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RESOURCE ARTICLE

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Validation of loop-mediated isothermal amplification for fast and portable sex determination across the phylogeny of birds

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Abstract

PCR is a universal tool for the multiplication of specific DNA sequences. For example, PCR-based sex determination is widely used, and a diversity of primer sets is available. However, this protocol requires thermal cycling and electrophoresis, so results are typically obtained in laboratories and several days after sampling. Loop-mediated isothermal amplification (LAMP) is an alternative to PCR that can take molecular ecology outside the laboratory. Although its application has been successfully probed for sex determination in three species of a single avian Family (raptors, Accipitridae), its generality remains untested and suitable primers across taxa are lacking. We designed and tested the first LAMP-based primer set for sex determination across the modern birds (NEO-W) based on a fragment of the gene chromo-helicase-DNA-binding protein located on the female-specific W chromosome. As nucleotide identity is expected to increase among more related taxa, taxonomically targeted primers were also developed for the Order Falconiformes and Families Psittacidae, Ciconiidae, Estrildidae and Icteridae as examples. NEO-W successfully determined sex in a subset of 21 species within 17 Families and 10 Orders and is therefore a candidate primer for all modern birds. Primer sets designed specifically for the selected taxa correctly assigned sex to the evaluated species. A short troubleshooting guide for new LAMP users is provided to identify false negatives and optimize LAMP reactions. This study represents the crucial next step towards the use of LAMP for molecular sex determination in birds and other applications in molecular ecology.

KEYWORDS
aves, CHD-W, CHD-Z, loop-mediated isothermal amplification (LAMP), neognathae, sexual chromosomes, ultraconserved element

1 | INTRODUCTION

The determination of the sex of individuals is often key in ecological and evolutionary studies, as well as for commercial and conservation purposes. Thus, several methods have been developed to sex individuals based on behaviour (Gray & Hamer, 2001), vocalizations (Volodin et al., 2009), morphology (Reynolds, Martin, Wallace, Wearn, & Hughes, 2008), genitals and sex organs (Maron & Myers, 1984; Richner, 1989) and hormone levels (Bercovitz, Czekala, & Lasley, 1978). However, these methods can have high error rates and sometimes are technically complex or highly invasive. In recent decades, the development of molecular techniques facilitated an accurate and relatively harmless sex determination, using small tissue samples obtained through low invasive methods (e.g., blood extraction) or even noninvasive sampling (e.g., moulted feathers, fur and faeces). In birds, sex determination is based on chromosomal differences between males (homogametic sex, two copies of Z chromosome) and females (heterogametic sex, one copy of Z and W

chromosomes; Harris & Walters, 1982). PCR-based techniques for sex determination mostly rely on the amplification of the sex-chromosome-specific gene chromo-helicase-DNA-binding protein (*CHD*), located on the sexual W and Z chromosomes (CHD-W and CHD-Z, respectively; Fridolfsson & Ellegren, 1999; Griffiths, Double, Orr, & Dawson, 1998). CHD-W and CHD-Z show different intron sizes and nucleotide composition, so the PCR amplification using specific primers and posterior fragment isolation by electrophoretic methods serves for sex determination. This standard methodology is nowadays widely used across bird taxa (Morinha, Cabral, & Bastos, 2012; Vucicevic et al., 2013), and different primers sets have been developed, based on differences in nucleotide composition in regions where PCR primers anneal (Fridolfsson & Ellegren, 1999; Griffiths et al., 1998; Lee et al., 2010; Wang & Zhang, 2009; Wang, Zhou, Lin, Fang, & Chen, 2011).

A major constraint of PCR protocols is that they require thermal cycling (for denaturation, primers annealing and DNA synthesis) and electrophoresis (to visualize PCR results). Therefore, the sex is not known until samples have been analysed in specialized laboratories, usually away from the sampling areas. This requires the transport of samples and usually implies that sex determination may take several days after collecting the samples. Although high-resolution melting analysis is an accurate technique for sex determination in birds (Faux, McInnes, & Jarman, 2014; Morinha et al., 2013), hand-held devices for quantitative PCRs required for a portable solution are still expensive and made these protocols unaffordable for most laboratories. Loop-mediated isothermal amplification (LAMP; Notomi, Mori, Tomita, & Kanda, 2015; Notomi et al., 2000) can lift these constraints on time, space and resources. Lee (2017) reviewed the application of LAMP method concluding that it "promises to revolutionise how molecular ecology is practised in the field," not only for sex determination but potentially also for other applications, for example, the detection of cryptic species or the monitoring of diseases and parasites. Furthermore, as LAMP is reported to be ten times more sensitive than PCR (Hamburger et al., 2013), LAMP might bring advantages in the analysis of samples containing little DNA, such as noninvasive samples, museum samples or eDNA, and may do so both in the laboratory and in the field (Lee, 2017). Finally, because LAMP is an easily implemented yet accurate DNA amplification and diagnosis tool, it can be used by researchers in parts of the world with the only need of basic molecular biology equipment.

LAMP is a single tube technique for amplification and synthesis of DNA using a single temperature. This is due to the *Bst* polymerase, an enzyme that allows an auto-cycling DNA strand displacement and, therefore, it does not require the denaturation, annealing and extension cycling of DNA as in PCR. LAMP requires two pairs of primers that recognize six different regions flanking the target region to synthesize a product of stem–loop DNA amplicons in the same strand (see Supplementary Material S1 and fig. 1 in Tomita, Mori, Kanda, & Notomi, 2008). In birds, sex determination based on LAMP requires a female-specific and a control molecular marker. The first primer set targets a fragment of the CHD-W, so positive LAMP reactions will be characteristic of females. The second primer set should target a DNA fragment located in any region shared between male and female genomes (e.g., CHD-Z or a highly conserved autosomal sequence) and it is used as a positive control for DNA quality and/or to monitor LAMP reaction (i.e., a lack of a positive result means the assay failed), avoiding false negatives.

The development of a fully operational field technique based on LAMP is possible by three main features. First, the reactions are isothermal so do not require a thermal cycler. Instead, a thermoblock or water bath (which can be, e.g., connected to the lighter or battery of a vehicle) is used for incubation at a single temperature. Second, LAMP products can be stained and easily checked by the unaided eye using turbidity (Mori, Nagamine, Tomita, & Notomi, 2001), pH-sensitive dyes (Tanner, Zhang, & Evans, 2015) or metal indicators (Tomita et al., 2008). Consequently, it does not require any special laboratory techniques such as electrophoresis, and can provide rapid results in situ. Third, primers of LAMP can be vacuum-dried and hydrated directly with LAMP reagents mixed prior to LAMP reaction. These reagents can be mixed with stabilizers (sucrose) to preserve enzyme activity, so no special storage conditions (e.g., low temperatures) are required. The latter is an advantage not only for fieldwork, but also because it facilitates enormously the shipment of reagents. Centeno-Cuadros, Abbasi, and Nathan (2017) validated these three main advantages over PCR-based methods for sex determination in three raptor species from the Family Accipitridae [*Gyps fulvus* (Hablizl, 1783), *Neophron percnopterus* (Linnaeus, 1758) and *Milvus migrans* (Boddaert, 1783)], and showed the suitability of this (relatively) easy, portable and quick (less than 90 min, including DNA extraction) technique for sex determination under field conditions. However, whether this technique can be adapted to determine sex in other species from other Families and Orders of birds and thereby can claim to be a general, competing technique for molecular sexing, remains unknown.

Here, we design and test primer sets for LAMP-based sex determination across a broad range of taxa of Neognathae, the clade including all modern birds with the exception of the flightless ratites (ostriches, kiwis, etc.) and the tinamous. We first designed and tested a highly conserved primer set for species across 12 taxonomic Orders (Anseriformes, Caprimulgiformes, Charadriiformes, Falconiformes, Gruiformes, Passeriformes, Pelecaniformes, Podicepediformes, Procelariformes, Psittaciformes, Strigiformes and Suliformes) (Objective 1). In addition, we designed a primer set to amplify an ultraconserved element to be used as a positive control for DNA amplification, to help discard false negatives (i.e., misleading males) (Objective 2). The optimization of this technique and the resulting successful application to a subset of Orders suggest that the LAMP-based approach is potentially extensible to all modern birds (Neognathae). As nucleotide identity is expected to increase among more closely related taxa (thus facilitating primer design), we also designed specific primer sets for LAMP reactions for particular Orders and Families (Objective 3). We first focused on diurnal raptors (Order Falconiformes) and parrots (Family Psittacidae) because of their major conservation and commercial interests. About one-third of the parrot species of the world are threatened with extinction, as well as

a number of raptor species (Donald, Collar, Marsden, & Pain, 2010), and knowing the sex of individuals can be crucial for both ex situ and in situ research and conservation programmes (Centeno-Cuadros et al., 2017; Lambertucci, Carrete, Speziale, Hiraldo, & Donázar, 2013). Additionally, parrots are among the birds most often bred in captivity to supply international pet markets, and captive-bred raptors are increasingly demanded for falconry (Abellán, Carrete, Anadón, Cardador, & Tella, 2016). Consequently, thousands of these birds bred by commercial and private aviculturists are weekly sexed by commercial laboratories just in Europe. We next designed primer sets for sex determination in Ciconiidae, Estrildidae and Icteridae and use this to explain how new, taxonomically targeted primers can be developed. We tested for the reliability of sex determining by LAMP in 24 individuals of the pied flycatcher (*Ficedula hypoleuca*) (Pallas, 1764) which were also sexed by PCR (Objective 4). Combined, our results reinforce and exemplify the ease of LAMP for sex determination, also when applying the same primer set to different taxonomic levels. Last, we also provide a short troubleshooting guide for new LAMP users.

2 | MATERIAL AND METHODS

2.1 | A "primer" on primer design for LAMP

We designed the forward and backward internal primers (FIP and BIP, respectively) to produce a looped structure every time a new FIP/BIP anneals and synthesizes a new DNA sequence. Two external primers (F3/B3) are involved in the strand displacement, which is directly related to the formation of the loop caused by FIP/BIP. As our interest was focused on primer design applicable across different taxonomic levels (Orders and Families), we first aligned all available sequences per taxon and choose one of the following two approaches. When the DNA sequence length and distribution of polymorphic sites met the standards required by PRIMER EXPLORER version 4 software (Eiken Chemical Co., Ltd., Japan; http://primerexplorer.jp/e/), we used the "designing common primer using multiple alignment" option to target the newly designed primers to relatively well-conserved regions in the consensus sequences within the taxonomic level under consideration. However, when highly polymorphic regions across relatively short fragments did not meet these standards, we used PRIMER3 (Untergasser et al., 2012) and recommendations by Tomita et al. (2008) to design alternative custom-made primers ("manual method"). Independent of the method applied for primer design, primer selection always requires a number of LAMP experiments for optimization of time and temperature prior to rejection or acceptance of any primer set for sex determination.

2.2 | Primer design for sex determination

The female-specific marker for sex determination of species within the superorder Neognathae (primer set "NEO-W," see Tables 1 and 2) was designed based on 92 sequences of CHD-W available in GenBank, corresponding to species from 12 Orders (Falconiformes, Passeriformes, Pelecaniformes, Psittaciformes, Strigiformes, Columbiformes, Galliformes, Charadriiformes, Gruiformes, Musophagiformes and Apterygiformes; see Supplementary Material S2). These sequences showed a 92% identity on average, and they are mapped between positions 2,246 and 2,445 of the first domain of the CHD1-W of *Gallus gallus* (Linnaeus, 1758) (GenBank Acc. No. AF181826). Primers for sex determination in Falconiformes (primer set FAL-W) were designed based on CHD-W sequences of all raptor species found in GenBank (see Supplementary Material S3). NEO-W and FAL-W primers were designed using the "manual approach" and PRIMER EXPLORER version 4, respectively.

We used a 461-bp fragment of highly conserved DNA in the so-called ultraconserved element (UCE) (99.9% identity) located on chromosome 6 of birds (UCE4126 in McCormack et al., 2013) to design a primer set (UCE, Table 1) to be used as a positive control for DNA quality and/or to monitor LAMP reactions. Similarly, we also designed specific primers to be used as positive control for Falconiformes (primer set FAL-Z) based on CHD-Z sequences of all raptor species found in GenBank (see Supplementary Material S3).

We extracted DNA from blood samples of one male and a female of 42 species to find conserved regions within bird families and target LAMP primers. A "salting-out" protocol (Müllenbach, Lagoda, & Welter, 1989) was used with an addition of LiCl precipitation and chloroform extraction for protein removal (Table 2). Next, we ran a single PCR per sample for sex determination using PCR conditions described in Centeno-Cuadros et al. (2017) and primers reported in Table 2. The specific fragment of females (CHD-W) of Ciconiidae, Estrildidae, Icteridae and Psittacidae was isolated from the agarose gel using the Isolate II PCR and Gel Kit (Bioline) following the manufacturer's instructions. Sequencing and assembling conditions are described in Centeno-Cuadros et al. (2017). We designed primer sets based on the consensus sequence obtained after aligning CHD-W sequences per species within Families (Ciconiidae, Estrildidae, Icteridae and Psittacidae, hereafter primer sets CIC-W, EST-W, ICT-W and PSI-W, respectively) and after aligning CHD-Z sequences (primer sets CIC-Z and ICT-Z) (Table 2) (GenBank Accession nos KY441616–KY441639). PSI-W and EST-W were designed manually, whereas primer sets CIC-W, CIC-Z, ICT-W and ICT-Z were designed using PRIMER EXPLORER version 4.

2.3 | Optimization of LAMP reactions

Our aim here was to validate the application of different primer sets across taxa so we did not test the portability of LAMP and its application under field conditions, as already shown in Centeno-Cuadros et al. (2017). Accordingly, all LAMP reactions were performed under controlled laboratory conditions using DNA templates of one male and one female (PCR-based sex determination from previous studies) of 42 species (from 12 Orders and 23 Families; see Table 2). All working dilutions were set to 25–50 ng/μl. Each LAMP reaction included dNTP 0.4 mM, betaine 1 M, 8 units of Bst 2.0 (New England Biolabs), 1× enzyme buffer (20 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 50 mM KCl, 2 mM $MgSO_4$, 0.1% Tween®-20, pH 8.8), 1.6 and 0.2 μM

TABLE 1 Primer sets designed for sex determination within different taxa of Neognathae (names within parentheses)

Primer set	Primer	Primer sequence (5′–3′)
CIC-W (Fam. Ciconiidae)	F3	CTCAATTGCCAAAACAATTGG
	B3	GAATTTTGCTGGTAGTAGCC
	FIP	CTGGCAATTACTATATGCCAAACAGTATTT**TTTT**GGGGATAAGAGTAATGTAACACT
	BIP	AACTTCCATAAATCTTTCTACAAAAAGGAC**TTTT**AGAAACCTTGATCTTTACCACTT
CIC-Z (Fam. Ciconiidae)	F3	TCACAGAAGATGGAGATTCC
	B3	CAACAGAGTTCTGATTTTCTCA
	FIP	GCCAAGAAGCTTTGGTCTTGAAC**TTTT**CTCTGGACAACTTGTTCAGT
	BIP	ACTACCACCAAGATTCATACCTGA**TTTT**CAGATGGTGAGGATGCTG
EST-W (Fam. Estrildidae)	F3	ACTCCTATTATAGTCTATCCGGAGCA
	B3	CCTTTTCAGGTAAGAATTTTGCTGG
	FIP	GAAGTGTTACATTATTCTTATTCCTCCCCC**TTTT**TTGAATTCTCAGTTGCCAAACCAAT
	BIP	GTGTCCTTTTTGTAGAAAGATTTATGAAAG**TTTT**AGAAGCCTTGATTTTTACCATTTTATCTT
FAL-W (Order Falconiformes)	F3	AAATGTTTTAGTCACGTAGCT
	B3	TCTGCATCGCTAAATCCTT
	FIP	CTTCTGCTCCTACTGCGTTTC**TTTT**TTAATCTGAAATTCCAGATCAGCT
	BIP	ATTCTGGATCTGATAGTGACTCCA**TTTT**ATTTTCTCGACGAGTAGTTCG
FAL-Z (Order Falconiformes)	F3	CATATTTTTGACAGGCTAGGTAA
	B3	CGCTAAATCCTTTAATATTTTCTCG
	FIP	TCACTTCCATTAAAGCTGATCTGG**TTTT**GTTGTTAATCACGTAGCTTTGA
	BIP	CGCAGTAGGAGCAGAAGATACT**TTTT**CACGTTTTTTAGGCCGTT
ICT-W (Fam. Icteridae)	F3	TGCCAAACCAATTGGGTG
	B3	TCAGGTAAGAATTTTGCTGG
	FIP	GACTAGTAATTCCTATATGCCTAATAGTAT**TTTT**GGGGATATAAGAATAATGTAACACT
	BIP	AACTTTCATAAATCTTTCTACAGAAAGGAC**TTTT**CAAGAAGCCTTGATTTTTACCAT
ICT-Z (Fam. Icteridae)	F3	CATAATCATAAACAAAGCTCAAAGG
	B3	TCTTTACTGTGTGGGTGTC
	FIP	CASCATGCTTTAGCTGTCCC**TTTT**ACTGCTGAAAGTCATCTTGTC
	BIP	CCAAGGTCATGTCCAAGTAGCT**TTTT**AAAGCACTGGAACAGGTT
NEO-W (Neognathae)	F3	CATGTAGCTTTGAACTACTTAATCT
	B3	TGCATCGCTAAATCCTTT
	FIP	GAGTCACTATCAGATCCAGAATATCTTC**TTTT**GATCAGCTTTAATGGAAGTGAA
	BIP	AGTGACTCCATCTCAGAAAGAAAAC**TTTT**CTCGGTCTTCCACGTTTT
PSI-W (Fam. Psittacidae)	F3	CAGTTTCCCTTTCAGGTAAG
	B3	TCAGTTGCCAAAACAATGG
	FIP	TTCTTCACAAAGGACACTTTTC**TTTT**TGTAGTAGCCAAGAAGCCTT
	BIP	AGGAAAAGACTGGCAATTACTATATGCTAA**TTTT**GGGGAGATAAGATTAATGTAACA
UCE (Neoaves)	F3	GGGAAACAAGGATAAAATTACTCC
	B3	TGCCCAGAAAATTCCATTC
	FIP	CGAGTGTGTTAAGCACAGTTTTATT**TTTT**ATGGTTAATGACCTATAGTATCTCC
	BIP	GAGGACTGTTCTGCAGGGTATT**TTTT**TGCTATCTGATTCGAAAAGTC

F3 = forward external primer; B3 = backward external primer; FIP = forward internal primer composed by F1c and F2 primers connected by TTTT (bold); BIP = backward internal primer composed by B1c and B2 primers connected by TTTT (bold).

of internal and external primers (FIP-BIP and F3-B3, respectively), 2 μl of DNA extract and fill with molecular biology grade H_2O to complete 25 μl. If unspecific or no amplification was observed in LAMP reactions, we prepared a new enzyme buffer (EB2) composed by 100 mM Tris-HCl (pH 8.3), 35 mM $MgCl_2$ and 250 mM KCl and ran LAMP as follows. Time and temperature during incubation of LAMP reactions are the most critical factors to set up LAMP conditions. Although the optimal temperature for enzymatic activity of Bst is described as 65°C, most of our previous experiments revealed that LAMP commonly works in a range of temperatures between 57

TABLE 2 Incubation temperatures for LAMP reactions of different primer sets for a set of species across different families and orders

Order	Family	Scientific name	Common name	PCR[a]	Primer set (T)	
					Sex	C+
Anseriformes	Anatidae	*Anas platyrhynchos*	Common Mallard	[1]	NEO-W (58[b])	—
	Anhimidae	*Chauna torquata*	Southern Screamer	[1]	NEO-W (58–61[b])	UCE (57–63)
Caprimulgiformes	Apodidae	*Tachymarptis melba*	Alpine Swift	[2]	NEO-W (55)	UCE (59–63)
Charadriiformes	Glareolidae	*Cursorius cursor*	Cream-coloured Courser	M5 [3]; P8 [4]	NEO-W (67)	—
	Laridae	*Larus michahellis*	Yellow-legged Gull	M5 [3]; P8 [4]	—	UCE (57–63)
Falconiformes	Accipitridae	*Accipiter gentilis*	Eurasian Goshawk	[2]	FAL-W (63)	—
		Aquila chrysaetos	Golden Eagle	[2]	FAL-W (61)	—
		Buteo buteo	Common Buzzard	[1]	FAL-W (59–63)	—
		Gypaetus barbatus	Bearded Vulture	[1]	FAL-W (59–63)	—
		Gyps fulvus	Griffon Vulture	[1]	FAL-W (62–63)	—
		Gyps rueppellii	Rüppell's Vulture	[1]	FAL-W (59–63)	—
		Milvus migrans	Black Kite	[2]	FAL-W (62–63)	FAL-Z (59)
		Milvus milvus	Red Kite	[2]	FAL-W (61)	—
		Neophron percnopterus	Egyptian Vulture	M5 [3]; P8 [4]	FAL-W (60–63)	—
	Cathartidae	*Cathartes aura*	Turkey Vulture	[1]	FAL-W (59–63)	—
	Falconidae	*Falco peregrinus*	Peregrine Falcon	[5]	NEO-W (53/58–61[b])	UCE (61)
Gruiformes	Gruidae	*Anthropoides virgo*	Demoiselle Crane	[2]	—	UCE (57–61)
Passeriformes	Alaudidae	*Galerida theklae*	Thekla Lark	[7]	NEO-W (56–61[b])	—
	Corvidae	*Corvus corax*	Common Raven	P2 [6]; P8 [4]	NEO-W (58–64[b])	—
	Estrildidae	*Estrilda melpoda*	Orange-cheeked Waxbill	[7]	NEO-W (56–60[b]), EST-W (56–63)	—
	Icteridae	*Agelaioides badius*	Bay-winged Cowbird	[7]	NEO-W (56–63[b]) ICT-W (60–63)	ICT-Z (62–65)
	Muscicapidae	*Ficedula hypoleuca*	European Pied Flycatcher	P2 [6]; P8 [4]	NEO-W (59/56–61[b])	—
	Sturnidae	*Leucopsar rotschildi*	Bali Myna	[7]	NEO-W (56–63[b])	—
Pelecaniformes	Ciconiidae	*Ciconia ciconia*	White Stork	[1]	CIC-W (59–65)	CIC-Z (63)
	Threskiornithidae	*Plegadis falcinellus*	Glossy Ibis	[2]	—	UCE (57–63)
Podicepediformes	Podicipedidae	*Podiceps nigricollis*	Black-necked Grebe	M5 [3]; P8 [4]	NEO-W (63/55–61[b])	UCE (59–63)
Procellariiformes	Procellariidae	*Calonectris diomedea*	Scopoli's Shearwater	[1]	NEO-W (59/55–61[b])	—

(Continues)

TABLE 2 (Continued)

Order	Family	Scientific name	Common name	PCR[a]	Primer set (T)	
					Sex	C+
Psittaciformes	Cacatuidae	*Nymphicus hollandicus*	Cockatiel	M5 [3]; P8 [4]	NEO-W (63)	—
	Psittacidae	*Anodorhynchus leari*	Lear's Macaw	M5 [3]; P8 [4]	PSI-W (59–63)	—
		Primolius auricollis	Yellow-collared Macaw	M5 [3]; P8 [4]	PSI-W (61)	—
		Ara chloropterus	Red-and-green Macaw	P2 [6]; P8 [4]	PSI-W (59)	—
		Ara macao	Scarlet Macaw	[3]	NEO-W (61[b])	—
		Aratinga solstitialis	Sun Parakeet	[1]	PSI-W (59)	—
		Psilopsiagon aymara	Grey-hooded Parakeet	[1]	PSI-W (60–63)	—
		Cyanoliseus patagonus	Burrowing Parrot	[1]	NEO-W (63[b])	—
		Aratinga nenday	Nanday Parakeet	[1]	PSI-W (60–62)	—
		Neophema pulchella	Turquoise Parrot	P2 [6]; P8 [4]	NEO-W (59/65[b])	UCE (61)
		Pionus maximiliani	Scaly-headed Parrot	M5 [3]; P8 [4]	NEO-W (55–61[b])	UCE (57–63)
		Psittacus erithacus	Grey Parrot	[1]	PSI-W (60)	—
Strigiformes	Strigidae	*Athene cunicularia*	Burrowing Owl	[2]	NEO-W (65)	UCE (57–63)
		Bubo bubo	Eurasian Eagle-owl	[5]	NEO-W (55/61[b])	UCE (59)
Suliformes	Sulidae	*Sula bassana*	Northern Gannet	[2]	NEO-W (51/58–61[b])	UCE (61/57–63[b])

PCR: reference for PCR-based sex determination. PRIMER SET (T): name and range of incubation temperatures (between parentheses) for each successful primer set tested on one male and one female. SEX: primer set for sex determination. C+: primer set to be used as positive control.

[a][1] Fridolfsson and Ellegren (1999). [2] Han, Kim, Kim, Park, and Na (2009). [3] Bantock, Prys-Jones, and Lee (2008). [4] Griffiths et al. (1998). [5] Ellegren (1996). [6] Griffiths, Daan, and Dijkstra (1996). [7] Lee et al. (2010).

[b]The LAMP reaction was run with the enzyme buffer EB2 (see text).

and 63°C. Therefore, we first fixed the incubation time to 60 min and tried every primer set in a range of temperatures (57, 59, 61, 63°C). When we observed weak or unspecific amplification (potential false positives), we then tried different combinations of lower or higher temperatures and shorter or longer incubation times (45 or 80 min). LAMP products were isolated in a 2.5% agarose gel and then stained with 5 μl of 1:50 diluted Sybr Green I Nucleic Acid Stain (Life Technologies) to visualize for the unaided eye changes in the colour of the content within PCR microtubes. Consequently, reaction mixes changed from orange (negative reaction) to yellow-green (positive reaction) due to its interaction with magnesium pyrophosphate generated during DNA synthesis (Mori et al., 2001).

2.4 | Validation test of LAMP

We tested the accuracy and robustness of LAMP-based sex determination of 24 individuals of known (PCR-based) sex of the European Pied Flycatcher by running LAMP reactions using the conditions described above, primer set NEO-W and an incubation time of 80 min at 59°C. Sex determination based on LAMP results was done blind with respect to known sex of individuals.

3 | RESULTS

3.1 | NEO-W and UCE: highly conserved primer sets for sex determination using LAMP (Objectives 1 and 2)

NEO-W, the primer set designed to target the most conserved region of the CHD-W fragment across sequences available in GenBank (see Supplementary Material S2), correctly assigned sex to 21 species within 17 Families and 10 Orders (Anseriformes, Caprimulgiformes, Charadriiformes, Falconiformes, Passeriformes, Podicipediformes, Procelariformes, Psittaciformes, Strigiformes and Suliformes;

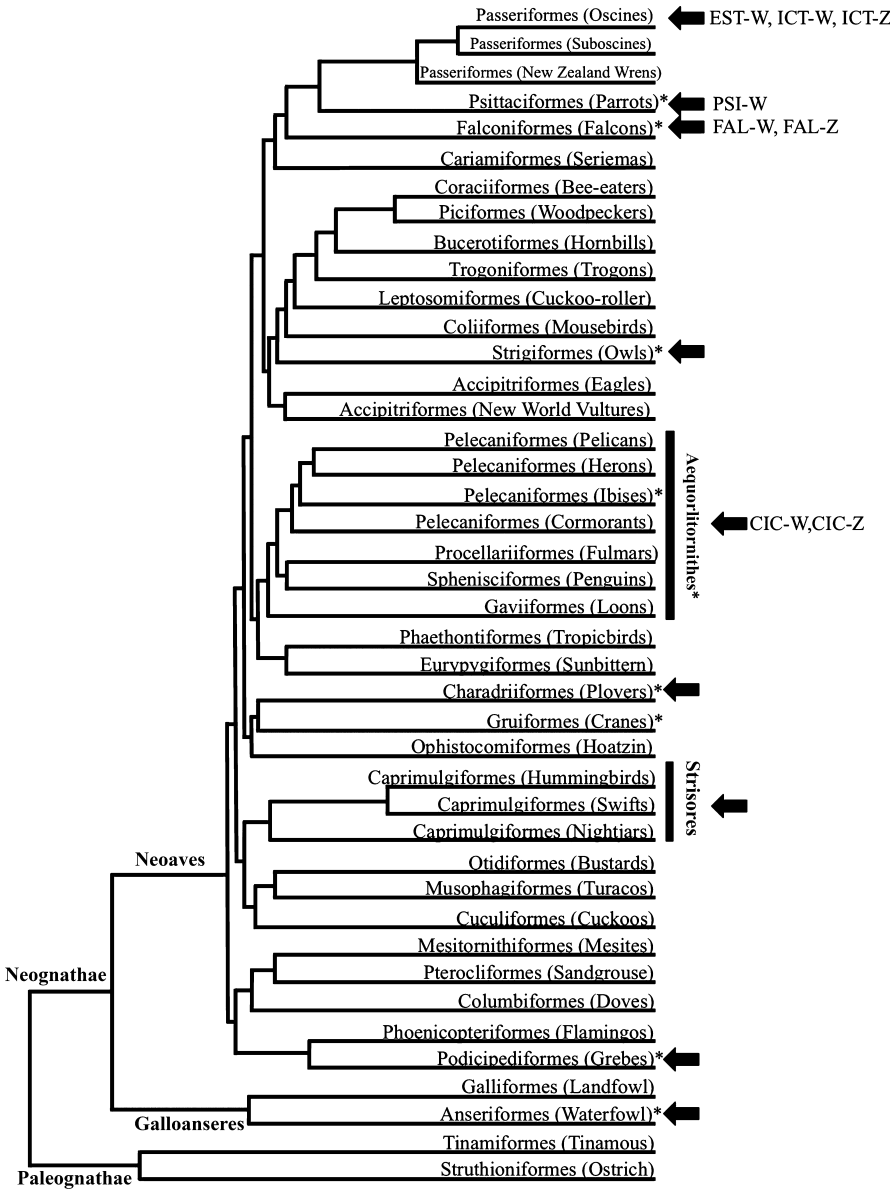

FIGURE 1 LAMP-based sex determination across the genome-scale phylogeny of birds (modified from Jarvis et al., 2014). The original total evidence nucleotide tree (TENT) by Jarvis et al. (2014) was built using the Exascale Maximum Likelihood (ExaML) code for phylogenetic inference applied to a data set partitioned in introns, UCEs, and first and second exon codon positions (third position excluded). Black arrows indicate bird Orders where primer set NEO-W differentiated females from males. Names on the right side of black arrows are successful primer sets for specific bird Families (Estrildidae: EST-W; Icteridae: ICT-W, ICT-Z; Psittacidae: PSI-W; Ciconiidae: CIC-W, CIC-Z) and the Order Falconiformes (FAL-W, FAL-Z). An asterisk denotes positive amplification using primer set UCE. Aequorlitornithes and Strisores are Neoavian sister clades supported by Prum et al. (2015) where species of Ciconiidae (Order Pelecaniformes), Sulidae (Order Suliformes) (Aequorlitornithes) and Apodidae (Order Caprimulgiformes) (Strisores) are included

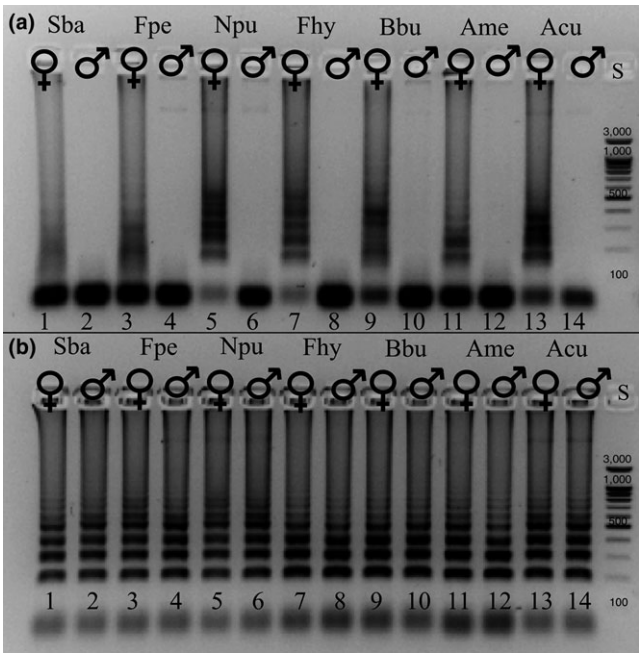

FIGURE 2 Electrophoresis (2.5% agarose) of LAMP products obtained using the primers set NEO-W (a) and UCE (b). Samples: female and male of Northern Gannet (*Sula bassana*) (Sba), Peregrine Falcon (*Falco peregrinus*) (Fpe), Turquoise Parrot (*Neophema pulchella*) (Npu), European Pied Flycatcher (*Ficedula hypoleuca*) (Fhy), Eurasian Eagle-owl (*Bubo bubo*) (Bbu), Alpine Swift (*Apus/Tachymarptis melba*) (Ame) and Burrowing Owl (*Athene cunicularia)* (Acu). Odd-numbered lanes (1 to 13) (a) show the laddered pattern characteristic of LAMP products as a result of the amplification of CHD-W in females. Even-numbered lanes (2 to 14) correspond to males lacking the sexual W chromosome and, therefore, showing no amplification using this primer set. Primer set UCE (b) amplifies in both females and males. S: size standard from 100 to 1,000 bp. and a last fragment of 3,000 bp

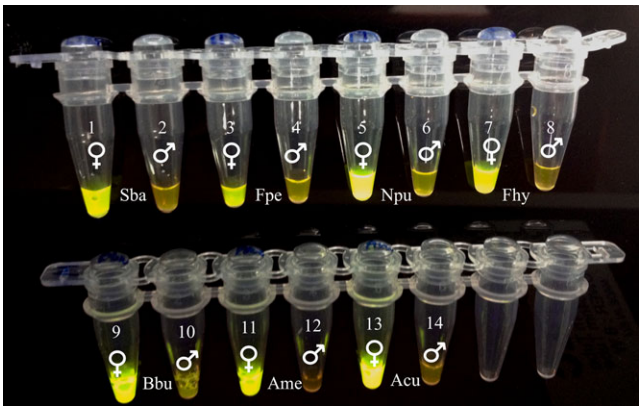

FIGURE 3 LAMP-based sex determination and visualization with the unaided eye in seven different avian Orders. (See Figure 2 for name codes)

see Figure 1). We hypothesize that despite primers anneal in highly conserved regions, the annealing conditions vary between taxa due to the nucleotide variation, which influences relevant LAMP standards such as melting temperature, change in free energy and GC content. The primer set designed to target the ultraconserved element (UCE, see Section 3.2) amplified in nine of these ten Orders (all orders except Procelariformes) as well as in Gruiformes and Pelecaniformes (see Table 2; Figures 1, 2 and 3). Even though UCE was designed based on Neoaves species (i.e., all Neognathae except Galloanseres—Galliformes and Anseriformes), this marker successfully amplified in the Southern screamer (*Chauna torquata*) (Oken, 1816) of the Order Anseriformes, suggesting its suitability for all modern birds.

3.2 | Applying primer sets for particular Orders and Families (Objective 3)

Primer sets designed specifically for the families Ciconiidae, Estrildidae, Icteridae and Psittacidae (hereafter CIC-W, EST-W, ICT-W and PSI-W, respectively) and the Order Falconiformes (FAL-W) correctly assigned sex to the evaluated species. Nonetheless, with the exception of Ciconiidae [CIC-W was tested only in *Ciconia ciconia*) (Linnaeus, 1758)], sex determination in at least one species within the taxon under evaluation was also possible using NEO-W (see Table 2).

The optimal temperature for incubation was not constant across taxa and markers and needed to be determined experimentally. For example, it varied between markers (e.g., CIC-W and CIC-Z required different temperatures), between markers within species (e.g., CIC-W amplified in White Storks at any temperature between 59 and 65°C, whereas amplification with CIC-Z was restricted to 63°C) and within markers between species (e.g., NEO-W discriminates males from females in Northern Gannet (*Sula bassana*) (Linnaeus, 1758) and Burrowing Parrot (*Cyanoliseus patagonus*) (Vieillot, 1818) after incubation at 51 and 63°C, respectively).

Overall, using any of the CHD-W-based primer sets presented in this work, we observed clear sex-based differential amplification across species of 20 Families of birds (all tested families except Gruidae, Laridae and Threskiornithidae) within 11 Orders (all tested orders except Gruiformes) (Tables 1 and 2; Figure 1). The combination of any of the female-specific markers with an ultraconserved element located within chromosome 6 (UCE) or any of the newly designed markers located on the Z chromosome of Ciconiidae, Icteridae or Falconiformes (CIC-Z, ICT-Z and FAL-Z, respectively) allowed for the molecular discrimination between males and females. For a better organization of our results, we only show LAMP primer sets for taxa where sex determination was achieved in at least one species.

3.3 | Reliability of LAMP (Objective 4)

With respect to reliability and error rate, sex determination of the 24 individuals of *Ficedula hypoleuca* (Order Passeriformes) using NEO-W fully matched PCR-based sex determination and accurately assigned sex to all individuals (15 males and 9 females).

4 | DISCUSSION

We have optimized the first LAMP-based sex determination and show it to be applicable to a range of species across the bird phylogeny and most likely to all modern bird species (Neognathae). A full battery of primer sets is now accessible to determine sex of individuals across different taxonomic levels. The aim of this study was not to just provide a list of LAMP conditions per species, because there are more than ten thousand bird species and because optimal LAMP conditions for a given species may vary between laboratories and the amount and source of DNA and inhibitors (see below), as is the case for PCR. Instead, we selected 12 Orders and designed a primer set based on conserved genetic similarity within CHD-W (NEO-W). This primer set yielded a high probability of successful molecular discrimination between females and males for species across the avian phylogeny. Primer set NEO-W was confirmed as a marker for sex determination, and is expected to be valid for a high proportion of the avian diversity. Additionally, we also show how primer sets can be developed and tested for lower taxonomic levels, so that LAMP can be tailored to specific study systems (see Section 3.2). The validation and reliability of LAMP applied to DNA extracted from feathers or faecal samples have been tested elsewhere (Centeno-Cuadros et al., 2017; Cho, Kang, & Park, 2006; Salant, Abbasi, & Hamburger, 2012). Here, we place our results into context and provide a few guidelines for troubleshooting for newly tested samples/species.

The primer set NEO-W (designed and based on genetic similarity within CHD-W between 12 Orders) correctly identified sex across 10 of 12 tested avian Orders, including songbirds (Passeriformes, comprising more than a half of the species within Aves; Figure 1). Although there are 42 Orders within Neognathae (Jarvis et al., 2014), this is a promising result due to the relatively high success rate (see Table 2), and also because the ten Orders tested here are distributed along the bird phylogeny, reinforcing the choice and identity of the CHD-W region selected for this work. Likewise, primer set UCE amplified an ultraconserved element located in chromosome 6 in both males and females in at least one species in 10 of the 12 tested orders (see Table 2) and poses it as a suitable marker to be used as positive control in LAMP-based sex determination. For those species that did not yield positive amplification using primer set UCE, we suggest to use the guidelines reported above for primer design based on any of the ultraconserved elements reported in McCormack et al. (2013).

We also developed and successfully tested some primers for specific Orders and Families that include species with conservation and economic interests, such as raptors (Falconiformes), parrots (Psittacidae), storks (Ciconiidae), estrildid finches (Estrildidae) and New World Blackbirds (Icteridae). Success rate in LAMP-based sex determination increased significantly when LAMP primers were designed to taxonomic levels lower than Orders. For example, primer set PSI-W was tested in 11 species of Psittacidae (see Table 2) and it efficiently discriminated between females and males in seven species. Nonetheless, caution must be taken prior to rejection of any primer set, as a failure in a LAMP-based sex determination can be attributed to lack of further optimization of the technique (incubation time and temperature) (see Section 4.1 below). The Family Psittacidae comprises most of the species within the Order Psittaciformes and the average divergence among genera occurred between 7.5 and 18 Mya (Tavares, Yamashita, & Miyaki, 2004). Another specific primer set for raptors (FAL-W) determined sex in all the nine species of Accipitridae tested, a Family showing ca. 20% of nucleotide divergence in cytochrome *b* (min: 5.0%; max: 34.6%) (Lerner & Mindell, 2005). Interestingly, FAL-W also worked in *Cathartes aura* (Linnaeus, 1758) (Family Cathartidae) confirming the validity of this primer set across taxa within the Order Falconiformes.

A useful technique to determine the sex of individuals (i) should be able to distinguish between males and females, (ii) should do so for all individuals and (iii) should do so without errors. Indeed, the application of NEO-W to a sample of 24 individuals of the European Pied Flycatcher revealed the correct sex of all tested individuals, thereby confirming that LAMP-based sex determination fulfils these three criteria.

The comparison of economic costs/benefits of LAMP over PCR it is not straightforward. PCR-based sex determination is linked to indirect costs such as storage and transportation of samples to molecular laboratories, which makes difficult the comparison between PCR and LAMP. Besides, the cost of some basic reagents and instruments required for both techniques are difficult to estimate (e.g., the production of ultrapure water, power consumption of thermocycler per run. . .). Considering only the price per reaction, we estimated the price per reaction for PCR-based sex determination on 0.50 €/sample (including the electrophoresis in an agarose gel) whereas this price rises to 0.80 € using LAMP (including Sybr Green) [but see, e.g., Pooja, Sudesh, Poonam, Joginder, and Suresh (2014)]. As LAMP-based sex determination requires two reactions (to amplify the sexual marker and the positive control, respectively), the final cost of a LAMP-based sex determination rises to 1.60 € per sample. Consequently, whether LAMP-based sex determination in situ truly worth this three times investment will depend on the study system, access to laboratory facilities and urge. LAMP reactions can be optimized to save *Bst* and Betaine and a different method for the detection of the amplified product will cheapen this technique (e.g., Goto, Honda, Ogura, Nomoto, & Hanaki, 2009). We believe the growing use of LAMP will decrease the cost difference between both techniques. Until then, the choice of LAMP over PCR will be linked to the requirement of an in situ diagnosis tool than economic reasons.

4.1 | Troubleshooting LAMP

This article summarizes the main experiences obtained after running several thousand LAMP reactions using different primer sets, samples and DNA extracts. Despite the growing application of LAMP to other disciplines (e.g., Abbasi, King, Muchiri, & Hamburger, 2010; Fukuta et al., 2014; Nyan et al., 2014) and the technical descriptions of the whole procedure (reviewed in e.g., Zhang, Lowe, & Gooding, 2014), we found almost no how-to or troubleshooting guides helping

new LAMP users to set up LAMP reactions in their study systems (but see, e.g., Notomi et al., 2000, 2015; Tomita et al., 2008). In this section, we provide some basic guidelines to facilitate the implementation of this technique within the Life Sciences.

4.1.1 | DNA

We have observed false-negative LAMP reactions due to DNA excess and/or presence of inhibitors, commonly found when DNA extractions are not followed by purification [e.g., hotshot NaOH protocols (Truett et al., 2000)]. For this reason, we strongly recommend to run LAMP reactions initially under controlled laboratory conditions (especially, purified DNA of known concentration). Only once optimal LAMP reactions are experimentally determined and false positives/negatives can be discarded under controlled conditions, nonpurified DNA and/or dilutions or unknown DNA concentrations can be tested. The optimization of LAMP on noninvasive samples and/or nonpurified DNA extracts based on the newly designed primers fell beyond the scope of this work and further work should be done in this direction. Nonetheless, LAMP has been already applied on noninvasive samples such as feathers (Centeno-Cuadros et al., 2017) and results are promising when applied to other noninvasive sample sources (e.g., faeces; Cho et al., 2006; Salant et al., 2012). It must be noted, though, that crucial parameters such as incubation time or temperature might change depending on the amount of template/inhibitors added. Because of this, future work to deploy LAMP under field conditions must focus on developing easy, fast, clean and yield-controlled DNA extraction protocols for biological tissues (see e.g., Abu Almakarem, Heilman, Conger, Shtarkman, & Rogers, 2012 for DNA extraction of plant and fungus tissues).

4.1.2 | Primers

As in PCR, primers for LAMP reactions must meet some standards related to melting temperature (T_M), change in free energy (ΔG, related to the stability at the end of the primers), guanine–cytosine content (GC content) and distance between primers (see PrimerExplorer manual at http://primerexplorer.jp/e/ and recommendations therein). LAMP-based amplification of the targeted CHD-W fragment will be influenced by a combination of any of these parameters and, ultimately, to the nucleotide differences between primer/template DNA sequences. Whereas primer design is a straightforward procedure using the PrimerExplorer engine in most of the cases, the user must consider the "manual approach" (see Section 2.2) when dealing with relatively short sequences (e.g., <200 bp). In these situations, we recommend to select the F3/B3 primers at the most upstream/downstream location with a T_M between 55 and 65°C (this temperature will differ from the incubation temperature used for LAMP reaction). F2/B2 and F1c/B1c should ideally be nested within the F3/B3 primers (although some overlap between the primers sequences might also work e.g., see figure 1 in Abbasi et al., 2010) and their T_M must be as similar as possible to the outer (F3/B3) primers. In our experience, running separate PCRs per primer pair (F3/B3 and FIP/BIP) before being used in LAMP reactions gives an idea about how different the annealing (incubation) temperatures might be in LAMP reactions. Nonetheless, outer and inner primer pairs differing in a few degrees on their annealing temperatures can still be used in a LAMP reaction. Last, due to the role of the outer (F3/B3) primers on the formation of loop structures, it is highly recommended to ensure a strong annealing between these primers and the template (which means choosing the more negative ΔG). However, this value would be directly related to the nucleotide composition of the target DNA region and sometimes there is no further choice of ΔG values (e.g., if there is no option to move upstream/downstream the template for the annealing of the primer). In this scenario, lowering the commonly used 1:8 ratio of the outer/inner primers may improve the rapidity and reproducibility of LAMP reactions.

4.1.3 | Incubation time and temperature

Undoubtedly, the optimization of LAMP reactions is necessary to find the optimal incubation time and temperature to discriminate between target and nontarget DNA regions. This temperature usually oscillates between 50 and 65°C and as a "rule of thumb" increasing concentrations of betaine or preservants (e.g., sucrose to stabilize the ready-mix reagents and storage at room temperature, see Hamburger et al. (2013) and Centeno-Cuadros et al. (2017)) tend to increase the incubation temperature. The incubation time must be taken cautiously: whereas false negatives can be avoided by increasing the time of incubation, false positives might also occur after an extended incubation time. These false-positive reactions are frequently explained as a nonspecific amplification or amplification in nontemplate samples due to improperly annealed primers and extendable primer secondary structures. Modifying the concentration of betaine (a reagent decreasing the formation of secondary structures that, therefore, facilitates the displacement activity of the *Bst* polymerase) may help to minimize the occurrence of false positives.

4.1.4 | *Bst* polymerase

The high strand displacement activity of this enzyme allows the auto-cycling strand displacement DNA synthesis required for isothermal amplifications. The wide range of possible future applications of LAMP is promoting the development of novel enzymes, including robust enzymatic activity even in high concentration of inhibitors. Caution must be taken when LAMP users wish to switch between *Bst* polymerases, as LAMP conditions may change considerably.

5 | CONCLUSIONS AND FUTURE WORK

We have successfully designed and tested 10 primer sets for loop-mediated isothermal amplification based on conserved regions of the

avian sexual Z and W chromosomes and an ultraconserved element on the autosomal chromosome 6 (Tables 1 and 2). The combination of these primers allows sex determination across different taxonomic levels within Neognathae, including the speciose songbirds (Passeriformes) and taxa of high conservation and economic interests such as raptors (Falconiformes) or parrots (Psittacidae). A generalist primer set (NEO-W) successfully identifies male and female individuals across most of the tested avian Orders. This finding validates the primer set as a good candidate for molecular sexing of birds across the avian phylogeny, and validates the targeted region on the W chromosome as suitable for a taxonomically more specific primer redesign where desired. Due to the simplicity, rapidity, accuracy and proven portability of LAMP, we are reporting here the first kit of molecular markers to allow researchers, wildlife ecologists, ornithologists, and commercial and private aviculturists to determine in situ the sex of most of individuals of birds in less than 2 hr (including DNA extraction, LAMP reaction and LAMP product staining). As far as we know, this is the first time LAMP primers have been designed and used to target a conserved DNA region in such a wide diversity of taxa. After the proof of principle reported by Centeno-Cuadros et al. (2017), this study thereby represents the crucial next step towards the application of LAMP for molecular sex determination in birds. Moreover, this application is extensible to other Classes (e.g., mammals species once the SRY marker is sequenced) and to other applications (e.g., detection of cryptic species or environmental DNA), confirming that LAMP is a taxonomically general, reliable and potent alternative to PCR.

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DATA ACCESSIBILITY

CHD-W and CHD-Z sequences obtained in this study are deposited in GenBank (Accession nos KY441616 to KY441639).

AUTHOR CONTRIBUTIONS

A.C.-C. conceived the study, designed the methodology and performed the laboratory work; J.L.T. and M.C. collected the samples; A.C.-C., M.C., M.D. and P.E. supported the project; A.C.-C. led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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