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Molecular identification of sex in the monomorphic breed of pigeons

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ABSTRACT

In many avian species, especially in monomorphic species and breeds, sex identification creates a serious problem, as they do not show any phenotypic differences. One of such breeds is the Wroclaw Meat Pigeon. In this study, molecular identification of sex with P2 and P8 primers used for the *CHD1* (*chromo-helicase-DNA-binding-protein*) gene amplification was performed. Peripheral blood samples were analyzed from 46 birds, and their DNA was isolated with the phenol-chloroform method. The fragments (370 bp *CHD1-Z*; 350 bp *CHD1-W*) obtained from the PCR were cut with the *Bsu*RI. Only the sequence in the Z chromosome was cut into fragments of 305 and 65 bp by the restriction enzyme. The difference between *CHD1-Z* and *CHD1-W* was visualized in 3% agarose gel. A single band was identified as male, whereas two bands (plus 1 invisible) were identified as female. Consequently, 23 specimens in each sex were identified.

Key words: *CHD1*, Monomorphic birds, PCR-RFLP, Sex identification.

INTRODUCTION

Bird species that display distinct sexual dimorphism do not pose difficulty in identifying their sex, which can be performed based on the size or plumage colour (Thanou *et al*., 2013). According to Griffiths *et al*., (1998), over 50% of the bird species exhibit monomorphism in relation to sex, which causes difficulty in determining their sex based on tertiary sexual characteristics. However, in many so-called monomorphic species, sex identification, especially prior to sexual maturity, is a complicated task, even for experienced specialists. In most species, even those typically different in appearance, the sex of the chicks is more difficult to be distinguished than that of adults (Dubiec and Zagalska-Neubauer, 2006). Sometimes, disorders related to sexual dimorphism may occur in birds, which make breeding work difficult (Grzegrzółka *et al*., 2016).

Previous methods of sex identification in monomorphic species relied on observations of behaviors and some anatomical differences. Direct methods of examining sex organs, such as cloacal endoscopy (Volodin *et al*., 2009), laparoscopy (Richner, 1989), and laparotomy (Maron and Myers, 1984) have been widely criticized for being invasive, stressful, and difficult to perform due to the size of the bird and influence of breeding season. Sex identification through karyotype analysis is an alternative method to those involving surgical procedures. Unlike mammals, female birds are heterogametic (ZW) and males are homogametic (ZZ) (Singh *et al*., 2014). The presence of a smaller chromosome (microchromosome-W) makes the differences between the ZZ and ZW chromosomes clearly visible, which allows the identification of sex. This heteromorphism, can be observed in most birds and used it to sex identification, the only exception being the ratites (flightless birds), such as emu or ostrich, in which both chromosomes are of the same size and shape (Smith *et al*., 2007; Dash *et al*., 2013). The karyotyping is not preferred for the identification of sex due to the duration and complexity of the entire process and due to the collection of biological material which can cause stress to the animal (Cerit and Avanus, 2007).

The development of a sex identification method based on DNA analysis allowed to limit the use of invasive methods. Several new molecular methods of sex identification in birds emerged that are based on hybridization (Longmire *et al*., 1993) and polymerase chain reaction (PCR); the latter including restriction fragment length polymorphism (RFLP) (Morinha *et al*., 2012) random amplified polymorphic DNA (RAPD) (Griffiths and Tiwari, 1993), amplified fragment length polymorphism (AFLP) (Morinha *et al*., 2012) and microsatellite sequences, mainly specific for W chromosome. Microsatellite sequences are specific to particular species, which limits the versatility and the frequency of their use in routine sex identification tests (Morinha *et al*., 2012).

Pigeons belong to the family *Columbidae* of the *Columbidoformes* order and can be found worldwide. In the case of 60% of all breeds of pigeons, appearance-based (phenotypic) sex identification is difficult (Wu *et al*., 2007). The Wroclaw Meat Pigeon is one of such monomorphic breeds (Fig 1). According to the standard, it belongs to pigeons of group 1 - formed. This breed was created around 1998 by prof. B. Nowicki at the former Agricultural Academy

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in Wroclaw in Poland and resulted from the crossing of the four already-existing breeds: Homing Pigeon, Polish Lynx, King, and American Meat Pigeon (Pawlina, 2011). The appearance of the Wroclaw Meat Pigeon is characterized by a large head, orange-colored eyes and narrow brows (Fig 1). Its beak is medium long and usually black, the same color as the bird. These pigeons are large and weigh between 600 and 900 grams. Their neck is short and strong and the breast is wide and the tail is narrow and the legs (shanks) are not feathered (Pawlina, 2011). Due to the lack of obvious tertiary sex characteristics between male and female birds, sex identification is difficult.

The gene that enabled to distinguish avian sexes for the first time was the highly conserved and universal *CHD1* (*chromo-helicase-DNA-binding-protein-1*) gene, located on both Z and W avian chromosomes (Griffiths and Tiwari, 1995). The discovery of differences in the length of nucleotide sequence of one of the introns, as well as of differences in the nucleotide sequence in one of the exons of *CHD1* gene, allows for the indisputable determination of sex in most species of birds (Fridolfsson and Ellegren, 1999). Furthermore, the use of a single set of primers in the PCR was shown to be sufficient to identify the gene in all flighted bird species (*Neognathae*) (Griffiths *et al*., 1996).

Therefore, the aim of present study was to establish the method of molecular sex identification that facilitate the proper pairing of breeding birds. For this purpose, the PCR-RFLP method and agarose gel electrophoresis were employed.

MATERIALS AND METHODS

Sex identification was performed on a population of 46 Wroclaw Meat Pigeons (Polish breed), owned by a private breeder in Poland (52°182′12″N -21°09′38″E). Peripheral blood samples were collected intravitally from the wing vein for routine veterinary examination in accordance with the Act from 15 January 2015 (Journal of Laws, 2015, item 266, art. 1.2). The blood samples were stored in 2 ml probes with K_2EDTA anticoagulant at -20°C. Genomic DNA was isolated with the phenol-chloroform method. The concentration of isolated DNA and its purity were determined with a Nano Drop 2000 (Thermo Scientific) spectrophotometer.

CHD1 gene fragment was PCR-amplified using P2 (5′ -TCTGCATCGCTAAATCCTTT-3′) and P8 (5′ -CTCCC AAGGATGAGRAAYTG-3′) primers (Griffiths *et al*., 1998). Briefly, 17 µl of the total reaction mixture contained 10 µl of 2 × REDTaq ReadyMix (1.5 units Taq DNA polymerase, 10 mM Tris-HCl, 50 mM $MgCl_2$, pH 8.3, 0.001% gelatin, 0.2 mM dNTP (dATP, dCTP, dGTP, TTP) stabilizers) (Sigma), P2/P8 primers (20 pmol μl^{-1}) 0.1 µl each, and 6.8 µl of MilliQ water. PCR was run in a thermocycler (Triblock Thermocycler-Biometra) according to the following thermal profile: primary denaturation in 95°C × 5 min; (94°C, 30 s; 48°C, 45 s; 72°C, 45 s) × 42; final elongation in 72°C × 5 min. The resulting PCR-amplified product was visualized in 1.5% high-resolution Nusieve GTG agarose (Lonza) gel in the presence of ethidium bromide. Bioinformatic analysis was conducted in order to determine the site of the cut on the *CHD1* gene by the *Bsu*RI enzyme. The restriction site analysis of the sequences was conducted by using Webcutter 2.0 program (http:// www.firstmarket. com/cutter/cut2.html). Then the amplified *CHD1* fragment was cut with the *Bsu*RI restriction enzyme. Then, 30 µl of Thermo scientific FastDigest *Bsu*RI reaction mixture was added, followed by horizontal electrophoresis in 3% high-resolution Nusieve GTG agarose (Lonza) gel. As a standard of mass in the agarose gel electrophoresis, pUC Mix Marker 8 (Fermentas) was used.

RESULTS AND DISCUSSION

PCR-amplified products of *CHD1* gene fragment with P2 and P8 primers were obtained for all the samples tested. The amplified products were separated in a 1.5% agarose gel (Fig 2). At this stage, we could not clearly determine the sex of the birds. In this study, PCR products of 370 bp fragment were obtained for all 46 birds.

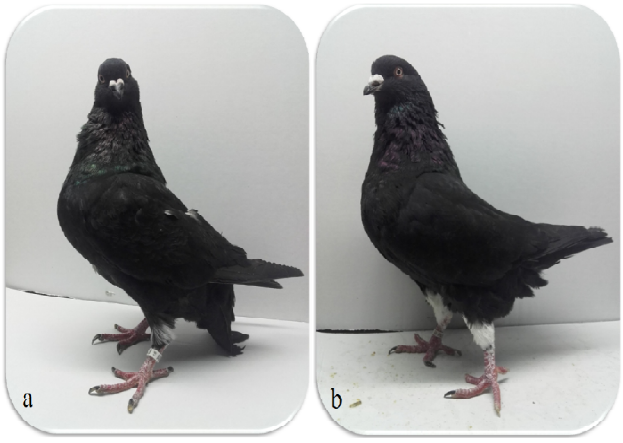

Fig 1: Wroclaw Meat Pigeons: (a) male, (b) female (Photo by M. Miąsko).

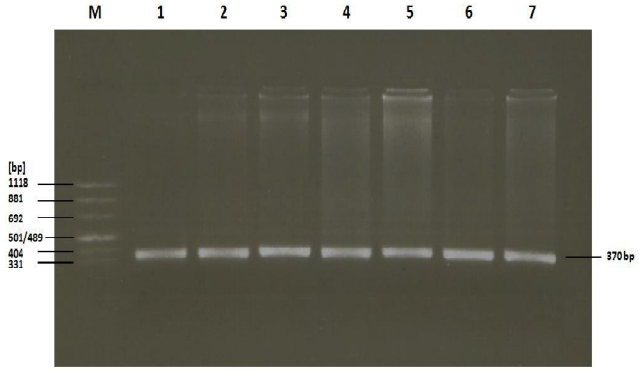

Fig 2: Electrophoretic separation of the PCR product in 1.5% agarose gel - Nusieve GTG Agarose (Lonza), M - pUC Mix Marker 8 (Fermentas), lanes 1-7 PCR product of appr. 370 bp length.

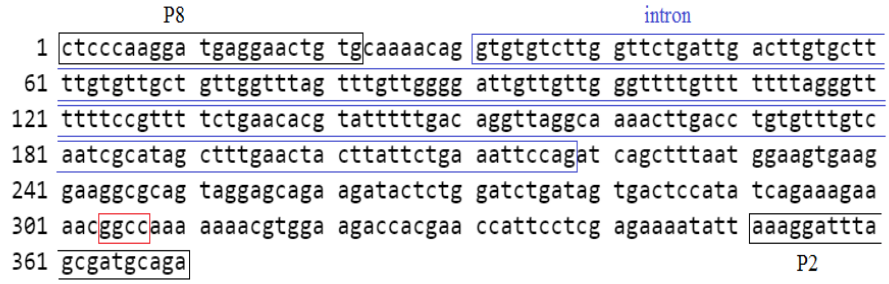

Fig 3: *CHD-Z* gene sequence (350 bp), intron – intron 9 sequence, P2 and P8 - primer binding sites in PCR, gg/cc – *Bsu*RI-recognized sequence.

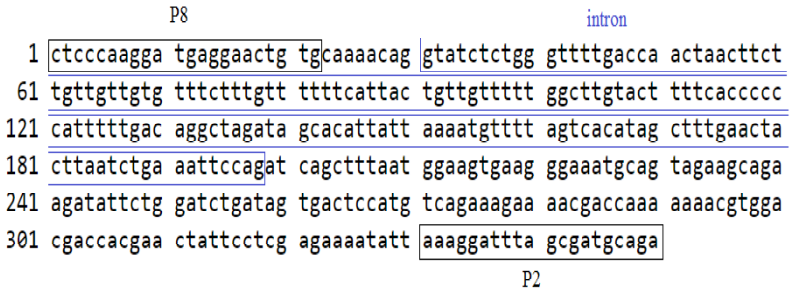

Fig 4: *CHD-W* gene sequence (350 bp), intron – intron 9 sequence, P2 and P8 – primer binding sites in PCR.

Results of the bioinformatic analysis: Bioinformatic analysis was performed on the *CHD1-Z* (GU289184.1) (370 bp) and *CHD1-W* (GU289183.1) (350 bp) gene sequences obtained from the NCBI GenBank for the rock pigeon (*Columba livia*). Both variants of the gene contain two coding fragments (exon 9 – 30 bp; exon 10 – 152 bp) and a noncoding fragment (intron 9), differing in length of 20 bp between the *CHD1-Z* and *CHD1-W* genes (Fig 3 and 4).

In order to compare the coding sequences of exon 9 of *CHD1-Z* and *CHD1-W* genes, the BLAST program (https://blast.ncbi.nlm.nih.gov) was used. Exon 9 contains 152 bp, of which 142 bp are identical to each other, which means 93% of the sequences are similar (Fig 5).

This shows that the 7% differences between the sequences of exon 9 can be used to identify sex in pigeons.

Cutting with restriction enzyme – *in silico* analysis: Virtual restriction of both *CHD1-Z* and *CHD1-W* genes was performed with *Bsu*RI enzyme and Webcutter 2.0 program. *Bsu*RI-recognized sequence and the corresponding visualized sequence of the cleavage site were found only in the *CHD1-Z* gene; therefore, *Bsu*RI could be used to digest PCR amplification product in further analysis (Fig 6). *CHD1-Z* (370 bp) was digested with *Bsu*RI restriction enzyme, which resulted in two fragments: 305 bp and 65 bp. However, *CHD1-W* (350 bp) was not affected by *Bsu*RI (Fig 7). Thus, the results obtained in the practical part of this study on sex identification in Wroclaw Meat Pigeons were predicted earlier in the bioinformatic simulation.

Restriction analysis of the *CHD1* gene fragment: In this study, based on the bioinformatic analyzes we hypothesized that the use of *Bsu*RI restriction enzyme in PCR-RFLP and subsequent electrophoretic separation of cut PCR products in 3% agarose gel of high-resolution will enable unambiguous sex identification in the studied birds. The specific sequence, recognized by the restriction enzyme, was

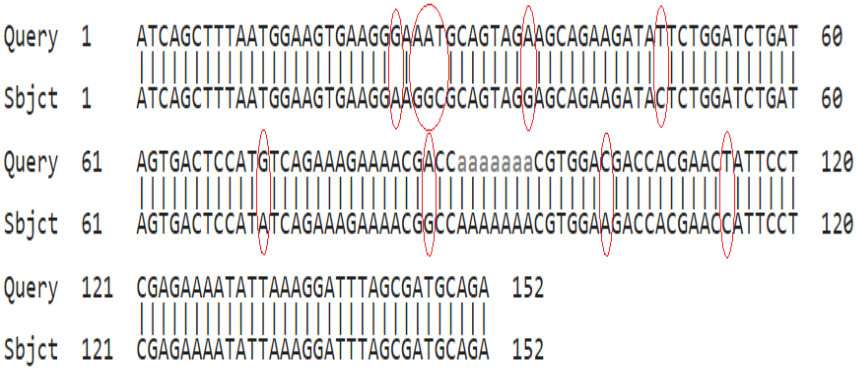

Fig 5: Comparison of exon 10 sequences (152 bp) of the *CHD1-Z* and *CHD1-W* gene, Query – query sequence –studied *CHD1-W* sequence, Sbjct – subject sequence – each *CHD1-Z* sequence (BLASTN 2.6.0+).

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ctcccaaggatgaggaactgtgcaaaacaggtgtgtcttggttctgattgacttgtgcttttgtgttgctgttgg  base pairs
gagggttcctactccttgacacgttttgtccacacagaaccaagactaactgaacacgaaaacacaacgacaacc   1  to  75

tttagtttgttggggattgttgttgggttttgttttttttagggttttttccgttttctgaacacgtatttttgac  base pairs
aaatcaaacaacccctaacaacaacccaaaacaaaaaaatcccaaaaaaggcaaaagacttgtgcataaaaactg  76 to 150

aggttaggcaaaacttgacctgtgtttgtcaatcgcatagctttgaactacttattctgaaattccagatcagct  base pairs
tccaatccgttttgaactggacacaaacagttagcgtatcgaaacttgatgaataagactttaaggtctagtcga  151 to 225

ttaatggaagtgaaggaaggcgcagtaggagcagaagatactctggatctgatagtgactccatatcagaaagaa  base pairs
aattaccttcacttccttccgcgtcatcctcgtcttctatgagacctagactatcactgaggtatagtctttctt  226 to 300

     BsuRI
aacggccaaaaaaacgtggaagaccacgaaccattcctcgagaaaatattaaaggatttagcgatgcaga     base pairs
ttgccggttttttgcaccttctggtgcttggtaaggagctcttttataatttcctaaatcgctacgtct  301 to 370
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Fig 6: *CHD-Z* gene sequence (370 bp), gg/cc sequence – *Bsu*RI cleavage site.

located only on the Z chromosome at 305 bp position; thus, *CHD1* gene sequence was cut into two fragments of 305 and 65 bp lengths (Fig 7). These fragments were separated in 3% agarose gel (Fig 8). Although the 65 bp fragment cannot be seen on the photo (contrary to what is shown in Fig 7), sex identification was possible due to the presence of 2 bands (350 and 305 bp length) in females and a single band of 305 bp length in males (Fig 8).

Three primer pairs are most commonly used to identify sex in birds: P2/P8 (Griffiths *et al*., 1998), 2550F/2718R (Fridolfsson and Ellegren, 1999), and 1237L/1272H (Kahn *et al*., 1998). In the studied population of Wroclaw Meat Pigeons using a pair of P2 and P8 primers, the *CHD1* gene was amplified in samples of 46 birds. Jensen *et al*. (2003), based on their research, found that both pair of primers: pair P2 and P8 and pair 1272H and 1237L for the sex identification in several bird species, including pigeons, can be used for amplification of the same intron. Considering that in case of using 1237L/1227H primers more non-specific fragments are obtained than when P2/P8 are used, the latter primers are preferred to be used in sex identification in many bird species. The difference between the two gene variants, *CHD1-Z* and *CHD1-W* in birds they studied was 10-40 bp (Jensen *et al*., 2003). Different set of primers – 2550F/2718R for the sex identification in pigeons was used by Jaiswal and Gottigalla (2016). The difference between the bands observed in electrophoresis ranged from 150 to 250 bp, whereas individual fragments of the gene were of 650 bp (*CHD1-Z*) and 400 bp (*CHD1-W*). In our study population, this difference was 20 bp (*CHD1-Z* 370 bp, *CHD1-W* 350 bp) and with the use of P2/P8, it was clearly visible in 1.5% agarose gel. Vucicievic *et al*., (2013) performed sex identification in 58 bird species by using both P2/P8 and 2550F/2718R pair of primers. With the use of P2/P8, they were unable to identify the sex in 16 species, including the pigeon (*Columba arquatrix*), for they obtained only a single band for all specimens of the species. Similarly, in the Wroclaw Meat Pigeon population studied with the use of P2/P8 primers, only a single band after electrophoretic separation in 1.5% agarose gel was obtained. According to Dubiec and Zagalska-Neubauer (2006), in some species, a single band can also be obtained for both sexes when 2550F/2718R pair of primers are used. However, as demonstrated by Kaherunnis *et al*. (2013), who used 2550F/2718R pair of primers, the sex of the pigeons they tested (*Columba livia*) was identifiable after electrophoretic separation of the PCR product in 1.5% agarose gel as the amplified fragments were of different length (CHD-Z 656 bp, CHD-W 448 bp) (Fridolfsson and Ellegren, 1999).

Sequence length [bp]		ZZ ♂	ZW ♀
350			
370	305	—	—
	65	—	—

Fig 7: Sex-related length of the *CHD1* gene PCR product sequences (in bp) after cutting with *Bsu*RI, as predicted in bioinformatics analysis.

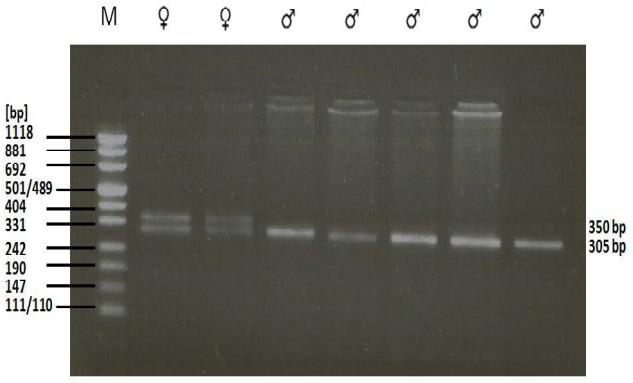

Fig 8: Electrophoretic separation of the *CHD1* gene PCR products in 3% agarose gel – Nusieve GTG Agarose (Lonza) cut with the restriction enzyme *Bsu*RI, M – pUC Mix Marker 8 (Fermentas).

The PCR-RFLP analysis has been proposed as a suitable strategy for molecular sexing in various bird species that have small differences between *CHD1-Z* and *CHD1-W* In addition, several researchers have described assigning the birds sex using specific restriction enzymes: *Mae*II, *Bsu*RI (*Hae*III) and *Dde*I (Griffiths and Tiwari, 1995; Griffiths *et al*., 1996; Bermúdez-Humarán *et al*., 2002), *Bsh*NI (Väli and Elts, 2002) in the *CHD1-Z* gene fragment, and *Asp*700I, which has restriction enzyme site in the *CHD1-W* gene fragment (Sacchi *et al*., 2004). In this study on Wroclaw Meat Pigeon, the *Bsu*RI restriction enzyme was used successfully, and the electrophoretic separation in 3% agarose gel with high-resolution allowed to uniquely identify sex in the studied birds. Sex differentiation of individuals creates the possibility of understanding the biology of reproduction in birds, which is crucial in the development of breeding programs, as well as in the protection of species. (Morinha *et al*., 2012).

CONCLUSION

PCR-RFLP technique for analysis of the *CHD1* gene along with the use of *Bsu*RI restrictase and subsequent electrophoretic separation of the obtained restriction fragments in 3% agarose gel led to clear identification of sex in all 46 monomorphic Wroclaw Meat Pigeons (23 specimens in each sex), which enabled the breeder to properly combine birds into breeding pairs.

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REFERENCES

Bermúdez-Humarán, L.G., Chávez-Zamarripa, P., Guzmán-Velasco, A., Leal-Garza, C.H., Montes de Oca-Luna, R. (2002). Loss of restriction site Ddei, used for avian molecular sexing in oreophasis derbianus. *Reprod. Domest. Anim*. **37**: 321-323.

Cerit, H., Avanus, K. (2007). Sex determination by CHDW and CHDZ genes of Avian Sex Chromosomes In *Nymphicus hollandicus*. *Turkish. J. Vet. Anim. Sci*. **31**: 371-374.

Dash, S., Kumarasamy, P., Malik, H., Thiagarajan, V. (2013). Phylogenetic analysis of ratite bird emu (*Dromaius novaehollandiae*) using sex specific sequences. *Indian J. Anim. Res*. **47**: 247-250.

Dubiec, A., Zagalska-Neubauer, M. (2006). Molecular techniques for sex identification in birds. *Biological Lett*. **43**(1): 3-12.

Fridolfsson, A.K., Ellegren, H. (1999). A Simple and Universal method for molecular sexing of non-ratite birds. *J. Avian Biol*. **30**: 116-121.

Griffiths, R., Daan, S., Dijkstra, C. (1996). Sex identification in birds using two CHD genes. *Proc. R. Soc. Lond., B, Biol. Sci*. **263**: 1249-1254.

Griffiths, R., Double, M., Orr, K., Dawson, R. (1998). A DNA test to sex most birds. *Mol. Ecol*. **7**: 1071-1075.

Griffiths, R., Tiwari, B. (1993). The isolation of molecular genetic markers for the identification of sex. *Proc. Natl. Acad. Sci. U.S.A*., **90**: 8324-8326.

Griffiths, R., Tiwari, B. (1995). Sex of the last Wild Spix's macaw. *Nature*. **375**: 454.

Grzegrzółka, B., Gruszczyńska, J., Domagała, E., Słonecki, R., Bartyzel, B.J., Czerniawska-Piątkowska, E. (2016). Morphological assessment of sexual dimorphism abnormality cases in Japanese quail (*Coturnix japonica*). *Reprod. Domest. Anim*. **15** Suppl. 2: 96.

Jaiswal, N.R., Gottigalla, B.Y. (2016). Sex identification in pigeons using CHD based molecular markers. *PARIPEX-Indian J. Res*. **5**: 209-210.

Jensen, T., Pernasetti, F.M., Durrant, B. (2003). Conditions for rapid sex determination in 47 avian species by PCR of Genomic DNA From Blood, Shell-Membrane Blood Vessels and Feathers. *Zoo Biol*. **22**: 561-571.

Journal of Laws, (2015). Ustawa z dnia 15 stycznia 2015 r. o ochronie zwierząt wykorzystywanych do celów naukowych lub edukacyjnych [In Polish] Dz. U. 2015, item 266, 1-25.

Kahn, N.W., John, J.S., Quinn, T.W. (1998). Chromosome-specific intron size differences In the avian CHD gene pro vide an efficient metod for sex identification in birds. *Auk*. **115**(4): 1074-1078.

Khaerunnisa, I., Sari, E., Ulfah, M., Sumantri, J.C. (2013). Avian sex determination based on Chromo Helicase DNA-binding (CHD) genes using polymerase chain reaction (PCR). *Media Peternakan*. **36**(2): 85-90.

Longmire, J.L., Maltbie, M., Pavelka, R.W., Smith, L.M., Witte, S.M., Ryder, O.A., Ellsworth, D.L., Baker, R.J. (1993). Gender identification in birds using microsatellite DNA fingerprint analysis. *Auk*. **110**(2): 378-381.

Maron, J.L., Myers, J.P. (1984). A description and evaluation of two techniques for sexing wintering sanderlings. *J. Field Ornithol*. **55**(3): 336-342.

Morinha, F., Cabral, J.A., Bastos, E. (2012). Molecular sexing of birds: A comparative rewiev of polymerase chain re action (PCR)-based methods. *Theriogenology*. **78**: 703-714.

Pawlina, E. (2011). The Wroclaw Meat Pigeon bred at the Wrocław University of Life Siences. *Przegląd Hodowlany*. **10**: 28-30.

Richner, H. (1989). Avian laparoscopy as a field technique for sexing birds and an assessment of its effects on wild birds. *J. Field Ornithol*. **60**(2): 137-142.

Sacchi, P., Soglia, D., Maione, S., Meneguz, G., Campora, M., Rasero, R. (2004). A non-invasive test for sex identification in short-toed eagle (*Circaetus gallicus). Mol. Cell. Probes*. **18**: 193-196.

Singh, J., Mukhopadhyay, C.S., Brah, G.S., Saini, S. (2014). Z-chromosome linked dinucleotide STRs: association with reproduction traits in chicken. *Indian J. Anim. Res.* **48**(6): 521-526.

Smith, C.A., Roeszler, K.N., Hudson, Q.J., Sinclair, A.H. (2007). Avian sex determination: what, when and where? *Cytogenet. Genome. Res.* **117**: 165-173.

Thanou, E., Giokas, S., Goutner, V., Liordos, V., Fraguedakis-Tsolis, S. (2013). Efficiency and accuracy of PCR-based sex determination methods in the European Phalacrocoracidae. *Ann. Zool. Fennici.* **50**(1-2): 52-63.

Väli, Ü., Elts, J. (2002). Molecular sexing of Eurasian Woodcock *Scolopax rusticola*. *WSGB.* **98**: 48.

Volodin, I., Kaiser, M., Matrosova, E.V., Klenova, A., Filatova, O., Kholodova, M. (2009). The technique of noninvasive distant sexing for four monomorphic Dendrocygna whistlings duck species by their loud Whistler. *Bioacoustics.* **18**: 277-290.

Vucicevic, M., Stevanov-Pavlovic, M., Stevanovic, J., Bosnjak, J., Gajic, B., Aleksic, N., Stanimirovic, Z. (2013). Sex determination in 58 bird species and evaluation of CHD gene as a universal molecular marker in bird sexing. *Zoo Biol.* **32**: 269-276.

Wu, Ch., Horng, Y., Wang, R., Yang, K., Huang, M. (2007). A novel sex-specific DNA marker in Columbidae birds. *Theriogenology.* **67**: 328-333.