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TECHNICAL NOTE

A new primer-pair for sex identification of larks and wagtails

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Abstract Chromobox-helicase DNA-binding gene (*CHD1*) is the most reliable gene for sex identification in birds. However, the *CHD1* fragments of some species are difficult to amplify. In this study, we designed a new primer set (IntP2/IntP8) that targets a conserved region of *CHD1* gene. Firstly, we tested this protocol in Oriental skylark (*Alauda gulgula*). PCR amplification produced a single band (259 bp) for males and two bands (259 and 297 bp) for females. We then successfully conducted sex identification in other bird species including Tibetan lark (*Melanocorypha maxima*), Horned lark (*Eremophila alpestris*), Mongolian skylark (*Melanocorypha mongolica*), Asian short-toed lark (*Calandrella cheleensis*), Humes short-toed lark (*Calandrella acutirostris*), Crested lark (*Galerida cristata*), Yellow-headed wagtail (*Motacilla citreola*), White wagtail (*Motacilla alba*) and Ground tit (*Pseudopodoces humilis* Hume). Collectively, these findings support that IntP2/IntP8 primer set can be used to accurately determine sex identity in birds.

Keywords Sex identification · *CHD1* · Larks · Wagtails

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Birds are key components of an ecosystem and they are considered as indicator species for a range of environmental parameters (Wanless et al. 2007; Chambers 2008). The Qinghai-Tibetan Plateau (QTP) is a highly sensitive and fragile region to global climate change and human activities (Wang et al. 2005). Because of its ecological and environmental importance, QTP has attracted attention from national and international eco-science communities. Recently, influences of climate changes on alpine plant and agricultural system have been documented (Jin et al. 2009; Li et al. 2013; Wang et al. 2007), however, little is known about the population dynamics of avian populations inhabiting the QTP. It has been shown that Larks are sensitive to the degradation and the fragmentation of natural habitat, due to the lesser song-matching patterns in the limited availability of suitable habitat and harder habitat selection (Heath et al. 1994; Anthony et al. 2008; Laiolo and Tella 2005; Morgado et al. 2010). The Oriental skylark (*Alauda gulgula*) is distributed across the QTP and hence may serve as a potential model for investigating the impact of environmental change on bird populations. Evaluating sex ratio fluctuation and sex-biased dispersal of the Oriental skylark can shed light on the population and growth dynamics of this species to environmental change. This requires a simple and accurate sex identification protocol.

However, more than 50 % birds in the world are monomorphic (Bermúdez-Humarán et al. 2002), which makes it difficult to distinguish male and female on basis of

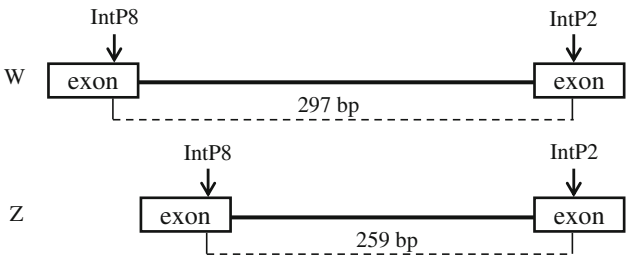

Fig. 1 A schematic diagram of *CHD1* DNA fragments used for sex identification using IntP8/IntP2 primer pair. *Arrows* show the approximate binding site for each primer. *Dark lines* represent the intron amplified by IntP8/IntP2 in *CHD1-Z* and *CHD1-W*, respectively. *Numbers* (bp) above *dotted lines* indicate the fragment sizes of Oriental skylark basing on PCR product sequencing

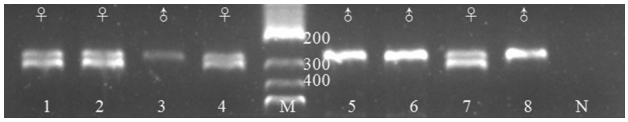

Fig. 2 PCR products of Oriental skylark (*Alauda gulgula*) separated on a 3 % agarose gel. *Lanes 1–4* were the samples of know gender, *lanes 5–8* were unknown. *N* negative control without template DNA, *M* molecular marker (DL20)

morphological and behavior analyses. To overcome these difficulties, researchers have developed accurate, rapid and noninvasive molecular sexing methods. Chromobox-helicase DNA-binding gene (*CHD*) is a sex-linked gene (Griffiths and Tiwari 1995) and it has been widely used for sexing in birds (Griffiths et al. 1998). Using a highly conserved primer set flanking the intron of *CHD1*, PCR amplification produces one common fragment (*CHD1-Z*) for both sexes and another band specific for females (*CHD1-W*). Numerous primer pairs have been designed for sex identification in birds, the primer P2 isolated from scarlet macaw (*Cyanopsitta spixii*), was designed by Griffiths and Tiwari (1995). Using a sequence alignment of the mouse *CHD1* gene (Delmas et al. 1993) and the chicken *CHD-Z* gene (Griffiths and Korn 1997), Griffiths et al. (1998) designed the forward primer P8. Using the chicken *CHD1W* gene, Fridolfsson and Ellegren (1999) developed the primer-pair 2550F/2718R. These two primer sets can be used in most if not all bird populations for sex identification (Tian et al. 2006; Yu et al. 2011; Ong and Vellayan 2008).

We attempted to use P2/P8 or 2550F/2718R for sex identification in Oriental skylark, however, P2/P8 did not amplify the target fragment in some samples while 2550F/2718R produced a single band in the female after we optimized the PCR conditions. This was likely due to polymorphisms within primer binding sites and preferential amplification of the shorter allele of *CHD1* (Robertson and Gemmell 2006; Shizuka and Lyon 2008; Wang et al. 2010). In this study, we aligned *CHD1W* gene of Eurasian skylark *Alauda arvensis* Linnaeus (JX456406) (Morinha et al. 2013) and designed a new primer pair that target a conserved region of *CHD1-W* gene across species using Primer Premier 5 (PREMIER Biosoft international, Palo Alto, CA, USA). We named this primer pair IntP2 (5′-GTCACTATCAGATCCAGARTATCTTC-3′) and IntP8 (5′-CTYCCAAGRATGAGRAACTGT-3′) respectively (Fig. 1).

We tested the accuracy and efficiency of this primer pair in different birds found in the QTP. A total of 41 samples of 10 species from 3 avian families were tested, including 14 samples with known gender (Table 1). DNA extraction from whole blood was performed by standard phenol–chloroform method (Maniatis et al. 1982).

Amplifications were carried out in a total volume of 25 μL in Veriti TM 96-Well Thermal Cycler (Applied Biosystems). Each 25 μL reaction contained 50-150 ng of genomic DNA, 0.5 μL of each primer (10 mM), 0.2 μL Easy Taq DNA polymerase (500 U, 5 U/μL), 2.5 μL 10× PCR buffer, 1.5 μL dNTPs (2.5 mM). The reaction profile was 95 °C for 4 min, then 36 cycles of 95 °C for 45 s, 51.5 °C for 50 s, 72 °C for 1 min, followed by 72 °C for 8 min. 2.0–2.5 μL PCR product was loaded on 3 % agarose gel stained with ethidium bromide.

Table 1 Avain species tested in this study

Order	Family	Species	Common name	N_1	N
Passeriformes	Alaudidae	*Alauda gulgula*	Oriental skylark	4	26
Passeriformes	Alaudidae	*Melanocorypha maxima*	Tibetan lark	1	2
Passeriformes	Alaudidae	*Melanocorypha mongolica*	Mongolian skylark	1	2
Passeriformes	Alaudidae	*Calandrella cheleensis*	Asian short-toed lark	1	1
Passeriformes	Alaudidae	*Calandrella acutirostris*	Humes short-toed lark	1	1
Passeriformes	Alaudidae	*Galerida cristata*	Crested lark	1	1
Passeriformes	Alaudidae	*Eremophila alpestris*	Horned larkl	1	2
Passeriformes	Passeridae	*Motacilla citreola*	Yellow-headed wagtail	1	1
Passeriformes	Passeridae	*Motacilla alba*	White wagtail	1	1
Passeriformes	Pseudopodocidae	*Pseudopodoces humilis*	Ground tit	2	4

N_1: number of individuals with known gender; N: number of total individuals used for sex identification

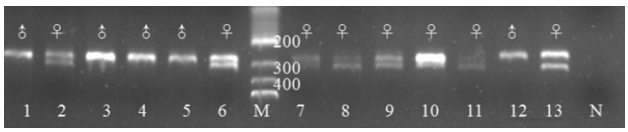

Fig. 3 Sex identification of Passeriform species using new primer pairs. *1*, *2* Tibetan lark (*Melanocorypha maxima*), *3*, *4* Mongolian sky lark (*Melanocorypha mongolica*), *5*, *6* Horned lark (*Eremophila alpestris*); *7* Asian short-toed lark (*Calandrella cheleensis*), *8* Humes short-toed lark (*Calandrella acutirostris*), *9* Crested lark (*Galerida cristata*), *10* Yellow-headedwagtail (*Motacilla citreola*), *11* White wagtail (*Motacilla alba*), *12*, *13* Ground tit [*Pseudopodoces humilis* (Hume)]. *N* negative control without template DNA, *M* molecular marker (DL20)

A single band was detected in male Oriental skylark (259 bp) and two bands (259 and 297 bp) in females (Fig. 2). Sequencing the purified PCR products revealed that the 259 bp band was from *CHD1-Z* section and the 297 bp band from *CHD1-W* region. *CHD1-Z* of Oriental skylark exhibited a 97 % sequence similarity with that of Eurasian skylark, and *CHD1-W* shared a 99 % identity with that of Eurasian skylark. Using the same primer-pair, we successfully amplified the *CHD1* homologous of Horned lark, Ground tit and validated with sequencing. In Ground tit, a 235 bp Z-band for males, 235 bp Z- and 294 bp W-band for females were amplified, respectively. While in Horned lark, a 257 bp Z-band for males, and 257 bp Z- and 297 bp W-band for female were evident, respectively.

Furthermore, we conducted sex identification using this primer pair in larks and wagtails. PCR products from five species of Alaudidae (Mongolian skylark, n = 2; Tibetan lark, n = 2; Asian short-toed lark, n = 1; Humes short-toed lark, n = 1; Crested lark, n = 1) and two species of Motacillida (Yellow-headed wagtail, n = 1; White wagtail, n = 1) included one band in the male and two different bands in the female (Fig. 3). Collectively, our results suggest that the new primer pair is effective and accurate for sex identification in some species of birds.

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