutra lutra*) spraints (faeces). These assays can also be used in determining an optimum microsatellite panel and can be employed as cost-saving screening tools for downstream genetic testing including microsatellite genotyping and haplotype analysis. The techniques are shown to work efficiently with *L. lutra* DNA isolated from tissue, hair, spraint, blood and anal jelly samples.

Keywords: *Lutra*, noninvasive, otter, real-time PCR, spraint

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Introduction

The detection of some mammals such as the Eurasian otter (*Lutra lutra*) can be challenging due to low densities or elusive behaviour with traditional capture–mark–recapture approaches causing practical and ethical difficulties (Oliveira *et al.* 2010) . A cost-effective approach to assess species presence/absence is to rely on faecal surveys carried out by conservation groups and the increasingly valuable citizen scientists. DNA-based assays for noninvasive genetic sampling (NGS) have become reliable tools for species identification, molecular sex-typing and individual identification by microsatellite genotyping using spraint DNA (Hansen & Jacobsen 1999; Dallas *et al.* 2000; Prigioni *et al.* 2006; Mucci & Randi 2007; Lanszki *et al.* 2008; Hájková *et al.* 2009).

Application of the real-time (quantitative) polymerase chain reaction (qPCR) to NGS is a well-established technique (Moran *et al.* 2008; O'Reilly *et al.* 2008; Mullins *et al.* 2010; O'Meara *et al.* 2012). TaqMan® probe qPCR assays amplify small (50–150 bp) products and are precise and sensitive to low template DNA concentrations. This is particularly applicable to otter (*Lontra* and *Lutra* spp.) spraints where DNA analyses remain challenging–low success rates are thought to be due to a faster rate of DNA degradation than that seen with other carnivore species (Dallas *et al.* 2003; Hájková *et al.* 2009; Mowry *et al.* 2011). The qPCR approach allows high sample throughput, multiplex PCR capabilities and DNA quantitation while post-PCR processing is not required. The qPCR approach enables a greater degree of stringency in DNA quantitation compared to alternative methods such as UV absorbance or fluorometry that cannot differentiate between host and prey DNA in a faecal sample.

PCR amplification of mitochondrial DNA (mtDNA) is a common approach to species identification (O'Reilly *et al.* 2008). The DNA sequence of the mtDNA control region (CR) demonstrates high homology within a species but variability between species makes it ideal for a

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DNA-based species identification test. Primers and probes for qPCR assays are constructed to incorporate these base pair differences between species to achieve assay specificity. A species identification assay is often used in conjunction with the amplification of the nuclear DNA (nDNA) X–Y chromosome homologous zinc finger (ZF) genes for sex-typing samples where primers and probes are designed to amplify chromosome-specific regions of each homologue (Aasen & Medrano 1990; Shaw *et al.* 2003; Mullins *et al.* 2010).

In this study, the design of two novel TaqMan® MGB probe qPCR assays for the species and sex identification of *L. lutra* spraints is presented. The practical specificity of the species assay was confirmed against noninvasively collected samples from a range of sympatric species. Both assays have been applied to NGS projects in Ireland and the UK. Due to the quantitative and qualitative nature of qPCR, the ability to use these qPCR assays as cost-saving tools to screen and identify samples suitable for downstream genetic analyses is also discussed.

Materials and methods

Sample collection

Muscle tissue samples (Ireland: male: n = 5, female: n = 3; Wales: male: n = 10: female: n = 11; England: male: n = 10: female: n = 9) and hair samples (Cork, Ireland: male: n = 2, female: n = 7) were obtained from road kill animals and those captured during GPS tracking studies. Faecal samples were collected during volunteer-based surveys in Ireland (Meath, Dublin, Kildare, Kilkenny, Waterford, Cork, Kerry: n = 854), North and West Wales (Conwy Valley, Anglesey, Snowdonia, Ceredigion: n = 733) and East Midlands, England (Derbyshire: n = 25). Tissue samples were stored in 95% ethanol and spraints and hair were collected in sealable plastic bags. All samples were stored at −20 °C postcollection and upon arrival in the laboratory.

DNA isolation

DNA was isolated from *L. lutra* tissue and hair using the ZR Genomic DNA™–Tissue MicroPrep (Zymo Research) according to the Solid Tissue and Hair protocols, respectively, with Zymo-Spin™ II columns. To isolate DNA from spraints or anal jelly, approximately 0.2 g of spraint/jelly was transferred to 1 mL of Stool Transport And Recovery (STAR) Buffer (Roche), vortexed and allowed to stand at room temperature for ≥30 min. The samples were centrifuged at 1000 ***g*** for 60 s and 150 μL of supernatant was removed for DNA isolation using the Solid Tissue protocol starting at Step 3. DNA was eluted in 150 μL H_2O for all sample types and was stored at −20 °C.

Assay design

For species identification, a 109-bp fragment of the *L. lutra* mtDNA CR (AY860354) was targeted with primers PM3F (5′-CTTGCCCCATGCATATAAGCA-3′, Mullins *et al.* 2010) and LLR (5′-GCAAGGATTGATGGTTTCTCG-3′) and a TaqMan® MGB VIC-labelled probe LLP (5′-VIC-TATGGTTGATTTTACATGTATCCACMGB-3′). Primer and probe sequences were designed towards conserved sequences in the *L. lutra* haplotypes (EU294255-EU294258, FJ971618-FJ971622, HQ113947, AJ006174, AJ006175 and AJ006178) available from GenBank. The molecular sex-typing of spraints was based on amplification of a 79-bp and a 75-bp fragment of the final intron regions of the *L. lutra* ZFX (AB491606) and ZFY (AB491597) genes, respectively. These targets were assayed using chromosome-specific primers LLXF (5′-CGCAGAGCAACCCTGTCATAA-3′), GOXR (5′-GG AGGGACTGAGGTTGGTTACC-3′), LLYF (5′-AACAGA ATCTTGTCCAGAAACTTCATT-3′), LLYR (5′-CTCCC GTCTGTTCGTTTTAGATTG-3′) and TaqMan® MGB VIC- or 6-FAM-labelled probes MMX (5′-VIC-CCTGG TCTGAAAACT-3′, Mullins *et al.* 2010) and LLYP (5′-6-FAM-CCACCCATAACACTCC-MGB-3′). Novel primers and probes were designed using Primer Express 2 software from Applied Biosystems (Life Technologies). Primers were purchased from Eurofins MWG Operon and probes from Applied Biosystems.

Real-time PCR

Each PCR contained 5 μL FastStart Universal Probe Master (Rox) (Roche), 2 μM each primer, 0.95 μM probe (s), template (1 μL purified spraint/jelly/hair DNA or 40 pg tissue DNA) and H_2O to a final volume of 10 μL in Microamp® Optical 96-well reaction plates (Applied Biosystems). Duplicate PCRs were performed on samples for molecular sex-typing (Lynch & Brown 2006) and both ZFX and ZFY primer and probe sets were combined in duplex PCRs. A known male otter tissue DNA sample (40 pg) was used as a positive control and 1 μL H_2O was used as a negative control instead of DNA with each qPCR plate. PCR controls were also assayed in duplicate. The PCR conditions were 50 °C for 2 min, 95 °C for 10 min, followed by 40 or 50 cycles of 95 °C for 15 s and 60 °C for 1 min for the species or sex-typing assays, respectively. All assays were carried out using an Applied Biosystems 7300 Real-Time PCR System, and Applied Biosystems Sequence Detection Software (SDS 1.2.3.) was used for data analysis.

Assay validation

The qPCR assays developed in this study were validated using DNA isolated from the tissue of known-sex otters. The efficiencies of the reactions were verified using serially diluted (three replicates across a 5-log dilution series of template: 4000–0.4 pg DNA) otter tissue DNA. The PCR efficiency (E) was calculated using the slope of the standard curve (m) as $E = 10^{(-1/m)} - 1$ (Applied Biosystems). Sex identification of these samples was verified by amplification of a 70-bp fragment of the male-specific SRY gene using the primers (Lut-SRY F, Lut-SRY R) and PCR conditions described by Dallas *et al.* (2000). Each reaction contained 5 μL GoTaq® Hot Start Green Master Mix (Promega), 2 μM each primer, 100 ng DNA and H_2O to a final reaction volume of 10 μL. PCR products were resolved by agarose gel electrophoresis and visualized under UV light using the GeneSnap V6.10 image analysis system (SynGene). To determine cross-species amplification, DNA (100 pg tissue DNA, 1 μL hair/scat DNA) of red fox (*Vulpes vulpes*), pine marten (*Martes martes*), American mink (*Neovison vison*), stoat (*Mustela erminea*), weasel (*Mustela nivalis*), European polecat (*Mustela putorius*), European badger (*Meles meles*), cat (*Felis catus*) or dog (*Canis lupis familiaris*), species that may be (incorrectly) collected during a given survey, were analysed using the otter species qPCR assay.

Identification of nonotter species

Faecal samples that did not show positive amplification using the otter species qPCR assay were analysed using the primers (PM-FOR, PM-REV) and SYBR qPCR conditions described by O'Reilly *et al.* (2008) that, in addition to otter, can detect a range of nonotter species including *V. vulpes*, *M. martes*, *N. vison*, *M. erminea*, *M. nivalis*, *M. putorius*, *M. meles*, *F. catus* and *C. lupis familiaris*. Each PCR contained 5 μL FastStart Universal SYBR Master (Rox) (Roche), 2 μM each primer, 1 μL purified faecal DNA and H_2O to a final volume of 10 μL. Samples presenting a $C_T \leq 30$, an indicator of good-quality amplifiable DNA (O'Reilly *et al.* 2008), were amplified with primers LMS3 (5′-TCCCTAAGACTCAAGGAAG AAGCA-3′, Statham 2005) and PM-REV targeting a 317-bp fragment of the unknown species mtDNA CR. Each PCR contained 5 μL GoTaq® Hot Start Green Master Mix (Promega), 2 μM each primer, 1 μL purified faecal DNA and H_2O to a final reaction volume of 10 μL. The PCR conditions were 95 °C for 2 min, 50 cycles of 95 °C for 30 s, 57 °C for 30 s and 72 °C for 30 s, followed by 72 °C for 10 min (Statham 2005). PCR products were resolved by agarose gel electrophoresis and visualised as above. PCR products were cleaned using microCLEAN (Microzone Ltd.) according to the manufacturer's instructions. DNA sequence analysis of products was carried out using the ABI BigDye Sequencing kit v3.1 (Applied Biosystems) according to the manufacturer's instructions followed by capillary electrophoresis on an ABI PRISM 310® Genetic Analyser (Applied Biosystems) with the standard run module. DNA sequence data obtained were used to query the National Centre for Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (BLAST) program (http://www.ncbi.nlm.nih.gov/BLAST/) to identify the unknown species (Altschul *et al.* 1990).

DNA screening for microsatellite panel selection

The data generated by the sex-typing qPCR assay was used for the evaluation and selection of *L. lutra* microsatellite primers for our studies. Increasing ZFX and ZFY C_T values reflected reducing quantities of nDNA template. Primer sets Lut435, Lut833, Lut604, Lut701, Lut818 (Dallas & Piertney 1998), Lut457, Lut615 (Dallas *et al.* 2002), 04OT04, 04OT05, 04OT14, 04OT17 and 04OT22 (Huang *et al.* 2005) were used to assay spraint DNA with ZFX C_Ts ranging from 29 to 40 (n = 4 per C_T). The 12 loci are known to be variable among Eurasian otter populations and were selected based on the number of alleles and length of amplicon. Primers were purchased from Eurofins MWG Operon with all forward primers fluorescently labelled with 6-FAM. For each primer set, PCRs contained 5 μL GoTaq® Hot Start Green Master Mix (Promega), 2 μM each primer (1 μL), template (1 μL tissue DNA, 1 μL hair DNA or 4 μL spraint DNA) and H_2O to a final volume of 10 μL. The PCR conditions were: 95 °C for 5 min, 40 cycles of 95 °C for 30 s, 57 °C for 90 s and 72 °C for 30 s, followed by 60 °C for 30 min. Fragment analysis was carried out on an ABI PRISM 310® Genetic Analyser (Applied Biosystems) according to the manufacturer's instructions with the standard run module. Alleles were scored with GS500 LIZ™ size standard using GENEMAPPER software version 3.7 (Applied Biosystems).

The primer sets Lut435, 04OT05, Lut457, Lut833, 04OT17, 04OT22, 04OT14, Lut701 and Lut818 were subsequently chosen with the following modifications. Lut833-r and 04OT17-r were redesigned as Lut833mini-r (5′-GAGGGTGAATGTCCATACTA-3′) and 04OT17mini-r (5′-CTTCAGTAGAAAGTGCACTTA-3′) to amplify shorter fragments of 157–169 bp and 124–128 bp, respectively and to increase their success with noninvasive samples. Lut818, 04OT14 and 04OT17 were modified according to Brownstein *et al.* (1996) to include 5′-GTTTCTT sequences on the reverse primers to promote nontemplated nucleotide addition. The nine loci were amplified in one to three multiplex reactions depending on the DNA source. Spraint DNA required

three multiplex reactions as A: 04OT14 (0.6 μM, 6-FAM), Lut435 (4 μM, NED™), 04OT17 (3 μM, VIC®) and 04OT22 (1 μM, 6-FAM), B: Lut833 (0.5 μM, ATTO550), 04OT05 (0.75 μM VIC®) and Lut701 (0.6 μM, 6-FAM) and C: Lut457 (4 μM, PET™) and Lut818 (5 μM, PET™). Hair DNA required two multiplex reactions where Lut457 and Lut818 were included in multiplexes A and B, respectively. Tissue DNA required one multiplex reaction where all nine primer sets were added in one reaction. The concentrations stated are for both forward and reverse primers of each locus. These optimized concentrations and fluorescent dyes enable multiplex reactions and the simultaneous fragment analysis of all nine loci as pooled amplicons. Forward primers labelled with VIC®, PET™ or NED™ were purchased from Applied Biosystems and those with ATTO550 and 6-FAM from Eurofins MWG Operon. All PCRs were duplicated to obtain consensus genotypes and followed the microsatellite PCR conditions as above.

Results

Assay validation and application

Both qPCR assays were validated using tissue DNA isolated from known-sex otters. Figure 1(a) shows the qPCR amplification curves of standardised tissue DNA samples analysed with the otter species identification assay and a standard curve derived from these data is shown in Figure 1(b). The standard curve of the Log input DNA concentration vs. C_T values highlights the efficiency of the reaction where a slope (m) of $-3.3 \pm 10\%$ reflects a PCR efficiency of $100\% \pm 10\%$, and an R^2 value > 0.99 provides confidence in correlating the C_T values of serially diluted template (Applied Biosystems). The species C_T values ranged from 20.15 to 33.14 with E = 99% (m = −3.289, R^2 = 0.999). This was replicated for the sex-typing assay where both ZFX and ZFY amplification was seen to remain linear in the 4000–4 pg range. For female tissue DNA, ZFX C_T values ranged from 26.98 to 36.49 with E = 93% (m = −3.17, R^2 = 0.996). For male tissue DNA, ZFX C_T values ranged from 27.74 to 38.34 and ZFY C_T values ranged from 27.51 to 37.85 with E = 92.7% (m = −3.51, R^2 = 0.999) and E = 95.7% (m = −3.43, R^2 = 0.994), respectively. Female samples were identified by the amplification of the ZFX target and male samples by the amplification of both ZFX and ZFY targets. The sex identification matched the results given by the SRY gene amplification. The species assay did not amplify DNA of *V. vulpes*, *M. martes*, *N. vison*, *M. erminea*, *M. nivalis*, *M. putorius*, *M. meles*, *F. catus* or *C. lupis familiaris*.

The species qPCR assay genetically identified 1394 of the 1612 faecal samples as being *L. lutra*. Of these samples, 514 were sex-typed as female, 415 were male and

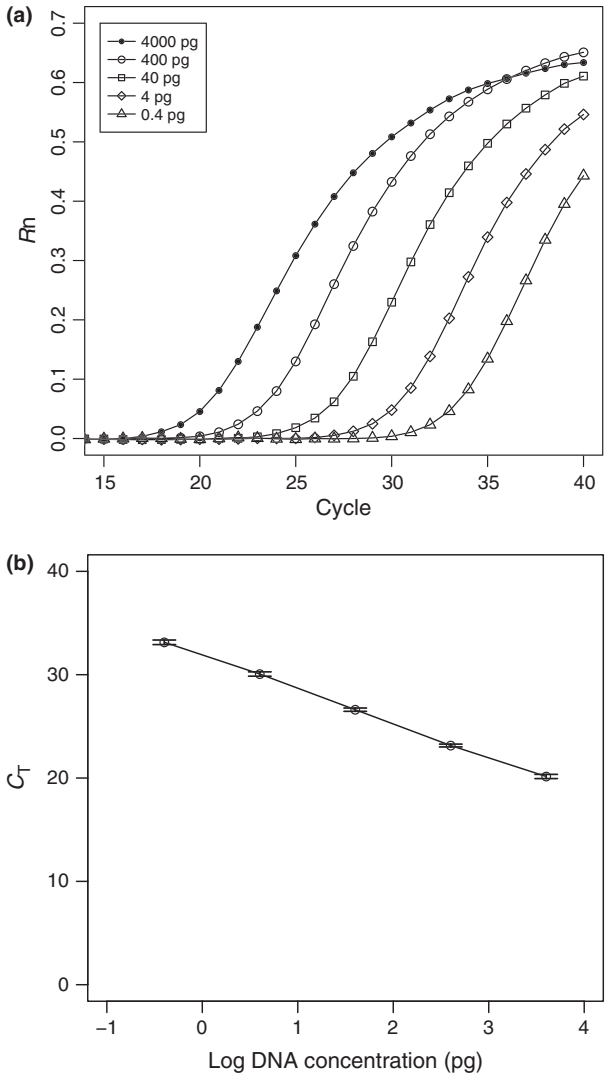

Fig. 1 Quantification of *Lutra lutra* mtDNA using the species identification qPCR assay developed in this study. (a) Amplification plots of serially diluted *L. lutra* tissue DNA and (b) standard curve derived from amplification data.

287 samples were undetermined, most likely due to degraded nDNA template. As mtDNA is more abundant than nDNA in cells, the C_T values recorded from the species assay were expectedly lower than those of the sex-typing assay. This reflected the inverse proportionality between C_T value and input DNA concentration. A species $C_T \leq 32$ was selected as the indicator threshold value above which a sex-typing assay would most likely fail and as a result, 178 samples were not tested. A plot of C_T data (Fig. 2) generated by the 929 sex-typed samples of species C_T values (LL) against corresponding ZFX C_T values (ZFX) demonstrates the overall trend of decreasing mtDNA detection equating to decreasing nDNA detection. Linear regression analysis on the data highlights variation in the detectability of the mtDNA and nDNA

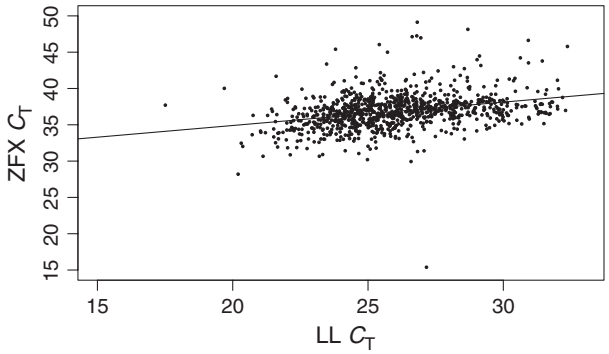

Fig. 2 Plot of qPCR data with species (LL) C_T values plotted against corresponding ZFX C_T values. m = 0.322, r = 0.318, $P < 0.001$.

targets relative to each other as slope m ≠ 1. The reason for this remains unclear and could be an effect of (i) a greater rate of mtDNA degradation compared to nDNA, or vice versa; (ii) equal rates of DNA degradation but an earlier loss of the larger mtDNA target; (iii) differential amplification of the targets due to relative amplicon size or; (iv) differential amplification of the targets due to dietary differences between individuals. Additional research is needed to explore these possibilities.

Identification of nonotter species

Two hundred and eighteen samples did not amplify with the otter species assay. Using the PM SYBR assay, 178 samples were found to contain good-quality amplifiable DNA ($C_T \leq 30$) and the remainder (n = 40) were either of poor-quality DNA or a species that cannot be amplified with the PM-FOR/PM-REV primers. Samples with a $C_T \leq 25$ (n = 68) were amplified with the LMS3/PM-REV primers and DNA sequence analysis of the resulting amplicons identified *N. vison* (n = 31), *M. martes* (n = 4) and *V. vulpes* (n = 4). Twenty-nine samples remained unidentified due to poor-quality DNA.

DNA screening for microsatellite panel selection

Twelve published *L. lutra* microsatellite primer sets were used to assay spraint DNA samples determined to have ZFX C_Ts ranging from 29 to 40. Figure 3 represents the positive/negative PCR amplification of each primer set across the range of DNA samples. For example, at ZFX C_T = 37, eight primer sets demonstrated successful PCRs with positive amplification of target, or, 04OT04 showed positive amplification with spraint DNA ZFX C_T = 29–37. The rate of PCR success is inversely proportional to the C_T values and is a reflection of nDNA input quantity and quality. This plot therefore highlights the primer sets most suited to NGS for *L. lutra*. In terms of

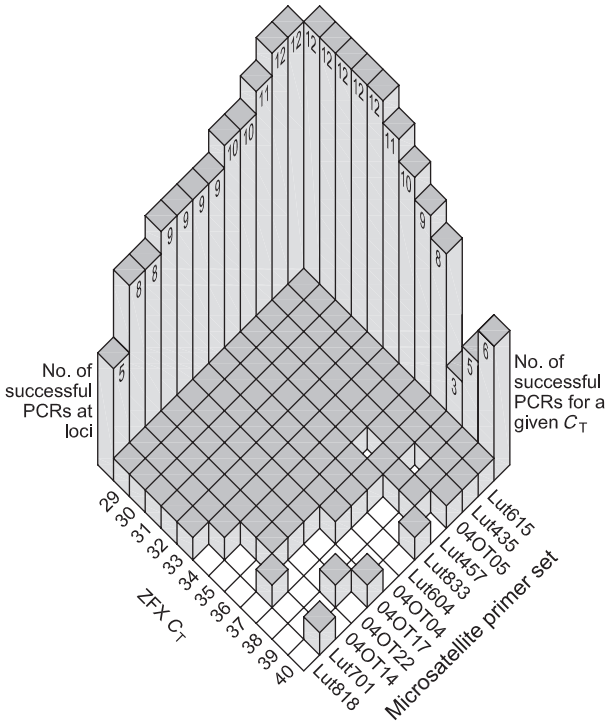

Fig. 3 Three-dimensional column chart representation of PCR amplification with each microsatellite primer set with spraint DNA ZFX C_Ts 29–40. Successful PCRs are denoted by shaded boxes with totals given per locus and per ZFX C_T.

sample throughput and cost management, the plot identifies the ZFX C_T above which the microsatellite genotyping of samples would most likely result in failed PCRs. This was identified as ZFX C_T 36. The choice of primer sets within the ≤C_T 36 section was based on the optimum combinatory evaluation of PCR success, number of alleles and amplicon size. The primer sets above this limit were assessed by the same criteria and those showing potential application based on a greater number of alleles but limited by amplicon size were redesigned (Lut833-mini-r and 04OT17mini-r) to improve PCR success.

Discussion

The noninvasive approach to monitor mammals, especially elusive species, is an increasingly applied technique in obtaining information on the structure, size, genetic diversity and relatedness of a population (Waits & Paetkau 2005; Arandjelovic *et al*. 2010; Oliveira *et al*. 2010; Ruiz-González *et al*. 2012). In addition to the advantages of qPCR (e.g. precision, sensitivity, specificity), the design and application of assays presented in this paper offered more information. The C_T values obtained from the qPCR assays not only confirmed the species or sex, but acted as indicators of the quantity and quality of DNA extracted from the otter spraint, specifi-

cally otter DNA. This was the first time that the qPCR assays were seen to indirectly act as quality control points for the downstream processing of samples. The use of different fluorescent reporter dyes on the X and Y chromosome-specific probes enabled multiplex PCRs that not only reduced labour and costs, but allowed amplification of the ZFX target to act as a built-in internal reaction control. Similar to the species assay, the ZFX C_T values indicated nDNA abundance and were used to establish those samples suitable ($C_T \leq 35$–36) for microsatellite genotyping as both methodologies are based on the amplification of nDNA. The data presented demonstrate an 86.5% success rate in the genetic identification of *L. lutra* spraints from the faecal sample set of this study. The remaining samples were determined to have DNA degraded beyond the limits of PCR amplification or were identified as other species by DNA sequence analysis. Of the positively identified otter samples, 66.4% were assayed with the sex-typing qPCR assay with 76.4% of these successfully identified as male or female. The success rate of the sex-typing qPCR assay would have been adversely affected by not using the prescreening approach prior to analysis. First by confirming otter species and second by identifying a species C_T screening threshold avoided success rates of just 57.6% or 66.6%, respectively.

It has been reported that only very fresh (<1 day old) spraints yield DNA suitable for microsatellite genotyping with a broad success range (Hájková *et al.* 2009 and references therein). While fresh/old (>1 day old) spraints can be discriminated with reasonable confidence, it is impossible to visually inspect the DNA quantity and quality of a given sample. Within the approximately 1600 faecal sample set of this study, we have found that neither fresh spraints categorically yield good-quality, amplifiable DNA nor do old (dried) spraints yield poor DNA. Furthermore, a diet rich in fish may reduce PCR success with faecal DNA samples as seen with the brown bear (*Ursus arctos*) where Murphy *et al.* (2003) suggest either the high lipid and low fibre content lowers intestinal cell sloughing or by-products interfere with the DNA isolation protocol chemistry. The qPCR assays designed for this study have the power to firmly evaluate these criteria while providing species and sex information. This again shows the importance and value of the qPCR approach to control overhead expenses (Hájková *et al.* 2009).

Collectively, the methodologies discussed in this study demonstrate an efficient approach to facilitate high-throughput analysis and critical evaluation of *L. lutra* samples from NGS projects. The samples tested to date cover a relatively broad geographical range and the primer and probe sequences of the species assay are conserved in the *L. lutra* haplotypes distributed across Europe (Stanton *et al.* 2009; Finnegan & O'Néill 2010). The application of the assays to either Eurasian otters from other regions or other otter species may require minor modifications in the primer/probe designs due to novel interpopulation or interspecies nucleotide polymorphisms. The forward and reverse primers of the species qPCR assay are suitable for use with the sea otter (*Enhydra lutris*) (AB497073) and the Asian small-clawed otter (*Aonyx cinerea*) (JQ038824) with one and two nucleotide polymorphism redesigns required, respectively, in the probe sequences to achieve assay specificity. Furthermore, as the LMS3/PM-REV primers amplify the mtDNA CR that identifies *L. lutra* haplotypes, this demonstrates the fail-safe capacity of our methodologies and workflow to discover novel haplotypes if and when they arise. SNPs in the sex-typing assay primers and probes may be identified by DNA sequence analysis of the ZF gene final intron regions. To date, the molecular sex-typing assay has been successfully used with DNA isolated from spraints of the giant otter (*Pteronura brasiliensis*) (Pickles RS & O'Reilly C, unpublished data).

The otter population in Ireland is considered of international importance due to its widespread distribution throughout the country (Ottino & Giller 2004) and its relatively stable demographic history compared to the rest of Europe (Finnegan & O'Néill 2010) due to an opportunistic diet in an extensive suitable habitat (Reid *et al.* 2013). Internationally, however, populations suffered major declines in recent decades (Mucci *et al.* 2010) but with legislative protection are now recovering, as seen in Wales and parts of England (Hobbs *et al.* 2011). The readily usable suite of DNA-based assays presented here may now be applied to new programmes to monitor these recovering populations involving citizen scientists over the coming years and beyond. The overall approach may also be adapted to other species.

Conclusions

This study has shown the successful application of real-time PCR for the molecular species and sex-typing of otter (*L. lutra*) spraints. These are species-specific assays that, when used in conjunction with microsatellite genotyping and haplotyping, represent a set of readily applicable molecular ecology tools to monitor otter populations by noninvasive genetic sampling. In addition to the sensitivity and ease of technique offered by real-time PCR, the assays also have a built-in critical control capacity for sample processing.

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C.O.R. and P.D.T. were the principle investigators and supervised the experiments; D.O.N., P.D.T. and C.O.R. designed the analytical tools; D.O.N. performed the majority of the laboratory work and analysed the data; D.B.O.M. supported the laboratory work and contributed to data analysis; E.A.C. contributed samples; L.C. managed the project; D.O.N. and C.O.R. drafted the article; all authors revised the article.

Data accessibility

Data used for this study are presented within the article.