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

A. Centeno-Cuadros^{1,2,*}, J.L. Tella², M. Delibes², P. Edelaar¹ and M. Carrete³

¹ Department of Molecular Biology and Biochemical Engineering, University Pablo de Olavide. Seville (Spain)

² Department of Conservation Biology, Estación Biológica de Doñana (EBD-CSIC), Avenida Américo Vespucio, s/n, Isla de la Cartuja, 41092. Seville (Spain)

³ Department of Physical, Chemical and Natural Systems, University Pablo de Olavide. Seville (Spain)

* Corresponding author: Alejandro Centeno-Cuadros (email: acenteno@upo.es)

Running title: Fast and portable sex determination of birds.

Keywords: Loop-mediated isothermal amplification, CHD-W, CHD-Z, Ultra-Conserved Element, Neognathae, Aves, sexual chromosomes.

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. Since 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.

38 **Introduction**

39 The determination of the sex of individuals is often key in ecological and
40 evolutionary studies, as well as for commercial and conservation purposes. Thus,
41 several methods have been developed to sex individuals based on behavior (Gray &
42 Hamer 2001), vocalizations (Volodin *et al.* 2009), morphology (Reynolds *et al.* 2007),
43 genitals and sex organs (Maron & Myers 1984; Richner 1989) and hormone levels
44 (Bercovitz *et al.* 1978). However, these methods can have high error rates and
45 sometimes are technically complex or highly invasive. In recent decades, the
46 development of molecular techniques facilitated an accurate and relatively harmless sex
47 determination, using small tissue samples obtained through low invasive methods (e.g.,
48 blood extraction) or even non-invasive sampling (e.g., molted feathers, fur and feces).
49 In birds, sex determination is based on chromosomal differences between males
50 (homogametic sex, two copies of Z chromosome) and females (heterogametic sex, one
51 copy of Z and W chromosomes) (Harris & Walters 1982). PCR-based techniques for
52 sex determination mostly rely on the amplification of the sex-chromosome-specific
53 gene Chromo-Helicase-DNA binding protein (*CHD*), located on the sexual W and Z
54 chromosomes (*CHD-W* and *CHD-Z*, respectively) (Griffiths *et al.* 1998; Fridolfsson &
55 Ellegren 1999). *CHD-W* and *CHD-Z* show different intron sizes and nucleotide
56 composition, so the PCR amplification using specific primers and posterior fragment
57 isolation by electrophoretic methods serves for sex determination. This standard
58 methodology is nowadays widely used across bird taxa (Morinha *et al.* 2012; Vucicevic
59 *et al.* 2013) and different primers sets have been developed, based on differences in
60 nucleotide composition in regions where PCR primers anneal (Griffiths *et al.* 1998;
61 Fridolfsson & Ellegren 1999; Wang & Zhang 2009; Lee *et al.* 2010; Wang *et al.* 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. Despite high-resolution melting analysis is an accurate technique for sex determination in birds (Morinha *et al.* 2013; Faux *et al.* 2014), 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 *et al.* 2000, 2015) 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, e.g. the detection of cryptic species or the monitoring of diseases and parasites. Furthermore, since 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 non-invasive samples, museum samples or eDNA, and may do so both in the lab or 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 *et al.* 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 thermo-block 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 *et al.* 2001), pH-sensitive dyes (Tanner *et al.* 2015) or metal indicators (Tomita *et al.* 2008). Consequently, it does not require any special lab 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 *et al.* (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 minutes, 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, Podicipediformes, Procellariiformes, Psittaciformes, Strigiformes and Suliformes) (Objective 1). In addition we designed a primer set to amplify an ultra-conserved 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). Since 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 *et al.* 2010), and knowing the sex of individuals can be crucial for both *ex situ* and *in situ* research and conservation programs (Lambertucci *et al.* 2013; Centeno-Cuadros *et al.* 2017). 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 *et al.* 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.

Material and methods

A “primer” on primer design for LAMP

We designed the forward and backwards-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. Since our interest was focused on primer design applicable across different taxonomic levels (Orders and Families), we first aligned all available sequences per taxon (see below) 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 v.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.

Primer design for sex determination

The female-specific marker for sex determination of species within the superorder Neognathae (primer set “NEO-W”, see Table 1 and Table 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 2246 and 2445 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 v.4, respectively.

We used a 461 bp fragment of highly conserved DNA in the so-called ultra-conserved 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 (Müllénbach *et al.* 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 numbers 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 v.4.

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 lab 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 1M, 8 units of Bst 2.0 (New England Biolabs), 1x enzyme buffer (20 mM Tris-HCl, 10 mM (NH₄)₂SO₄, 50 mM KCl, 2 mM MgSO₄, 0.1% Tween[®] 20, pH 8.8), 1.6 μM and 0.2 μM of internal and external primers (FIP-BIP and F3-B3, respectively), 2 μL of DNA extract and fill with molecular biology grade H₂O 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 MgCl₂ 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°C and 63°C. Therefore, we first fixed the incubation time to 60 minutes and tried every primer set in a range of temperatures (57°C, 59°C, 61°C, 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 minutes). 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 color 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).

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 minutes at 59°C. Sex determination based on LAMP results were done blind with respect to known sex of individuals.

Results

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, Procellariiformes, Psittaciformes, Strigiformes and Suliformes) (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 (see below). The primer set designed to target the ultra-conserved element (UCE, see Material and Methods) amplified in nine out of these ten Orders (all orders except Procellariiformes) as well as in Gruiformes and Pelecaniformes (see Figure 1, Figure 2 and Figure 3 and Table 2). 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.

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°C 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°C 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) (Figure 1, Table 1, Table 2). The combination of any of the female-specific markers with an ultra-conserved 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.

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).

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 Material and Methods). The validation and reliability of LAMP applied to DNA extracted from feathers or fecal samples has been tested elsewhere (Cho *et al.*

2006; Salant *et al.* 2012; Centeno-Cuadros *et al.* 2017). 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 out 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 ultra-conserved element located in chromosome 6 in both males and females in at least one species in 10 out 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 ultra-conserved 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, since a failure in a LAMP-based sex determination can be attributed to lack of further optimization of the

technique (incubation time and temperature) (see “Troubleshooting LAMP” 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 *et al.* 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, (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 fulfills 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
ultra-pure 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 *et al.* (2014)].
Since 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 lab 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 *et al.* 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.

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 *et al.* 2010; Nyan *et al.* 2014; Fukuta *et al.* 2014) and the technical descriptions of the whole procedure (reviewed in e.g. Zhang *et al.* 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.

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 lab 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, non-purified DNA and/or dilutions or unknown DNA

concentrations can be tested. The optimization of LAMP on non-invasive samples and/or non-purified 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 non-invasive samples such as feathers (Centeno-Cuadros *et al.* 2017) and results are promising when applied to other non-invasive sample sources (e.g. feces) (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 *et al.* 2012 for DNA extraction of plant and fungus tissues).

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 Material and Methods) 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 55-65°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.

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 non-target DNA regions. This temperature usually oscillates between 50-65°C and as a “rule of thumb” increasing concentrations of betaine or preservatives (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 non-template 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.

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, since LAMP conditions may change considerably.

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 ultra-conserved element on the autosomal chromosome 6 (Table 1 and Table 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 two hours (including DNA extraction, LAMP reaction

and LAMP product staining). As far as we known, 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.

Acknowledgements

We are in debt with Mónica Gutiérrez and Ana Píriz for their help, support, laboratory assistance, expertise and encouragement during the experimental design and laboratory work. We also thank David Canal for providing samples of the European Pied Flycatcher and the Laboratorio de Ecología Molecular, Estación Biológica de Doñana, CSIC (LEM-EBD) for logistical support. This work was funded by the Severo Ochoa Award and Fundación REPSOL. A.C-C. was granted by the University Pablo de Olavide. P.E. acknowledges the Spanish Ministry of Economy and Competitiveness for financial support (RYC-2011-07889, with support from the European Regional Development Fund).

REFERENCES

- Abbasi I, King CH, Muchiri EM, Hamburger J (2010) Detection of *Schistosoma mansoni* and *Schistosoma haematobium* DNA by loop-mediated isothermal amplification: identification of infected snails from early prepatency. *The American Journal of Tropical Medicine and Hygiene*, **83**, 427–32.
- Abellán P, Carrete M, Anadón JD, Cardador L, Tella JL (2016) Non-random patterns and temporal trends (1912-2012) in the transport, introduction and establishment

477 of exotic birds in Spain and Portugal. *Diversity and Distributions*, **22**, 263–273.

478 Abu Almakarem AS, Heilman KL, Conger HL *et al.* (2012) Extraction of DNA from
479 plant and fungus tissues in situ. *BMC Research Notes*, **5**, 266.

480 Bantock TM, Prys-Jones RP, Lee PLM (2008) New and improved molecular sexing
481 methods for museum bird specimens. *Molecular Ecology Resources*, **8**, 519–28.

482 Centeno-Cuadros A, Abbasi I, Nathan R (2017) Sex determination in the wild: a field
483 application of Loop-Mediated Isothermal Amplification successfully determines
484 sex across three raptor species. *Molecular Ecology Resources*, **17**, 153–160.

485 Cho H-S, Kang J-I, Park N-Y (2006) Detection of Canine Parvovirus in Fecal Samples
486 using Loop-Mediated Isothermal Amplification. *Journal of Veterinary Diagnostic
487 Investigation*, **18**, 81–84.

488 Donald PF, Collar NJ, Marsden SJ, Pain DJ (2010) *Facing extinction*. T & D Poyser,
489 London.

490 Ellegren H (1996) First Gene on the Avian W Chromosome (CHD) Provides a Tag for
491 Universal Sexing of Non-Ratite Birds. *Proceedings of the Royal Society of London
492 B: Biological Sciences*, **263**, 1635–1641.

493 Faux CE, McInnes JC, Jarman SN (2014) High-throughput real-time PCR and melt
494 curve analysis for sexing Southern Ocean seabirds using fecal samples.
495 *Theriogenology*, **81**, 870–874.

496 Fridolfsson A-K, Ellegren H (1999) A simple and universal method for molecular
497 sexing of non-ratite birds. *Journal of Avian Biology*, **30**, 116–121.

498 Fukuta S, Takahashi R, Kuroyanagi S *et al.* (2014) Development of loop-mediated
499 isothermal amplification assay for the detection of *Pythium myriotylum*. *Letters in
500 Applied Microbiology*, **59**, 49–57.

501 Goto M, Honda E, Ogura A, Nomoto A, Hanaki K-I (2009) Colorimetric detection of
502 loop-mediated isothermal amplification reaction by using hydroxy naphthol blue.
503 *BioTechniques*, **46**, 167–172.

504 Gray CM, Hamer KC (2001) Food-provisioning behaviour of male and female Manx
505 shearwaters, *Puffinus puffinus*. *Animal Behaviour*, **62**, 117–121.

506 Griffiths R, Daan S, Dijkstra C (1996) Sex identification in birds using two CHD genes.
507 *Proceedings of the Royal Society of London B: Biological Sciences*, **263**, 1251–
508 1256.

509 Griffiths R, Double MC, Orr K, Dawson RJG (1998) A DNA test to sex most birds.
510 *Molecular Ecology*, **7**, 1071–1075.

511 Hamburger J, Abbasi I, Kariuki C *et al.* (2013) Evaluation of loop-mediated isothermal
512 amplification suitable for molecular monitoring of schistosoma-infected snails in
513 field laboratories. *The American Journal of Tropical Medicine and Hygiene*, **88**,
514 344–51.

515 Han J-I, Kim J-H, Kim S, Park S-R, Na K-J (2009) A simple and improved DNA test

516 for avian sex determination. *The Auk*, **126**, 779–783.

517 Harris T, Walters C (1982) Chromosomal sexing of the Black Shouldered Kite (*Elanus*
518 *caeruleus*) (Aves: Accipitridae). *Genetica*, **60**, 19–20.

519 Jarvis ED, Mirarab S, Aberer AJ *et al.* (2014) Whole-genome analyses resolve early
520 branches in the tree of life of modern birds. *Science*, **346**, 1320–1331.

521 Lambertucci SA, Carrete M, Speziale KL, Hiraldo F, Donázar JA (2013) Population sex
522 ratios: another consideration in the reintroduction - reinforcement debate? *PloS one*,
523 **8**, e75821.

524 Lee P (2017) DNA amplification in the field: move over PCR, here comes LAMP.
525 *Molecular Ecology Resources*, **17**, 138–141.

526 Lee JC-I, Tsai L-C, Hwa P-Y *et al.* (2010) A novel strategy for avian species and
527 gender identification using the CHD gene. *Molecular and Cellular Probes*, **24**, 27–
528 31.

529 Lerner HRL, Mindell DP (2005) Phylogeny of eagles, Old World vultures, and other
530 Accipitridae based on nuclear and mitochondrial DNA. *Molecular Phylogenetics*
531 *and Evolution*, **37**, 327–346.

532 Maron JL, Myers J. (1984) A description and evaluation of two techniques for sexing
533 wintering sanderlings. *Journal of Field Ornithology*, **55**, 336–342.

534 McCormack JE, Harvey MG, Faircloth BC *et al.* (2013) A phylogeny of birds based on
535 over 1,500 loci collected by target enrichment and high-throughput sequencing.
536 *PloS One*, **8**, e54848.

537 Mori Y, Nagamine K, Tomita N, Notomi T (2001) Detection of loop-mediated
538 isothermal amplification reaction by turbidity derived from magnesium
539 pyrophosphate formation. *Biochemical and Biophysical Research Communications*,
540 **289**, 150–4.

541 Morinha F, Cabral JA, Bastos E (2012) Molecular sexing of birds: A comparative
542 review of polymerase chain reaction (PCR)-based methods. *Theriogenology*, **78**,
543 703–714.

544 Morinha F, Travassos P, Seixas F *et al.* (2013) High-resolution melting analysis for bird
545 sexing: a successful approach to molecular sex identification using different
546 biological samples. *Molecular ecology resources*, **13**, 473–83.

547 Müllenbach R, Lagoda PJ, Welter C (1989) An efficient salt-chloroform extraction of
548 DNA from blood and tissues. *Trends in Genetics*, **5**, 391.

549 Notomi T, Mori Y, Tomita N, Kanda H (2015) Loop-mediated isothermal amplification
550 (LAMP): principle, features, and future prospects. *Journal of Microbiology*, **53**, 1–
551 5.

552 Notomi T, Okayama H, Masubuchi H *et al.* (2000) Loop-mediated isothermal
553 amplification of DNA. *Nucleic Acids Research*, **28**, E63.

554 Nyan D-C, Ulitzky LE, Cehan N *et al.* (2014) Rapid Detection of Hepatitis B Virus in

555 Blood Plasma by a Specific and Sensitive Loop-Mediated Isothermal
556 Amplification Assay. *Clinical Infectious Diseases*, **59**, 16–23.

557 Pooja S, Sudesh D, Poonam K, Joginder SD, Suresh KG (2014) Loop-mediated
558 isothermal amplification (LAMP) based detection of bacteria: A Review. *African*
559 *Journal of Biotechnology*, **13**, 1920–1928.

560 Prum RO, Berv JS, Dornburg A *et al.* (2015) A comprehensive phylogeny of birds
561 (Aves) using targeted next-generation DNA sequencing. *Nature*, **526**, 569–573.

562 Richner H (1989) Avian laparoscopy as a field technique for sexing birds and an
563 assessment of its effects on wild birds. *Journal of Field Ornithology*, **60**, 137–142.

564 Salant H, Abbasi I, Hamburger J (2012) The development of a loop-mediated
565 isothermal amplification method (LAMP) for *Echinococcus granulosus*
566 coprodetction. *The American journal of tropical medicine and hygiene*, **87**, 883–7.

567 Tanner NA, Zhang Y, Evans Jr TC (2015) Visual detection of isothermal nucleic acid
568 amplification using pH-sensitive dyes. *BioTechniques*, **58**, 59–68.

569 Tavares ES, Yamashita C, Miyaki CY (2004) Phylogenetic relationships among some
570 Neotropical parrot genera (Psittacidae) based on mitochondrial sequences. *The Auk*,
571 **121**, 230–242.

572 Tomita N, Mori Y, Kanda H, Notomi T (2008) Loop-mediated isothermal amplification
573 (LAMP) of gene sequences and simple visual detection of products. *Nature*
574 *Protocols*, **3**, 877–882.

575 Truett GE, Heeger P, Mynatt RL *et al.* (2000) Preparation of PCR-quality mouse
576 genomic DNA with hot sodium hydroxide and tris (HotSHOT). *BioTechniques*, **29**,
577 52, 54.

578 Untergasser A, Cutcutache I, Koressaar T *et al.* (2012) Primer3--new capabilities and
579 interfaces. *Nucleic Acids Research*, **40**, e115.

580 Volodin I, Kaiser M, Matrosova V *et al.* (2009) The technique of noninvasive distant
581 sexing for four monomorphic *Dendrocygna* whistling duck species by their loud
582 whistles. *Bioacoustics*, **18**, 277–290.

583 Vucicevic M, Stevanov-Pavlovic M, Stevanovic J *et al.* (2013) Sex determination in 58
584 bird species and evaluation of CHD gene as a universal molecular marker in bird
585 sexing. *Zoobiology*, **32**, 269–76.

586 Wang N, Zhang Z-W (2009) The novel primers for sex identification in the brown
587 eared-pheasant and their application to other species. *Molecular Ecology*
588 *Resources*, **9**, 186–8.

589 Wang Z, Zhou X, Lin Q, Fang W, Chen X (2011) New primers for sex identification in
590 the Chinese egret and other ardeid species. *Molecular Ecology Resources*, **11**, 176–
591 9.

592 Zhang X, Lowe SB, Gooding JJ (2014) Brief review of monitoring methods for loop-
593 mediated isothermal amplification (LAMP). *Biosensors & Bioelectronics*, **61**, 491–
594 9.

596 **DATA ACCESSIBILITY**

597 CHD-W and CHD-Z sequences obtained in this study are deposited in GenBank

598 (Accession Numbers KY441616 to KY441639)

599

600 **AUTHOR CONTRIBUTIONS**

601 A.C-C. conceived the study, designed the methodology and performed the laboratory

602 work; J.L.T and M.C. collected the samples; A.C-C., M.C., M.D. and P.E. supported the

603 project; A.C-C. led the writing of the manuscript. All authors contributed critically to

604 the drafts and gave final approval for publication.

605

606

607 **Table 1.** Primer sets designed for sex determination within different taxa of Neognathae (names within parentheses). F3 = forward external
608 primer; B3 = backwards external primer; FIP = forward internal primer composed by F1c and F2 primers connected by TTTT (**bold**); BIP =
609 backward internal primer composed by B1c and B2 primers connected by TTTT (**bold**).

610	PRIMER SET	PRIMER	PRIMER SEQUENCE (5'-3')
611	CIC-W (Fam. Ciconiidae)	F3	CTCAATTGCCAAAACAATTGG
612		B3	GAATTTTGCTGGTAGTAGCC
613		FIP	CTGGCAATTACTATATGCCAAACAGTATTT TTTT TGGGGATAAGAGTAATGTAACACT
614		BIP	AACTTCCATAAATCTTTCTACAAAAGGACT TTTT AGAAACCTTGATCTTTACCACTT
615	CIC-Z (Fam. Ciconiidae)	F3	TCACAGAAGATGGAGATTCC
616		B3	CAACAGAGTTCTGATTTTCTCA
617		FIP	GCCAAGAAGCTTTGGTCTTGAAC TTTT CTCTGGACAACCTTGTTTCAGT
618		BIP	ACTACCACCAAGATTCATACCTGAT TTTT TCAGATGGTGAGGATGCTG
619	EST-W (Fam. Estrildidae)	F3	ACTCCTATTATAGTCTATCCGGAGCA
620		B3	CCTTTTCAGGTAAGAATTTTGCTGG
621		FIP	GAAGTGTTACATTATTCTTATTCCTCCCCC TTTT TTGAATTCTCAGTTGCCAAACCAAT
622		BIP	GTGTCCTTTTTGTAGAAAGATTTATGAAAG TTTT AGAAGCCTTGATTTTACCATTTTATCTT
623	FAL-W (Order Falconiformes)	F3	AAATGTTTTAGTCACGTAGCT
624		B3	TCTGCATCGCTAAATCCTT
625		FIP	CTTCTGCTCCTACTGCGTTTCT TTTT TAATCTGAAATTCCAGATCAGCT
626		BIP	ATTCTGGATCTGATAGTGACTCCAT TTTT ATTTTCTCGACGAGTAGTTCG
627	FAL-Z (Order Falconiformes)	F3	CATATTTTGGACAGGCTAGGTAA
628		B3	CGCTAAATCCTTTAATATTTTCTCG
629		FIP	TCACTTCCATTAAAGCTGATCTGG TTTT TGTTGTTAATCACGTAGCTTTGA
630		BIP	CGCAGTAGGAGCAGAAGATACT TTTT TCAGTTTTTTAGGCCGTT
631			
632			
633			
634			
635			

636	PRIMER SET	PRIMER	PRIMER SEQUENCE (5'-3')
637	ICT-W (Fam. Icteridae)	F3	TGCCAAACCAATTGGGTG
638		B3	TCAGGTAAGAATTTTGCTGG
639		FIP	GACTAGTAATTCCTATATGCCTAATAGTATTTTTGGGGATATAAGAATAATGTAACACT
640		BIP	AACTTTCATAAATCTTTCTACAGAAAGGACTTTTCAAGAAGCCTTGATTTTTACCAT
641	ICT-Z (Fam. Icteridae)	F3	CATAATCATAAACAAAGCTCAAAGG
642		B3	TCTTTACTGTGTGGGTGTC
643		FIP	CASCATGCTTTAGCTGTCCCTTTTACTGCTGAAAGTCATCTTGTC
644		BIP	CCAAGGTCATGTCCAAGTAGCTTTTAAAGCACTGGAACAGGTT
645	NEO-W (Neognathae)	F3	CATGTAGCTTTGAACTACTTAATCT
646		B3	TGCATCGCTAAATCCTTT
647		FIP	GAGTCACTATCAGATCCAGAATATCTTCTTTTGATCAGCTTTAATGGAAGTGAA
648		BIP	AGTGACTCCATCTCAGAAAGAAAACTTTCTCGGTCTTCCACGTTTT
649	PSI-W (Fam. Psittacidae)	F3	CAGTTTCCCTTTCAGGTAAG
650		B3	TCAGTTGCCAAAACAATGG
651		FIP	TTCTTCACAAAGGACACTTTTCTTTTGTAGTAGCCAAGAAGCCTT
652		BIP	AGGAAAAGACTGGCAATTACTATATGCTAATTTTGGGGAGATAAGATTAATGTAACA
653	UCE (Neoaves)	F3	GGGAAACAAGGATAAAATTACTCC
654		B3	TGCCCAGAAAATTCCATTC
655		FIP	CGAGTGTGTTAAGCACAGTTTTATTTTATGGTTAATGACCTATAGTATCTCC
656		BIP	GAGGACTGTTCTGCAGGGTATTTTGTGCTATCTGATTCGAAAAGTC
657			
658			
659			
660			
661			
662			

663

664 **Table 2.** Incubation temperatures for LAMP reactions of different primer sets for a set of species across different Families and Orders. **PCR:**
665 reference for PCR-based sex determination. **PRIMER SET (T):** name and range of incubation temperatures (between parentheses) for each
666 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.
667 The asterisk (*) indicates the LAMP reaction was run with the enzyme buffer EB2 (see text).

						PRIMER SET (T)	
	ORDER	FAMILY	SCIENTIFIC NAME	COMMON NAME	PCR^a	SEX	C+
668							
669							
670							
671	Anseriformes	Anatidae	<i>Anas platyrhynchos</i>	Common Mallard	[1]	NEO-W (58*)	-
672							
673		Anhimidae	<i>Chauna torquata</i>	Southern Screamer	[1]	NEO-W (58-61*)	UCE (57-63)
674							
675	Caprimulgiformes	Apodidae	<i>Tachymarptis melba</i>	Alpine Swift	[2]	NEO-W (55)	UCE (59-63)
676							
677	Charadriiformes	Glareolidae	<i>Cursorius cursor</i>	Cream-coloured Courser	M5 [3]; P8 [4]	NEO-W (67)	-
678							
679		Laridae	<i>Larus michahellis</i>	Yellow-legged Gull	M5 [3]; P8 [4]	-	UCE (57-63)
680							
681	Falconiformes	Accipitridae	<i>Accipiter gentilis</i>	Eurasian Goshawk	[2]	FAL-W (63)	-
682							
683			<i>Aquila chrysaetos</i>	Golden Eagle	[2]	FAL-W (61)	-
684							
685			<i>Buteo buteo</i>	Common Buzzard	[1]	FAL-W (59-63)	-
686							
687			<i>Gypaetus barbatus</i>	Bearded Vulture	[1]	FAL-W (59-63)	-
688							
689							

						PRIMER SET (T)	
	ORDER	FAMILY	SCIENTIFIC NAME	COMMON NAME	PCR ^a	SEX	C+
690	Falconiformes (cont.)	Accipitridae	<i>Gyps fulvus</i>	Griffon Vulture	[1]	FAL-W (62-63)	-
691			<i>Gyps rueppellii</i>	Rüppell's Vulture	[1]	FAL-W (59-63)	-
692			<i>Milvus migrans</i>	Black Kite	[2]	FAL-W (62-63)	FAL-Z (59)
693			<i>Milvus milvus</i>	Red Kite	[2]	FAL-W (61)	-
694			<i>Neophron percnopterus</i>	Egyptian Vulture	M5 [3]; P8 [4]	FAL-W (60-63)	-
695		Cathartidae	<i>Cathartes aura</i>	Turkey Vulture	[1]	FAL-W (59-63)	-
696			<i>Falco peregrinus</i>	Peregrine Falcon	[5]	NEO-W (53/58-61*)	UCE (61)
697		Gruidae	<i>Anthropoides virgo</i>	Demoiselle Crane	[2]	-	UCE (57-61)
698		Passeriformes	<i>Galerida theklae</i>	Thekla Lark	[7]	NEO-W (56-61*)	-
699			<i>Corvus corax</i>	Common Raven	P2 [6]; P8 [4]	NEO-W (58-64*)	-
700			<i>Estrilda melpoda</i>	Orange-cheeked Waxbill	[7]	NEO-W (56-60*), EST-W (56-63)	-
701							
702							
703							
704							
705							
706							
707							
708							
709							
710							
711							
712							
713							
714							
715							
716							
717							
718							
719							

720						PRIMER SET (T)	
721	ORDER	FAMILY	SCIENTIFIC NAME	COMMON NAME	PCR^a	SEX	C+
722	Passeriformes (cont.)	Icteridae	<i>Agelaioides badius</i>	Bay-winged Cowbird	[7]	NEO-W	ICT-Z
723						(56-63*)	(62-65)
724		Muscicapidae	<i>Ficedula hypoleuca</i>	European Pied Flycatcher	P2 [6]; P8 [4]	ICT-W	-
725						(60-63)	
726						NEO-W	-
727	Pelecaniformes	Sturnidae	<i>Leucopsar rotschildi</i>	Bali Myna	[7]	(59/56-61*)	
728						NEO-W	-
729		Ciconiidae	<i>Ciconia ciconia</i>	White Stork	[1]	(56-63*)	
730						CIC-W	CIC-Z
731						(59-65)	(63)
732	Podicipediformes	Threskiornithidae	<i>Plegadis falcinellus</i>	Glossy Ibis	[2]	-	UCE
733							(57-63)
734		Podicipedidae	<i>Podiceps nigricollis</i>	Black-necked Grebe	M5 [3]; P8 [4]	NEO-W	UCE
735						(63/55-61*)	(59-63)
736							
737	Procellariiformes	Procellariidae	<i>Calonectris diomedea</i>	Scopoli's Shearwater	[1]	NEO-W	-
738						(59/55-61*)	
739						NEO-W	-
740						(63)	
741						PSI-W	-
742	Psittaciformes	Psittacidae	<i>Anodorhynchus leari</i>	Lear's Macaw	M5 [3]; P8 [4]	(59-63)	
743						PSI-W	-
744			<i>Primolius auricollis</i>	Yellow-collared Macaw	M5 [3]; P8 [4]	(61)	
745			<i>Ara chloropterus</i>	Red-and-green Macaw	P2 [6]; P8 [4]	PSI-W	-
746			<i>Ara macao</i>	Scarlet Macaw	[3]	(59)	
747						NEO-W	-
748						(61*)	
749							

750						PRIMER SET (T)		
751	ORDER	FAMILY	SCIENTIFIC NAME	COMMON NAME	PCR ^a	SEX	C+	
752								
753	Psittaciformes (cont.)	Psittacidae (cont.)	<i>Aratinga solstitialis</i>	Sun Parakeet	[1]	PSI-W	-	
754							(59)	
755			<i>Psilopsiagon aymara</i>	Grey-hooded Parakeet	[1]	PSI-W	-	
756							(60-63)	
757			<i>Cyanoliseus patagonus</i>	Burrowing Parrot	[1]	NEO-W	-	
758							(63*)	
759			<i>Aratinga nenday</i>	Nanday Parakeet	[1]	PSI-W	-	
760							(60-62)	
761			<i>Neophema pulchella</i>	Turquoise Parrot	P2 [6]; P8 [4]	NEO-W	UCE	
762							(59/65*)	(61)
763			<i>Pionus maximiliani</i>	Scaly-headed Parrot	M5 [3]; P8 [4]	NEO-W	UCE	
764						(55-61*)	(57-63)	
765			<i>Psittacus erithacus</i>	Grey Parrot	[1]	PSI-W	-	
766						(60)		
767	Strigiformes	Strigidae	<i>Athene cunicularia</i>	Burrowing Owl	[2]	NEO-W	UCE	
768							(65)	(57-63)
769			<i>Bubo bubo</i>	Eurasian Eagle-owl	[5]	NEO-W	UCE	
770						(55/61*)	(59)	
771	Suliformes	Sulidae	<i>Sula bassana</i>	Northern Gannet	[2]	NEO-W	UCE	
772							(51/58-61*)	(61/57-63*)
773								
774								
775	^a [1] Fridolfsson and Ellegren (1999). [2] Han et al. (2009). [3] Bantock et al. (2008). [4] Griffiths et al. (1998). [5] Ellegren (1996).							
776	[6] Griffiths et al. (1996). [7] Lee et al. (2010).							

777 **Figure 1.** LAMP-based sex determination across the genome-scale phylogeny of birds
 778 (modified from Jarvis et al. 2014). The original Total Evidence Nucleotide Tree
 779 (TENT) by Jarvis et al. (2014) was built using the Exascale Maximum Likelihood
 780 (ExaML) code for phylogenetic inference applied to a dataset partitioned in introns,
 781 UCEs, and first and second exon codon positions (third position excluded). Black
 782 arrows indicate bird Orders where primer set NEO-W differentiated females from males.
 783 Names on the right side of black arrows are successful primer sets for specific bird
 784 Families (Estrildidae: EST-W; Icteridae: ICT-W, ICT-Z; Psittacidae: PSI-W;
 785 Ciconiidae: CIC-W, CIC-Z) and the Order Falconiformes (FAL-W, FAL-Z). An
 786 asterisk denotes positive amplification using primer set UCE. Aequorlitorornithes and
 787 Strisores are Neoavian sister clades supported by Prum et al. (2015) where species of
 788 Ciconiidae (Order Pelecaniformes), Sulidae (Order Suliformes) (Aequorlitorornithes) and
 789 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 ladder 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 1000 bp. and a last fragment of 3000 bp.

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