Sex determination in the wild: a field application of Loop-Mediated Isothermal Amplification successfully determines sex across three raptor species

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Abstract

PCR-based methods are the most common technique for sex determination of birds. Although these methods are fast, easy and accurate, they still require special facilities that preclude their application outdoors. Consequently, there is a time lag between sampling and obtaining results that impedes researchers to take decisions *in situ* and in real time considering individuals' sex. We present an outdoor technique for sex determination of birds based on the amplification of the duplicated sex-chromosome-specific gene Chromo-Helicase-DNA binding protein using a Loop-Mediated Isothermal Amplification (LAMP). We tested our method on Griffon Vulture (*Gyps fulvus*), Egyptian Vulture (*Neophron percnopterus*) and Black Kite (*Milvus migrans*) (family *Accipitridae*). We introduce the first fieldwork procedure for sex determination of animals in the wild, successfully applied to raptor species of three different subfamilies using the same specific LAMP primers. This molecular technique can be deployed directly in sampling areas since it only needs a voltage inverter to adapt a thermo-block to a car lighter and results can be obtained by the unaided eye based on colour change within the reaction tubes. Primers and reagents are prepared in advance to facilitate their storage at room temperature. We provide detailed guidelines how to implement this procedure, which is simpler (no electrophoresis required), cheaper and faster (results in ca. 90 minutes) than PCR-based laboratory methods. Our successful cross-species application across three different raptor subfamilies posits our set of markers as a promising tool for molecular sexing of other raptor families and our field protocol extensible to all bird species.

Introduction

Accurate sex determination is critical in wildlife management, captivity breeding programs and studies on behaviour, ecology and evolution. It is especially challenging in monomorphic bird species (i.e. species with no phenotypic differentiation between males and females) and nestling/juveniles or when samples are obtained without handling individuals (e.g. non-invasive sampling). Sex determination in birds relied originally on observational studies and palpation or expensive and complicated surgeries, hormones analyses or endoscopies. However, these techniques were mostly inaccurate, posed a threat for life or required expensive and complex facilities that hamper their extension. These issues have been partially solved by molecular techniques using karyotypes and, more recently, amplifying the duplicated 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). These PCR-based techniques are nowadays widely applied across non-ratite birds due to their relatively easy diagnosis by running an electrophoresis on an agarose gel. A visual examination of these gels generally shows a single band, corresponding to the double copy of the CHD-Z fragment and diagnostic of males, or a double band, corresponding to the CHD-Z and CHD-W copies differing in fragment sizes and characteristic of females (e.g. Griffiths *et al.* 1998; Fridolfsson & Ellegren 1999). A digestion with restriction enzymes is also needed in specific cases when CHD-Z and CHD-W do not differ in fragment size (reviewed in Morinha *et al.* 2012; Vucicevic *et al.* 2013). This easy and accurate technique based on PCR (but see Robertson & Gemmell 2006) has been widely applied not only for molecular sexing to fresh tissue samples (e.g. blood) but also to non-invasive samples such as museum bird specimens (Bantock *et al.* 2008), feathers (Horvath *et al.* 2005) or unincubated eggs

(Aslam *et al.* 2012). However, this technique urges for special and expensive equipment for thermal cycling and electrophoresis and entails specialized laboratories away from the study sites. Researchers and wildlife managers would therefore benefit from an accurate, portable and inexpensive molecular technique for sex determination that can be operated in the field and yield results in relatively short time.

Loop-mediated isothermal amplification (LAMP)

The loop-mediated isothermal amplification (LAMP) (Notomi *et al.* 2000) uses *Bst*, a DNA polymerase that contains a 5'-3' polymerase activity and owns a high strand displacement activity that allows an auto-cycling strand displacement DNA synthesis. In other words: no thermal cycling is needed. Two pairs of primers that recognize six different regions produce a final product of stem–loop DNAs with several inverted repeats of the target in the same strand. These products can be stained by using turbidity (Mori *et al.* 2001), pH-sensitive dyes (Tanner *et al.* 2015) or any metal indicator (Tomita *et al.* 2008) and make results easily checked by unaided eye. Because of its high-specificity and sensitivity to the target region, isothermal conditions and easy detection, LAMP appeared as a promising tool in molecular techniques that has been already applied in medicine (Nyan *et al.* 2014), microbiology (Fukuta *et al.* 2014) and parasitology (Abbasi *et al.* 2010) (among others) and applied in poultry or species with some commercial interest (Hsu *et al.* 2011; Chan *et al.* 2012; Kim *et al.* 2015). As far as we know, this technique has not been applied in ecology and evolution despite LAMP reactions can be run under field conditions.

A study case: movement ecology of Griffon Vultures in the Middle East

The population of Griffon Vulture (*Gyps fulvus*, Hablizl 1783) in the Middle East has dramatically decreased in the last decades mainly because of poisoning and human

disturbance. The Israeli Nature and Parks Authority in collaboration with the Hebrew University of Jerusalem started an intensive monitoring program to understand foraging and behavioural ecology of this species. It was shown how long-range forays (i.e. relatively short-term movements in which individuals depart from their regular foraging area, travel to remote locations and return to the original core area) were biased towards females after deploying high-resolution global positioning system and accelerometer tags (GPS-ACC tags) on adult birds. These long-range forays likely represent failed breeding attempts (Spiegel *et al.* 2015), but further studies focusing only in female birds are needed to disentangle among the causes and consequences of these movements for the population persistence. In this example, a large number of vultures are captured in a walk-in traps in a single monitoring/sampling/tagging effort, but only some of them (e.g. a tenth) are equipped with expensive GPS-ACC tags (Spiegel *et al.* 2013). A molecular technique for sex determination that could be fully applicable under field conditions would be, therefore, desirable to deploy GPS-ACC tags preferably in female birds while minimizing handling time of all captured birds.

In this study we introduce a molecular approach based on LAMP for sex determination of Griffon Vultures in 90 minutes (see BOX 1). Female-specific primers were designed to amplify a CHD fragment located in the W-chromosome and results were compared to those obtained under standard lab conditions as a reference. We developed a fully operational field technique for Griffon Vultures, using vacuum-dried primers and stabilizers to preserve enzyme activity. Furthermore, we successfully applied these primer sets also to Egyptian Vulture (*Neophron percnopterus*, Linnaeus 1758) and Black Kite (*Milvus migrans*, Boddaert 1783) and proved for the first time the utility of LAMP for sex determination in birds across species.

Material and Methods

We extracted DNA from fresh blood samples stored in absolute ethanol of four females and four males of Griffon Vultures, Egyptian Vultures and Black Kites using a NaOH based extraction protocol (Truett *et al.* 2000). We chose this protocol because it is simple and fast hence can also be implemented in the field. First, we run one PCR per sample under standard lab conditions for sex determination to be compared to our LAMP-based protocol using 1x BioTaq^{TM} buffer, $MgCl_2$ 3mM, dNTPs 0.2 mM, 2550F and 2718R primers 0.2 μM (Fridolfsson & Ellegren 1999) and 0.5 units of Taq polymerase added to 2 μL of 1:100 dilution of DNA in a final volume of 25 μL. Cycling conditions were as follows: 94ºC for 2 minutes, 55ºC for 30 seconds and 72ºC for 1 minute, followed by 35 cycles of 92ºC/30sec, 50ºC/30sec and 72ºC/45 sec and a final extension step of 5 minutes at 72ºC. Five microliters of PCR products of males of the three raptor species (CHD-Z) were then cleaned from excess of primers and nucleotides using 2 μL of an enzymatic mixture of Antarctic phosphatase and *Escherichia coli* exonuclease I incubated at 37ºC during 45 minutes followed by 80ºC/15 minutes for enzymes inactivation. Sequences were analysed for forward and reverse directions in an Applied Biosystems 3130 Genetic Analyzer using the same primers for amplification in the Applied Biosystems BigDye Terminator Cycle Sequencing Kit v. 1.1. Forward and reverse sequences for each PCR product were edited and assembled using using Geneious 8.0.5 (http://www.geneious.com) (Kearse *et al.* 2012) and uploaded into GenBank database (accession numbers KU563739-KU563741)

Sex determination by LAMP

Two sets of primers are required for sex determination (table 1). ACCIW targets CHD-W (specific of females) and positive LAMP reactions will be characteristic of females. ACCIZ targets CHD-Z and it is used as a positive control for DNA quality and/or to monitor LAMP reaction. Consequently, only females will amplify CHD-W (ACCIW+) and CHD-Z (ACCIZ+) and males will be ACCIW- confirmed only after rejecting any failure during LAMP reaction by amplifying CHD-Z (ACCIZ+, positive control). Otherwise (ACCIZ-) repetition of LAMP reactions will be required to ensure a correct sex determination.

LAMP primer design

We designed two specific primer sets of forward and backward external primers (F3/B3) and forward and backward internal primers (FIP/BIP) (figure 1) specific to Griffon Vultures based on a sequence of CHD-W (GenBank accession number: EU430640) and CHD-Z based on the three studied species (GenBank accession numbers: KU563739- KU563741, this study) (table 1). Primer selection for ACCIW required preliminary assays of primer design and optimization in experimental laboratory conditions following recommendations summarized in Tomita *et al.* (2008). Primers for ACCIZ were designed using Primer Explorer V4 software (Eiken Chemical Co., Ltd., Japan; http://primerexplorer.jp/e/). We prepared a primer-mix for each marker (ACCIW and ACCIZ) and eight reactions containing vacuum-dried primers in a final concentration of 1.6 μM of internal (FIP/BIP) and 0.2 μM of external (F3/B3) primers and stored them at room temperature.

LAMP reactions

We prepared a ready-mix for eight reactions as in Hamburger *et al.* (2013) to preserve enzyme activity at room temperature and make it portable and effective for field

conditions. This mix was composed by 1x enzyme buffer, dNTP 0.4mM, betaine 1M, 2% sucrose (used as stabilizer) and 8 units of Bst DNA polymerase (New England Biolabs) per reaction. The ready-mix can be stored for months at room temperature (Hamburger *et al.*, 2013) and although we did not evaluate it specifically, we observed amplifications days after their preparation. Prior to LAMP, we rehydrated the primer-mix with the same volume of molecular biology grade H_2O than before being vacuum-dried and transferred its whole content to the microtube containing the ready-mix. Finally, 23μL of this mix were pipetted to PCR-tubes and 2 μL of the 1:100 diluted DNA were added. LAMP reactions were incubated between 45 to 80 minutes and temperatures ranging between 55 to 69 ºC (table 1). LAMP-amplified products were detected by running a 2.5% agarose gel electrophoresis. We stained LAMP reactions with 5 μL of 1:50 diluted Sybr Green I Nucleic Acid Stain (Life Technologies) to allow an easy diagnosis of LAMP reactions by the unaided eye. This reagent changes the colour of the content within PCR microtubes from orange to yellow-green due to its interaction with residuals of magnesium pyrophosphate generated during DNA synthesis in LAMP (Mori *et al.* 2001). We also irradiated LAMP reactions with an UV portable lamp to detect yellow fluorescence in positive reactions.

Results

Optimization of LAMP reactions in Griffon Vultures

The combination of different reaction times (45, 60 and 80 minutes) and temperatures (55 ºC, 57 ºC, 59 ºC, 61 ºC, 63 ºC, 65 ºC, 67 ºC and 69 ºC) showed that ACCIW primers fully discriminated females from males when LAMP reactions were performed at 64 ºC for 80 minutes (table 1), as shown by the characteristic laddered pattern in the agarose

gel (figure 3). A different combination of time and temperature yielded unspecific amplifications in males (false positives) or lack of amplification in females (false negatives) (results not shown). On the other hand, ACCIZ showed a similar laddered pattern in all individuals after incubation at 59ºC during 80 minutes in both females and males. These results discard false negatives in ACCIW and therefore support male determination in those samples that did not amplify with the female-specific set of primers. Visualization of amplification products was possible at daylight when SYBR Green I was added within the microtubes (ACCIW: females in yellow and males in orange; ACCIZ: all individuals in yellow) (figure 4). The irradiation with 320 nm UV light stressed these results and only positive reactions irradiated fluorescence (figures 4C and 4D).

Cross-species amplification of LAMP primers

The two primer sets (ACCIW and ACCIZ) also amplified the targeted regions in Egyptian Vulture and Black Kite (table 1). The three studied raptor species needed 59 ºC to amplify ACCIZ, although Griffon Vultures and Black Kites required longer incubation times (80 minutes) than Egyptian Vultures (60 minutes) to ensure positive reactions in all samples. ACCIW, on the other hand, amplified CHD-W fragments of females of Egyptian Vultures at 63 ºC/60 minutes and Black Kites at 67 ºC/80 minutes.

Discussion

We have developed a simple and portable method for molecular sex determination of three raptor species of the family *Accipitridae* based on Loop Mediated Isothermal Amplification (LAMP). This procedure shows two main advantages over classical PCR-based sex determinations. First, it can be easily performed in the field because

DNA extraction (10 minutes) and two LAMP reactions (maximum 80 minutes in total) only need a water bath or thermo-block for incubation at a single temperature. All LAMP reagents (vacuum dried primers and a ready-mix including stabilizers) can be stored at room temperature for months (Hamburger et al. 2013). This is an advantage not only for fieldwork, but also facilitates enormously the shipment of reagents (no cool boxes nor dry ice are needed). Second, sex is determined with the unaided eye according to change of colour within the reaction tubes caused by the interaction of a fluorescent label and the pyrophosphate residuals produced during LAMP. We believe our work unties the indivisible link between molecular sex determination and fully equipped laboratories and allows for the first time sex determination of individuals (fledging to adults) in sampling areas located far away from wild populations.

The lab in the field

Logistic is one of the major issues to make a protocol applicable to field conditions. Using thermo-blocks instead of thermo-cyclers cheapens and facilitates enormously the procedure. However, reagents and primers used for biochemical reactions usually need special storage conditions such as freezing or cooling that complicate their shipment and delivery. The primer-mix (dehydrated primers) and ready-mix (reagents with stabilizers) prepared prior to their shipment and delivery allow long-term storage at room temperature and prevent their degradation. Although freeze-drying (i.e. lyophilisation) is widely accepted as the preferred technique for achieving long term storage of biological materials and oligonucleotides (Day & Stacey 2007), we chose vacuum-dried primers over lyophilized because i) they were kept safe at room temperature and ii) involved lower costs (vacuum driers). We also tested different concentrations of sucrose to stabilize the ready-mix reagents and keep them at room temperature and found that concentrations up to 2% worked optimally in the three tested

235 species. This concentration kept reagents at room temperature at least for seven days
236 and did not decreased the efficiency of LAMP reactions. We observed inhibition of
237 LAMP reactions as sucrose concentration increased above 8% of the reaction volume
238 (data not shown) in close agreement with the only and previous work optimizing
239 sucrose in LAMP reactions (Hamburger *et al.* 2013).

240 We recommend an initial effort to find an optimal DNA extraction protocol and dilution
241 of DNA template to decrease the proportion of inhibitors per reaction, especially if non-
242 invasive samples (e.g. feathers, faeces…) are used. We followed a hotshot NaOH
243 protocol for DNA extraction (Truett *et al.* 2000) from blood samples because it was
244 simple and fast (10 mins / 100ºC in NaOH 100 mM) so it can be easily performed in
245 field conditions. However, a 10 to 100 fold dilutions were needed not only to reduce the
246 amount of DNA, but also to decrease the proportion of inhibitors therein. We applied
247 our protocol to 23 individuals of Griffon Vulture (8 females and 15 males) in our lab to
248 evaluate the efficiency and accuracy of the method and correctly determined the sex of
249 20 individuals (87%) using a 1:100 dilution of template. The three samples left were
250 correctly assigned when DNA templates were diluted to 1:10. These results stress the
251 need to standardize the procedure with equal concentrations of DNA to minimize
252 inhibitions of LAMP reactions that bias sex determination. Sensitivity analyses of DNA
253 concentrations in LAMP show the high efficiency at very low DNA concentrations (e.g.
254 femtograms: 10^{-15} g), way below the expected concentration from DNA extractions
255 from nucleated red blood cells (e.g. Poon *et al.* 2006; Bonizzoni *et al.* 2009; Hamburger
256 *et al.* 2013). As far as we know, this is the first study testing outdoor conditions for
257 LAMP, despite it has always been cited as molecular technique that could be easily
258 taken to the field or, at most, taken to laboratories in hospitals with limited resources.

This protocol relies on the amplification of the female-specific CHD region located in the W chromosome (ACCIW) and the homologous region in the Z chromosome present in both males and females (ACCIZ). We recommend these two LAMP reactions in parallel with a negative control (i.e. free-template LAMP reactions) so ACCIW- and ACCIW+ reactions can be distinguished from false negatives and positives (respectively). False positives are among the most common flaws in LAMP reactions. This type I error is usually explained by cross-sample contaminations (LAMP is 10 to 100 times more sensitive than PCR) (Le *et al.* 2012) or background amplification (i.e. amplification in template-free reactions due to primer dimers) (Kimura *et al.* 2011; Wang *et al.* 2015). However, although false positives were found while optimizing the technique, we discarded these two explanations because we observed no amplification in template-free reactions.

LAMP is a relatively novel molecular technique for DNA amplification widely applied in medicine (Poon *et al.* 2006; Nyan *et al.* 2014; Fernández-Soto *et al.* 2014) and parasitology (Abbasi *et al.* 2010; Salant *et al.* 2012; Hamburger *et al.* 2013). It does not require any molecular background nor experience, only a few days of training (Hamburger *et al.* 2013; Cuadros *et al.* 2015). However, despite this great potential, the application of LAMP in life and environmental sciences has been focused in species with commercial interest (Hsu *et al.* 2011, 2012; Abdulmawjood *et al.* 2014; Kim *et al.* 2015). We believe that the proposed technique can be highly instrumental to studies in ecology, behaviour and evolution, as well as for conservation projects. For instance, LAMP has a great potential for species determination from faecal samples (especially challenging for elusive mammals) or to distinguish between sibling species that cannot easily be distinguished morphologically. LAMP has been applied for sex determination in rock pigeons (*Columba livia*) but, although suggested, it was not tested on other bird

284 species within the *Columbidae* family (Chan *et al.* 2012) nor set up for field conditions.
285 Chan et al. (2012) designed primers for sex determination based on CHD and for
286 positive control based on a fragment of the mitochondrial 18S ribosomal RNA gene.
287 This marker choice may overestimate the number of individuals assigned as males when
288 the sexual marker fails because mitochondrial genomes are by several orders of
289 magnitude than nuclear genomes and this may generate amplification bias favouring
290 mitochondrial genomes.

291 *Conclusions and perspectives*

292 We have optimized a LAMP-based protocol for sex determination suitable for fieldwork
293 for three raptor species belonging to three different subfamilies within *Accipitridae.*
294 This is a relatively large family with more than 300 species with an important role as
295 top predators essentially in all terrestrial ecosystems around the world. Our successful
296 LAMP application of the same primer sets across species of three different raptor
297 subfamilies is a promising fully operational tool for molecular sexing of raptors in field
298 conditions. To our knowledge, this is not only the first fully operational field
299 application of the LAMP sex-determination technique, but also the first demonstration
300 of the utility of this approach beyond the single-species level. Furthermore, this novel,
301 portable and accurate molecular technique for sex determination is simpler (it does not
302 require electrophoresis) and cheaper than PCR-based methods (Hamburger *et al.* 2013;
303 Pooja *et al.* 2014), it can provide results in less than 90 minutes and be applied during
304 regular fieldwork conditions. Moreover, the potential of the methodology described
305 here goes beyond its application to raptors (*Accipitridae*) and aims to be extended to
306 higher taxonomic levels within *Aves* based on the homology and relative well conserved
307 region of the widely used CHD gene for sex determination across taxa (Griffiths *et al.*
308 1998; Vucicevic *et al.* 2013). Our work opens a new tool kit for ecologists that has

309 remained almost unknown despite its extensive use in other disciplines (e.g. Nyan *et al.*
310 2014; Fukuta *et al.* 2014; Abbasi *et al.* 2010). To facilitate adoption of this tool, we
311 provide a "know how" guide to apply LAMP to projects where species identification or
312 sex determination is needed in real time and *in situ*. Future work in this direction will
313 facilitate enormously the work of wildlife managers and researchers as well as for
314 poultry and exotic bird breeders with important conservationist, economic and
315 commercial benefits for these collectives.

316

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DATA ACCESIBILITY

ACCIZ sequences specific of Griffon vulture, Black Kite and Egyptian vulture obtained in this study for LAMP primers design are deposited in GenBank (accession numbers: KU563739, KU563740 and KU563741, respectively). The alignment of these sequences is available at Dryad Project as doi:10.5061/dryad.4kr93.

AUTHOR CONTRIBUTION

A.C-C. conceived the study and A.C-C., R.N. and I.A. planned and designed the experiments. A.C-C. performed the laboratory work and drafted the manuscript and A.C-C. and R.N. edited the manuscript. All authors reviewed the final draft of the manuscript.

BOX 1. *Sex determination by LAMP: a workflow*

At the laboratory. Primers and reagents are prepared in advance in a laboratory (see text). These mixes do not require special storage conditions whereas the primer mix needs to be rehydrated before being added to the ready mix.

In the car. LAMP needs a thermo-block or water bath for incubation at a single temperature. Under field conditions, we suggest to use voltage inverters plugged in car lighters as the portable unit of power supply required for incubation.

At the field (after sampling) (see also figure 2). **Step 1**: DNA extraction using NaOH. We recommend dilutions with H_2O to avoid inhibitions during LAMP reactions. **Step 2**: two reactions per sample are required to ensure correct sex determination: ACCIZ (positive control) and ACCIW (sex determination) (see table 1 for specific temperature and time conditions). **Step 3**: addition of Sybr-Green and evaluation of colour change within the reaction tubes. **Step 4**: interpretation of the two LAMP reactions per sample and sex determination.

Table 1. LAMP primers to amplify CHD-W and CHD-Z. F3 = forward external primer; B3 = backwards 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).

			Temperature (ºC) / Time (minutes)		
Primer set	**Primer**	**Sequence (5'-3')**	***Gyps fulvus***	***Neophron percnopterus***	***Milvus migrans***
ACCIW	F3	TTTCACACATGGCACACC	64º/80’	63º/60’	67º/80’
	B3	GTTTTCTTTCTGAGATGGAGTC			
	FIP	AGTTCAAAGCTACGTGACTAAAACA**TTTT**TTCCCCCATTTTTGACAGG			
	BIP	ATTCCAGATCAGCTTTAATGGAAGT**TTTT**CAGATCCAGAATATCTTCTGCTC			
ACCIZ	F3	AMCAGCTGATATTGGAAGG	59º/80’	59º/60’	59º/80’
	B3	TTTCTTTASTYTGAGGGTGA			
	FIP	GGCAACYTGCTTTMRCTGTYG**TTTT**ACCTCTGGMTATSGTCTTG			
	BIP	CCAGGTGGCTTYTGAATGTCA**TTTT**GCRCTGGAACAAGTTGTC			

Figure 1. Schematic illustration of primer design for LAMP of ACCIW. A) Six different regions (forward: F1, F2 and F3; backward: B1, B2 and B3) and their complementary regions (forward: F1c, F2c and F3c; backward: B1c, B2c and B3c) are located on target DNA. B) Two outer (F3 and B3) and two inner primers (FIP and BIP) are used in each LAMP reaction. FIP (BIP) is composed by the F1c (B1c) sequence and the F2 (B2) sequence joined by a T-linker. C) Location of the F3/B3 and FIP/BIP primers along the CHD-W sequence. **(Note to the Editorial Board: colour online only)**

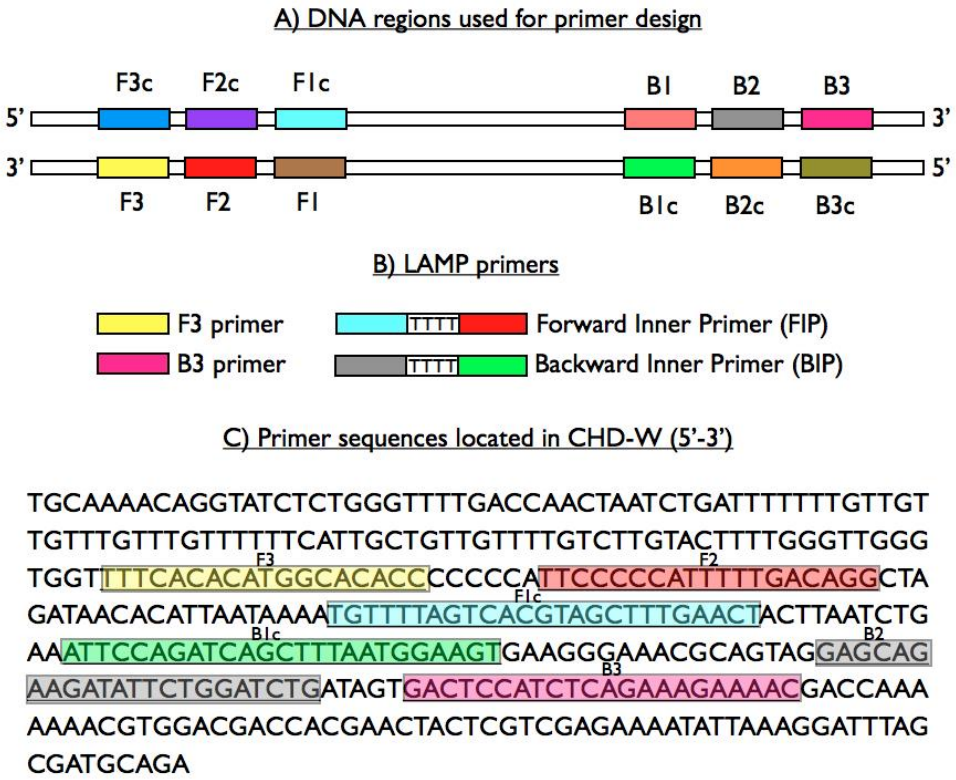

Figure 2. Workflow for LAMP-based sex determination. **(Note to the Editorial Board: colour online only)**

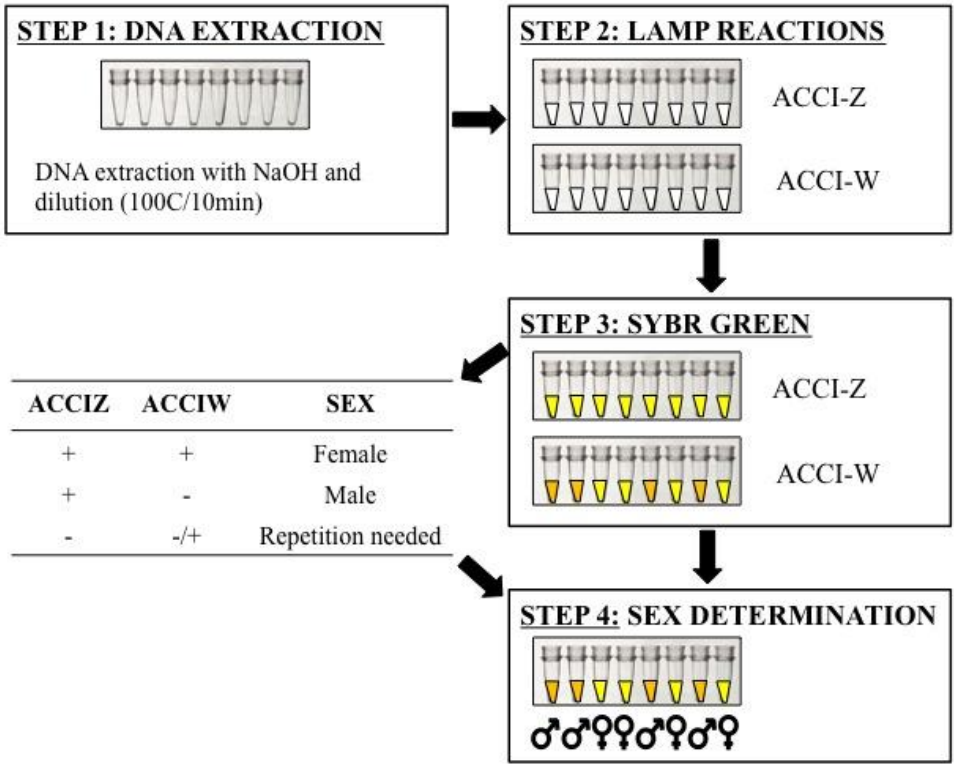

Figure 3. Agarose gel showing LAMP results tested in Griffon Vultures (*Gyps fulvus*). Primers sets ACCIW (A) and ACCIZ (B). S: 100 bp size standard.

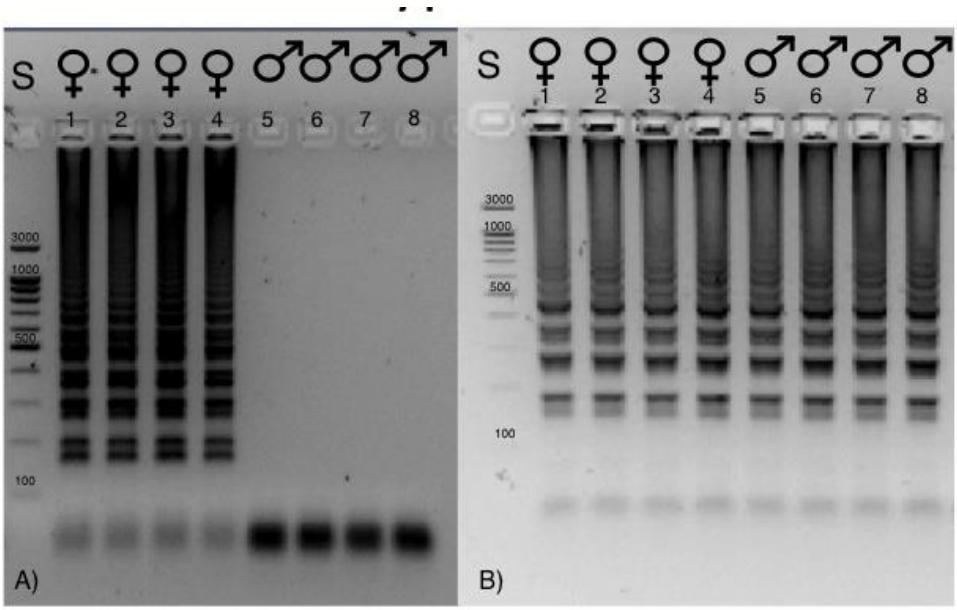

Figure 4. Visual detection of LAMP products using the set of primers ACCIW and ACCIZ. The colour of the reaction mix changed to yellow-green when LAMP reaction was positive and remained orange when there was no amplification after adding SYBR Green I. These colours can be observed with daylight (A and B) and after irradiating with a portable UV lamp at 320 nm. Samples 1 to 8 are the same as in figure 3. **(Note to the Editorial Board: colour online only)**