Standardization of a PCR-HRM assay for DNA sexing of birds

S Ndlovu

orcid.org/0000-0002-4903-5875

Previous qualification (B.Sc. Hons)

Dissertation submitted in *partial* fulfilment of the requirements for the *Masters* degree in *Biochemistry* at the North-West University

Supervisor: Prof R Louw

Graduation May 2018

25681915

ACKNOWLEDGEMENTS

I would like to express my deepest thanks to **Prof Roan Louw**, my study leader for taking part in useful decision making, and giving necessary advice, professional guidance and arrangement of all facilities to make life easier. I choose this moment to acknowledge his contribution gratefully.

It is my radiant sentiment to place on record my best regards, deepest sense of gratitude to **Dr Oksana Lavenets** for her precious guidance which was extremely valuable for my study both theoretically and practically.

I would like to express my deepest gratitude to **Prof Francois van der Westhuizen** for awarding me an opportunity to take part in this interesting field of study. In spite of being extraordinarily busy with his duties, he took time to hear, guide and keep me on the correct path and allowing me to carry out my project at their esteemed organization.

I would also like to thank my **family** for their love, support and offering me with the best they have to offer.

Lastly, I would like to thank the Lord for all the wonders he has performed in my life, and the opportunities he has blessed me with.

I perceive this opportunity as a big milestone in my career development. I will strive to use gained skills and knowledge in the best possible way, and I will continue to work on their improvement, in order to attain desired career objectives.

// ABSTRACT

Sex determination of birds is of great significance in many ecological and demographic studies, where sex ratio between sexes is important. Obtaining this information is difficult in many avian species since more than 50% of all bird species are sexually monomorphic. This presents a challenge for avian breeders and evolutionary studies since sex identification is vital in the field.

The problems associated with accurate avian gender determination in the field were conquered by the introduction of molecular techniques through the knowledge of sex linked genes. This molecular-based technique widely applied for sexing birds is based on intronic variation of the Chromo-helicase-DNA-binding (CHD) gene on the Z and W chromosomes. The intronic length variations between the CHD-Z and CHD-W resulted in the development of a polymerase chain reaction (PCR) approach that allow for sex discrimination. However, the classic PCR-based techniques are time consuming and laborious as they involve a minimum of three steps: DNA isolation, PCR and gel electrophoresis.

In this study a PCR-high resolution melt (HRM) assay was developed for the molecular gender identification of avian species. The HRM protocol for avian molecular discrimination was developed based on the difference in melting curve patterns of the CHD1 fragments. The new test was then compared to the conventional PCR assay using

numerous different bird species. Although the PCR-HRM assay performed well in Lovebird species, the assay showed poor PCR amplification in other species, which directly affected the accuracy of the HRM technique in these species. Therefore, the newly established PCR-HRM assay could not be recommended for implementation in a commercial DNA diagnostics laboratory.

Key words: CHD1 gene, PCR, DNA, HRM, high resolution melt, avian, sexing, birds

TABLE OF CONTENTS

ACKNOWLEDGEMENTS……………………………..……………………………...ii
ABSTRACT……………………………………………………………………….iii
LIST OF FIGURES……………………………………..………………..………....ix
LIST OF TABLES………………………………………...…………..…………xii
ABBREVIATIONS, SYMBOLS AND UNITS…………………..………………………xiii
CHAPTER 1: Introduction……………………………….………………………1

CHAPTER 2: Literature Review……………………………….…………...…...3
2.1 Introduction……………………………………………….………………3
2.2 Importance of gender identification in birds………………….…………..3
2.3 General methods for gender determination of birds……….………......4
2.3.1 Vent sexing……………………………………………………….………5
2.3.2 Laparoscopy…………………………………………………….………..5
2.3.3 Karyotyping…………………………………………………………......6
2.4 Development of DNA-based techniques for avian gender identification...7
2.4.1 Single Stranded Conformation Polymorphism (SSCP)………..….8
2.4.2 Restriction Fragment Length Polymorphism (RFLP)… ……..……9
2.4.3 Randomly Amplified Polymorphic DNA (RAPD)……………………9
2.4.4. Amplified Fragment Length Polymorphism (AFLP)…….………..10
2.4.5 Microsatellites……………………………………………..…….…11
2.4.6 Capillary electrophoresis……………………………………….………11
2.5 CHD-based sex identification…………………………………………….…12
2.6 Comparison of three commonly utilized CHD-linked primer sets….……15
2.7 Selection of a suitable sexing technique……………………………..18
2.8 Real-time PCR methods for sex determination…………………………...19
2.8.1 Real-time PCR using TaqMan probes……………………………..20
2.8.2 Real-time PCR combined with melting curve analysis..……..….21

TABLE OF CONTENTS continued…

2.8.3 HRM analysis……………………….…………………………..….22

2.9 Rational, guidelines from the industrial partner, aim and objectives as well as experimental strategy…………….…………...….….…………..25

2.9.1 Rational………………………...………..........................…..25

2.9.2 Guidelines from the industrial partner………..……………....…..26

2.9.3 Aim and objectives…………………………………………….……..27

2.9.3.1 Aim……………………………………...............27

2.9.3.2 Objectives………………………………….....…..27

2.9.4 Experimental strategy…………………………………..……...28

CHAPTER 3: Materials and methods……………………..………………….....…30

3.1 Materials………………………………………………….…………..…30

3.1.1 Sample collection………………………………..........……..…..30

3.1.2 List of species selected for inclusion in this study………………..30

3.1.3 Reagents and buffers……………………………………….....….31

3.2 Methods……………………….……………………………………..……31

3.2.1 DNA isolation………………..…………………………….……31

3.2.2 PCR-HRM using KAPA HRM Master-mix………...…...…………32

3.2.3 Conventional PCR……………………………………………………33

3.2.4 Post-PCR melting curve analysis……………………….......…...34

3.2.5 Post-PCR high resolution melt (HRM) analysis……….………..34

CHAPTER 4: Standardization of the analytical methods………………………..…36

4.1 Introduction…………………………...…....…………….…..………36

4.2 Evaluation and optimization of the conventional PCR protocol for avian molecular sexing…………………………….………………….…………….36

4.3 Optimization of the PCR-HRM assay………..……………….……………44

TABLE OF CONTENTS continued…

4.3.1 Utilization of the KAPA HRM Fast master-mix for the PCR-HRM assay ……………………………………………………………..46

4.3.1.1 Calibration of the Step-one plus real-time PCR instrument……………………………………………………………..46

4.3.1.2 KAPA HRM FAST Master mix assay optimization ……….....46

4.3.1.3 KAPA HRM FAST Master-mix optimization with different DNA concentrations ………………………………………...47

4.3.2 Establishing the EvaGreen dye PCR-HRM assay 49

4.3.2.1 Amplification of Fischer’s Lovebird samples ......…………....50

4.3.2.2 Melting curve analysis of Fischer’s Lovebird samples 51

4.3.2.3 High resolution melt (HRM) analysis of Fischer’s Lovebird samples ………………………………………………………..54

4.3.3 The effect of different primers on the EvaGreen dye PCR-HRM assay……………………………………………………………..59

4.3.3.1 Conventional PCR analysis of Peach-faced Lovebird samples amplified with P2/P8 and 2550F/2718R primer sets...….60

4.3.3.2 EvaGreen HRM analyses of Peach-faced Lovebird samples amplified with P2/P8 and 2550F/2718R primer sets ……………..63

CHAPTER: 5 Comparison of the conventional PCR and EvaGreen dye PCR-HRM assays in different avian species ……………………………...69

5.1 Introduction……………………………………………………………….69

5.2 Analysing different avian species samples using the standardized methods…………………………………………………………………….70

5.2.1 Fischer's Lovebird ……………………………………………...…70

5.2.2 Nyasa Lovebird …………………………………..………………..75

5.2.3 Black-cheeked Lovebird ……………………………..……………..79

5.2.4 Peach-faced Lovebird ……………………………………..…………..84

5.2.5 Green-cheeked Conure …………..…………………………………...89

TABLE OF CONTENTS continued…

5.2.6 Quaker parrot ………………..…….…………………………..……95

5.2.7 Lineolated parakeet ………………………………………………..…..98

5.3 Comparing the analytical methods using the results obtained …………102

CHAPTER 6: Conclusions…...105

6.1 Introduction…………………………………………………..………..105

6.2 Problem statement, aim and objectives……………………………….....105

6.3 Conclusions………………………………………………………….....106

6.3.1 Objective 1- Obtain samples of specific avian species to conduct the study …………………………………………………………106

6.3.2 Objective 2 – Evaluate the existing PCR protocol for avian molecular sexing, and apply it to determine the sex of the chosen samples …...…………………………………………..………107

6.3.3 Objective 3 – Design and optimize a PCR-HRM protocol for avian molecular sexing ……………..…………………………....…..…108

6.3.4 Objective 4 – Compare the accuracy of the PCR-HRM assay to the conventional PCR using different avian species ………………...109

6.4 Final conclusions and future recommendations…………..……….…...110

REFERENCES…………………………………………………………………...113

LIST OF FIGURES

Figure 2.1: Applications of PCR-based methods for avian gender determination…...7

Figure 2.2: Basic principles of avian molecular sexing based on CHD1 gene ……...14

Figure 2.3: Comparison of three commonly utilized avian molecular sexing primers for gender identification in Spotted Barbtail…………………………………...17

Figure 2.4: Diagrammatic representation of the function of TaqMan probes………..21

Figure 2.5: Experimental strategy workflow……..………………………………....…..28

Figure 4.1: Annealing temperature gradient gel image of a female Fischer's Lovebird sample…………………………………………………………………...37

Figure 4.2: PCR amplification of Galah Cockatoo and Fischer's Lovebird gel image...………………………………………………………………..…39

Figure 4.3: PCR amplification of Fischer's Lovebird samples with reduced annealing temperature……………………………………..…………………....41

Figure 4.4: PCR amplification of Galah Cockatoo samples gel image……..………..42

Figure 4.5: PCR amplification of Nyasa Lovebird samples gel image……….………43

Figure 4.6: The HRM analysis workflow illustrated in logical steps…………………...45

Figure 4.7: KAPA HRM FAST master-mix optimization gel image of Fischer's Lovebird samples……………………………………………………………….………47

Figure 4.8: KAPA HRM FAST master-mix gel image of different DNA concentrations of a Fischer's Lovebird sample …..…………………………………...…48

Figure 4.9: PCR amplification of Fischer's Lovebird samples gel image for EvaGreen melting curve analysis ………………………..……………………..……51

Figure 4.10: Melting curve analysis (MCA) using EvaGreen on Fischer's Lovebird samples…………………………………………………………...……..52

Figure 4.11: Raw data melt curve results for ten Fischer's Lovebird samples ..……..55

Figure 4.12: Aligned melt curve of ten Fischer's Lovebird samples ……...…………...56

Figure 4.13: HRM analysis data difference plot of ten Fischer's Lovebird samples ...58

Figure 4.14: PCR amplification of Peach-faced Lovebird samples with the P2/P8 primer set ………………………..…………..…..……………………….………61

LIST OF FIGURES continued…

Figure 4.15: PCR amplification of Peach-faced Lovebird, four females and one male amplified with 2550F/2718R primers gel image..…………………...……62

Figure 4.16: Aligned melt curves of ten Peach-faced Lovebird samples amplified with P2/P8 and 2550F/2718R primer sets ………...……………………..……64

Figure 4.17: Difference plots of ten Peach-faced Lovebird samples amplified with P2/P8 and 2550F/2718R primer sets …………………………….…......66

Figure 5.1: Agarose gel image of Fischer's Lovebird samples………………………...70

Figure 5.2: Derivative melt curves of Fischer's Lovebird………………..……..……...71

Figure 5.3: Aligned melt curves of Fischer's Lovebird samples……..………………..…73

Figure 5.4: HRM analysis data difference plot of Fischer's Lovebird samples……….74

Figure 5.5: Agarose gel image of Nyasa Lovebird samples…………………………...75

Figure 5.6: Derivative melt curves of Nyasa Lovebird…………………….……………76

Figure 5.7: Aligned melt curves of Nyasa Lovebird……….……………...……………77

Figure 5.8: HRM analysis data difference plot of Nyasa Lovebird………….……...78

Figure 5.9: Agarose gel image of Black-Cheeked Lovebird samples…………....…..80

Figure 5.10: Derivative melt curves of Black-cheeked Lovebird……...….………….....…81

Figure 5.11: Aligned melt curves of Black-cheeked Lovebird…………….…………….82

Figure 5.12: HRM analysis data difference plot of Black-Cheeked Lovebird sample……………………………………………………...………….83

Figure 5.13: Agarose gel image of Peach-faced Lovebird samples………...…………85

Figure 5.14: Derivative melt curves of Peach-faced Lovebird…………….……….....86

Figure 5.15: Aligned melt curves of Peach-faced Lovebird……………….……….…...87

Figure 5.16: HRM analysis data difference plot of Peach-faced Lovebird….…………88

Figure 5.17: Agarose gel image of Green-cheeked Conure samples………...………89

Figure 5.18: Derivative melt curves of Green-cheeked Conure………….…….………91

Figure 5.19: Aligned melt curves of Green-Cheeked Conure………………….………92

Figure 5.20: HRM analysis data difference plot of Green-cheeked Conure……..…..93

LIST OF FIGURES continued…

Figure 5.21: Agarose gel image of Quaker parrot samples……………………..………95
Figure 5.22: Aligned melt curves of Quaker parrot………………..……..………………96
Figure 5.23: HRM analysis data difference plot of Quaker parrot……..………..……….97
Figure 5.24: Agarose gel image of Lineolated parakeet samples……………..………..98
Figure 5.25: Derivative melt curve of Lineolated parakeet……………..……..…………99
Figure 5.26: Aligned melt curves of Lineolated parakeet…………………...…...……..100
Figure 5.27: HRM analysis data difference plot of Lineolated parakeet………………101

LIST OF TABLES

Table 2.1: Nucleotide sequences of CHD-linked primers applied to sex identification in birds……………………………………………………………..………..13

Table 2.2: Comparative description of the main advantages and limitations of sexing techniques based on their applicability in the field of molecular sexing of birds…………………………..…………………………………..……….24

Table 5.1: Evaluation of the PCR-HRM assay accuracy on different species……………………………………………………………..………102

ABBREVIATIONS, SYMBOLS AND UNITS

°C	Degree Celsius
3’	Three prime end
5’	Five prime end
CHD	Chromo-helicase DNA binding gene
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
dsDNA	double stranded DNA
EtBr	Ethidium bromide
HRM	High resolution melt
µL	Microliter
µM	Micromolar
ng/µl	Nanogram per microliter
NWU	North-West University
THRIP	Technology and Human Resources for Industry Program
PCR	Polymerase chain reaction
SNP	Single nucleotide polymorphism
ssDNA	single stranded DNA
MCA	Melting curve analysis
TAE	Buffer containing Tris base, acetic acid and EDTA
Tm	Melting temperature
v/v	volume per total volume
w/v	weight per total volume

CHAPTER 1: Introduction

The approach to accurately determine the sex of birds in a population is vital in order to obtain the sex ratio of the birds so as to be able to determine the effective size of the population, to manage, conserve wildlife and to design breeding programs for threatened species. The pairing of males and females can be ascertained based on the breeding programs designed with this knowledge. More than 50% of bird species are sexually monomorphic, having no distinguishable morphological differences between males and females. Therefore sexing of nestlings, juveniles and even adult birds of monomorphic species cannot be accurately determined by phenotypic traits.

Numerous methods have been used for sex determination in birds which include surgical sexing, behaviour signs, vent sexing etc. These traditional techniques present significant problems because the birds may suffer stress since they are subjected to invasive procedures. The accuracy of these methods also remains questionable. The development of DNA based sexing procedures have provided a less invasive option to sex birds. However, the standard conventional polymerase chain reaction (PCR) technique is still laborious and time consuming. Recently real-time PCR technologies for avian molecular sexing have demonstrated increased sensitivity and reduction in analysis time. Thus, a

need exists for a PCR-high resolution melt (HRM) assay for the accurate and cost effective molecular sex determination of avian samples in a diagnostic environment.

The structure of the thesis is as follows: Chapter 2 consists of a literature review, with a detailed illustration of the experimental approach, aim and objectives. In Chapter 3, all the analytical methods used in this study is described. In Chapter 4, the standardization of the analytical techniques are described. The standardized analytical techniques (conventional PCR and the PCR-HRM assay) are compared in Chapter 5 using seven different avian species. Chapter 6 is the concluding chapter where final conclusion are made, relative to the objectives set for the study, and future recommendations are made. Finally a list of references used in this study is given.

CHAPTER 2: Literature Review

2.1 Introduction

In this literature study, the importance of avian sexing will be given, the overview of various general traditional methods for sex determination of birds will be discussed and their limitations in detail to better understand the motive for the study. Furthermore, the development of DNA based techniques, the basic principles of avian molecular sexing based on the CHD1 gene and comparison of the three CHD-linked primers sets for avian molecular sexing will be discussed. The selection of a suitable sexing technique will be further motivated, and the advances in the avian molecular sexing techniques will be given in a chronological order. The HRM analysis, which is the most recently introduced technique, will be discussed.

2.2 Importance of gender identification in birds

Sex identification in avian species is one of the key points of avian breeding and evolutionary studies. Through the knowledge of sex identification genes, poultry breeding programmes can be applied more successfully. Reproduction is possible by keeping males and females together in avian breeding. If the breeders are not sure of the sex of the birds, they cannot get any new-borns from monomorphic birds. The time spent for reproduction process causes significant financial losses (Cerit and Avanus, 2007). Sex

identification in birds is necessary for the following reasons: It helps with management and conservation of avian species, the study of animal ecology, behaviour, population structure and life history. It has been reported that nestling turkeys, ducks, parrots, cranes, geese, owls and other monomorphic bird species pose a challenge to zoologists and breeders as their sexual morphology often cannot be distinguished. The challenge emanates from indistinguishable external sex organs in birds. The availability of this information is of fundamental importance towards wildlife conservation and management of protected species (Donohue and Dufty, 2006; Balkiz *et al*., 2007). Furthermore, it is often difficult or impossible to distinguish between males and females outside the breeding season in other species (Donohue and Dufty, 2006). Gender determination techniques help to maintain a balance of sex ratio (Studer-Thiersch, 1986) of small populations (Griffiths *et al*., 1998; Chang *et al*., 2008), in order to prevent declines in the genetically effective population size (Ryman and Laikre, 1991).

2.3 General Methods for gender determination of birds

Sex identification in avian species can be performed by many techniques, such as vent sexing, laparoscopy, steroid sexing, and DNA based techniques. However, the preference between these methods depends on laboratory facilities and the experience of the experimenters. The traditional methodologies based on different morphological entities in the sex determination of birds, are time consuming, expensive, and, in some cases, invasive and harmful (Jodice *et al*., 2000). To avoid these limitations, techniques for avian sexing were improved (Morinha *et al*., 2012). Additionally, sex identification

using molecular methods has proved to be a valuable tool in wildlife conservation, in addition to behaviour studies and breeding programmes (Dawson *et al*., 2001). Bird sexing is currently done worldwide using a common PCR test that is a time consuming test (PCR and gel-electrophoreses) that can only reveal the sex of the common bird species, generates biological hazards and is prone to contamination (open system). Since bird sexing is done globally, it is imperative to develop and apply a method that is more robust, generates much less contaminants, is relatively faster and less prone to contamination (closed system). In this section, general methods for gender identification of birds are given and discussed, as well as the introduction of DNA based sexing techniques.

2.3.1 Vent sexing

Several approaches to sex monomorphic species of birds have been used in the field (Boersma and Davies, 1987), including vent sexing, a method based on holding a day old chick upside down and examining the vent area (cloaca). Experts can correctly identify gender with 95% accuracy while the success rate is lower in non-specialist people with an accuracy of 60-70%.

2.3.2 Laparoscopy

Laparoscopy is a surgical technique based on the evaluation of physical characteristics of the reproductive tract. The method requires the bird to be placed under anaesthesia

so that an endoscope could be inserted through the skin. Although this is a good procedure in that it allows other internal organs to be viewed, it also poses a disadvantage in that it requires the necessity of anaesthesia and risk of accidental injury to the vital organs. This examination can be harmful and lethal to the birds (Tella and Torre, 1993).

2.3.3. Karyotyping

Karyotyping is a method based on exploring the sex chromosomes in birds. In birds, females are heterogametic (Z and W chromosomes) and males are homogametic (ZZ chromosomes) (Ellegren, 1996). The chromosomes are isolated from cultured cells which are commonly derived from a feather or blood cells. Most avian chromosomes are micro chromosomes, so it is difficult to count them accurately. Female birds on the other hand possess W chromosomes which are comparable in size with most micro chromosomes. The large-sized Z chromosome can be discriminated from the smaller W chromosome. The size differences between these chromosomes make it practical for sex identification (Archawaranon, 2004). The disadvantage of chromosome analyses is the difficulty in obtaining viable cells from the culture. It is a time consuming procedure (Cerit and Avanus, 2007).

The procedures mentioned above have limitations. Vent sexing is very intrusive for sensitive life stages of birds. Laparoscopy is a stressful and invasive procedure which could be fatal. Karyotyping is a time consuming procedure considering the difficulty in obtaining viable cells from the culture.

2.4 Development of DNA Based techniques for avian gender identification

DNA sexing has become the preferred method for determining the sex of monomorphic birds after developments in DNA technology. Most of the DNA techniques are based on the Polymerase Chain Reaction (PCR) method (Vucicevic *et al*., 2013). In the 1970s, DNA research was difficult, expensive and slow. With the new developments in DNA technology and techniques, genetic science was revolutionized by the invention of PCR (Cerit and Avanus, 2007). As such, DNA research became simpler, cheaper and faster. Figure 2.1 demonstrates the importance of the application of DNA based techniques in various fields.

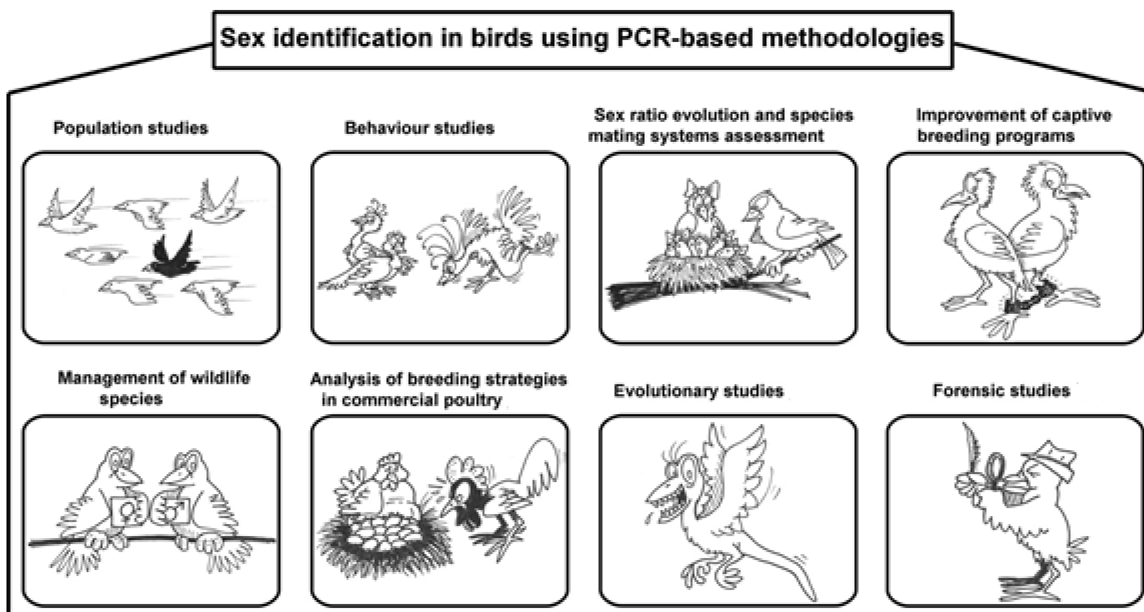

Figure 2.1: Applications of PCR-based methods for avian gender determination. The fundamental research fields dependent on the study are presented. Image adapted from Morinha *et al*. (2012).

2.4.1. Single Strand Conformation Polymorphism (SSCP)

SSCP is a technique that is based on the principle that single-stranded DNA (ssDNA) molecules form specific secondary conformations as dictated by their nucleotide sequence, under non-denaturing circumstances. The procedure involves PCR amplification of the target sequence, then the double-stranded PCR amplicons are denatured with heat at higher temperature and by introducing denaturants like formamide, dimethyl sulfoxide (DMSO) or sodium hydroxide. The ssDNA products are separated in a non-denaturing polyacrylamide gel electrophoresis (Morinha *et al*., 2012). The ssDNA products take on a specific conformation based on their primary sequence during electrophoresis. In theory, single base changes have an influence on the conformational structure of ssDNA molecules, causing visible mobility shifts. These differences impact the position and number of bands in polyacrylamide gel, making genotype assessment possible. This technique was applied successfully to determine the sex of some bird species. It was used in screening PCR products amplified from the CHD1 gene with P2/P8 primers (Morinha *et al*., 2011, Ramos *et al*., 2009). As a result of intronic variations between CHD1Z and CHD1W alleles, different conformational structures will occur. In this way, the band patterns of both males (ZZ) and females (ZW) can be analysed and studied in the polyacrylamide gel (Morinha *et al*., 2012). This technique is cost-effective but has a limitation in that it is time consuming as it requires a lot of optimization of several factors and thus, limiting the high-throughput applicability of the technique.

2.4.2. Restriction Fragment Length Polymorphism

Restriction Fragment Length Polymorphism (RFLP) is a technique that exploits variations in homologous DNA sequences. This technique involves the digestion of DNA by restriction enzymes and the resulting restriction fragments are separated by agarose gel electrophoresis. PCR-RFLP analysis has been suggested as an appropriate approach for molecular identification of sex of various bird species with small differences between CHD1 amplicons (Bermúdez *et al.*, 2002). The intronic variations in CHD1Z and CHD1W alleles amplified with universal primers, allowed for the identification of restriction sites in both fragments. The selection of suitable restriction enzymes (e.g. *Hae*III; *Dde*I; *Asp*700) allowed for the selective digestion of CHD1Z and CHD1W fragments. The fragments are analysed by agarose or polyacrylamide gel electrophoresis. The specific band patterns vary depending on the species and the restriction enzyme selected. This technique is simple but has some shortfalls in that the selection of suitable restriction enzymes is a complicated task because of the high nucleotide variability of CHD1 introns among different bird species. As a result, the development time is increased and the high-throughput applicability is reduced (Morinha *et al.*, 2012).

2.4.3 Randomly Amplified Polymorphic DNA

Randomly Amplified Polymorphic DNA (RAPD) is performed using PCR, but the segments of DNA that are amplified are random. No knowledge of the DNA sequence for the target genome is required. DNA fragments produced by PCR are called RAPD markers, which could be used in sex identification. If the selected RAPD marker is on the

W chromosome, a female specific allele, it would be amplified only in females (Welsh and McClelland, 1990). The reliability of RAPD markers is questionable. Their low reproducibility, sensitivity to reaction conditions and competition between different DNA fragments cause more weakly amplified bands. This method was criticized as species-specific by (Griffiths and Tiwari, 1993).

2.4.4 Amplified Fragment Length Polymorphism

Amplified Fragment Length Polymorphism (AFLP) uses restriction enzymes to digest genomic DNA, followed by the ligation of specific double-stranded oligonucleotide adapters to the sticky ends of all restriction fragments (Morinha *et al.*, 2012). These adapters are designed to avoid the reconstitution of the original restriction site after ligation. The adapter-restriction fragments are subsequently amplified by PCR under highly selective conditions with adapter-specific primers containing an extension of one to three nucleotides at the 3' end. The AFLP-PCR products are analysed with polyacrylamide gel electrophoresis for maximum resolution for the detection and selection of specific polymorphic marker. The AFLP technique was applied successfully by (Griffiths and Orr, 1999) in the isolation of sex-specific markers. The AFLP analysis has some disadvantages (time-consuming method development, relatively expensive and species-specific nature of markers) that reduce its routine applicability in avian sexing (Morinha *et al*, 2012).

2.4.5 Microsatellites

Microsatellites (sometimes referred to as variable number of tandem repeats) are short tandem repeated DNA sequences formed by repetitive motifs of 1 to 6bp found both in coding and non-coding regions of the genome (Morinha *et al.*, 2012). These motifs usually have a high variability in the number of repeat units. The potentialities of these highly polymorphic genetic markers have been explored in many fields of biological research (Olah *et al.*, 2016). In bird sexing, microsatellites are used as sex-specific markers that enable gender identification. Jones *et al.*, (2002) reported one sex-linked locus for sex identification in Whooping cranes. The PCR amplification of this microsatellite locus revealed the presence of a female-specific fragment from the W chromosome. The presence or absence of this sequence allowed the accurate sexing of females and males. For maximum resolution, polyacrylamide gel electrophoresis is applied. This molecular strategy requires species-specific markers, which increases the development time and intensiveness of labour, limiting its routine applicability in the gender differentiation of birds.

2.4.6 Capillary Electrophoresis

Capillary electrophoresis (CE) is an analytical method with great advantages (speed, high-throughput applicability, high resolution and sensitivity). The separation of DNA fragments occurs inside of a fused-silica capillary filled with a sieving matrix, under high voltages. The CE system performs a rapid size-based separation of the specific fragments and the DNA is detected by UV absorption. This method requires an internal

size standard that enables the accurate fragment size attribution based on its relative electrophoretic migration (Morinha *et al.*, 2012). This approach was developed based on the analysis of DNA fragments amplified from CHD1F/CHD1R primer pairs. The primer set CHD1F/CHD1R was designed from sequence alignments of 2550F/2718R products (Fridolfsson and Ellegren, 1999) to reduce the amplicon length. Smaller PCR products are more suitable for CE analysis. Thus, the CE electropherogram detects one amplification product for male sample (Z allele) and two amplification products for female samples (Z and W alleles) allowing accurate sex differentiation. The CE is a simple and rapid strategy for avian molecular sexing.

2.5 CHD- based sex identification

Current molecular techniques of non-ratite avian gender determination explore the sequence polymorphisms in the CHD gene. The CHD gene, encoding chromo-helicase DNA binding protein 1, was the first gene discovered on the avian W chromosome (CHD-W) (Cerit and Avanus, 2007; Ellegren, 1996). CHD-Z is situated on the Z chromosome and is found in both sexes. The CHD gene was the first gene reported as a suitable sex-linked marker for molecular sexing of non-ratite birds (Griffiths *et al.*, 1998).The most universal tag for sex typing is provided by the CHD gene. It is located in both chromosomes in almost all bird species except ratites (flightless birds), which have undifferentiated sex chromosomes. In birds, males are homogametic (ZZ) and females are heterogametic (ZW). The CHD 1 gene is highly conserved, but it contains some intronic variations between CHD1Z and CHD1W sequences, which allow the selection of

several specific primers for avian sexing based on PCR amplification of these regions (Morinha *et al.*, 2013). The PCR products migrate as a single band for males (ZZ) and females migrate as two bands (W and Z) due to unequal allele size in the intronic region (Dubeic and Zagalska-Meubauer, 2006).

The three CHD-related primer pairs used in sex identification were designed to flank the fragment of the gene with the intron. This allows discrimination between the products from the Z and W chromosomes on a gel (Dubeic and Zagalska-Meubauer, 2006). The three CHD-related primer pair sequences are given in Table 2.1.

Table 2.1: Nucleotide sequences of CHD-linked primers applied to sex identification in birds (Dubeic and Zagalska-Meubauer, 2006)

Primers	Nucleotide sequence	Source
P2 P8	5'-TCTGCATCGCTAAATCCTTT-3' 5'-CTCCCAAGGATGAGRAAYTG-3'	Griffiths *et al.*, 1998
2550F 2718R	5'-GTTACTGATTCGTCTACGAGA-3' 5'-ATTGAAATGATCCAGTGCTTG-3'	Fridolfsson & Ellegren, 1999
1237L 1272H	5'-GAGAAACTGTGCAAAACAG-3' 5'-TCCAGAATATCTTCTGCTCC-3'	Kahn *et al.*, 1998

In Figure 2.2, the basic principles of avian molecular sexing based on the CHD1 gene are illustrated and discussed.

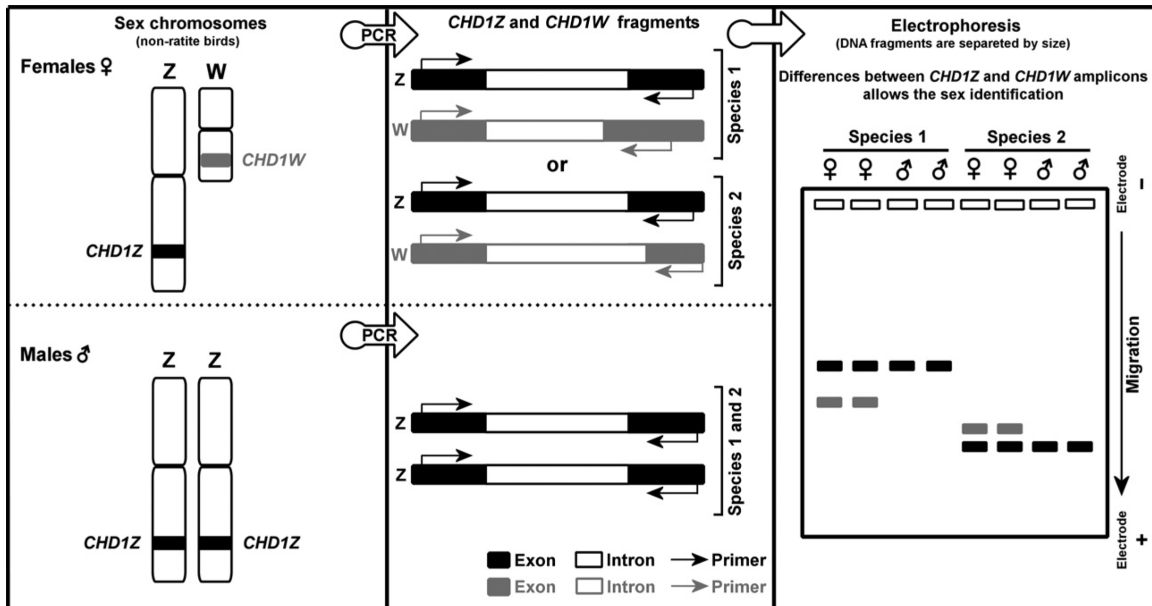

Figure 2.2: **Basic principles of avian molecular sexing based on CHD1 gene**. Males are homogametic (ZZ), that is, they have two copies of CHD1Z allele, while females are heterogametic (ZW), having one copy of each CHD1W and CHD1Z. The intronic variations between CHD1Z and CHD1W in non-ratite birds allow sex identification of birds using specific primers to amplify these particular regions. The PCR products migrate as single band for males (ZZ) and two bands for females (ZW) on agarose gel electrophoresis. Image adapted from (Morinha *et al.*, 2012).

Figure 2.2 demonstrates the basic principles of avian molecular sexing. The standard methodology is based on amplification by PCR of CHDZ and CHDW alleles using universal CHD1 primers, and subsequent electrophoresis. The CHD1 gene is highly conserved, however, it contains some intronic variations between CHDZ and CHDW sequences (Figure 2.2), which allow the selection of several specific primers for avian sexing based on the PCR amplification of these regions (Wang and Zhang 2009). In

theory, the amplification products migrate as a single band for males (CHDZ) and two bands in females (CHDZ and CHDW).

2.6 Comparison of three commonly utilized CHD-linked primer sets

The P2/P8 primer pair are universal primers for molecular sex identification. This primer pair has limitations in that it amplifies a sequence over an intron. Due to gender differences in the intron length, a smaller fragment is amplified from the Z chromosome than from the W chromosome. As a result, the shorter Z chromosome fragment may be preferentially amplified as observed in the Adelie, *Pygoscelis adeliae* (Dawson *et al*., 2001). Females are misidentified as males in this instance. The differences between fragments amplified with P2/P8 primers are usually small in most avian species, ranging from 10-80bp or even less 3 to 8bp in other species, which could lead to misidentification of species due to small intron differences between the Z and W fragments as shown in Figure 2.2. In contrast, the design of the 2550F/2718R primers is such that the amplified W fragment is the smaller one, so avoiding this potential problem (Dawson *et al*., 2001). The 2550F/2718R primers have been reported to produce only one band both in males and females in some species (Fridolfsson and Ellegren 1999). This occurs as a result of preferential amplification of the shorter allele from the W chromosome, which then results in no detectable product from the Z chromosome. In such cases, the birds can be sexed based on the differences in size of both amplified fragments. The single fragment in both males and females has been reported to be found in the *Anatidae, Coruidae, Scolopacidae, Falconidae* and *Accipiteridae* (Dubeic and Zagalska-Meubauer, 2006).

One advantage of the P2/P8 primers is that they have been shown to amplify the target regions in a large number of non-ratites and despite scoring difficulties in some species, sex could be allocated in all species in the study conducted by (Dawson *et al*., 2001). Compared to P2/P8 primer pair, the 2550F/2718R primer pair has been tested in far fewer species, and the amplified fragments are larger, and could be prone to even more polymorphisms. The larger difference between the sizes of the Z and W products obtained using 2550F/2718R makes it less likely that any polymorphism will lead to scoring error (Dawson *et al*., 2001).

The primers 1237L/1272H (Kahn *et al*., 1998) have been reported to produce a larger number of non-specific fragments than P2/P8 (Griffiths *et al*., 1998). It is advisable to make use of the latter pair. Both these sets of primers (1237L/1272H and P2/P8) target the same intron, but 2550F/2718R flank a different intron (Jensen *et al*., 2003). The differences between products amplified with 2550F/2718R ranges from 150 to 250bp, while products amplified with P2/P8 range from 10 to 80bp (Dubeic and Zagalska-Meubauer, 2006). Therefore, in some species where P2/P8 primers are used, it is recommended to make use of polyacrylamide gel rather than agarose, as it provides better resolution. Dawson *et al*. (2001) could not distinguish the Z and W fragments amplified with P2/P8 on agarose gels in the auklets.

Sex identification based on P2/P8 primers may be difficult in some species if there is a polymorphism in the Z chromosome. Such polymorphism has been documented in nearly twenty species. In order to avoid this limitation, the 2550F/2718R primers instead of P2/P8 should be used, as they flank a different intron, which is most probably responsible for a polymorphism of fragments from the Z chromosome, thus producing two distinct

products with significant length differences easily separated on agarose gels without the need of polyacrylamide gel (Dubeic and Zagalska-Meubauer, 2006). Some studies have revealed polymorphisms within the CHD1 gene in several species, potentially producing different sized introns in heterogametic males, and leading to misidentification of gender (Port and Greeney, 2012).

For confident sex assignment in non-ratite birds, it was concluded that both P2/P8 and 2550F/2718R sexing primers should initially be tested in order to identify the primers most appropriate for the study (Dawson *et al.*, 2001). In Figure 2.3, the differences between the three CHD-related primer pairs are demonstrated.

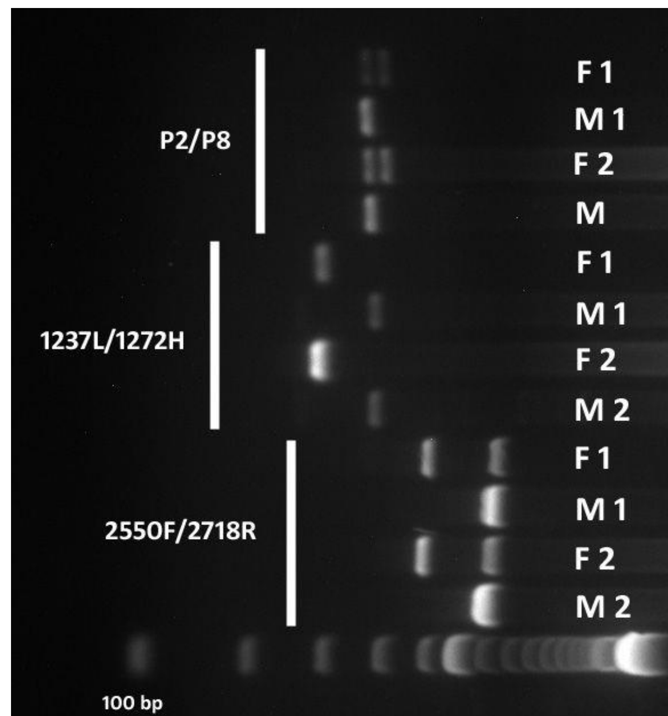

Figure 2.3: **Comparison of three commonly utilized avian molecular sexing primers for gender identification in Spotted Barbtail**. P2/P8 (Griffiths *et al.*, 1998), 1237L/1272H (Kahn *et al.*, 1998) and 2550F/2718R (Fridolfsson and Ellegren 1999).Each

primer set was tested using samples from four adult birds. Image adapted from (Port and Greeney, 2012).

Port and Greeney (2012) compared the three avian molecular sexing primer pairs. The results suggested that for Spotted Barbtail, the 2550F/2718R (Fridolfsson and Ellegren, 1999) and P2/P8 (Griffiths *et al*., 1998) primers provided the most reliable results. However for most individuals, all three pairs provided fragments suitable for sexing. In all individuals, a comparison of the results for each primer set suggested the sexing was unambiguous (Figure 2.3). For 2550F/2718R, males were identified by the presence of a single band between 600 and 700bp. Heterogametic females were identified by the presence of a second additional fragment of approximately 475bp. For P2/P8, males were identified by the presence of a single 380bp fragment while females had a second additional fragment of approximately 415bp (Figure 2.3). In contrast, 1237L/1272H produced single bands of differing lengths for males and females. Females were identified by the presence of bands of approximately 300bp in length while the amplification products for males were approximately 380bp in length.

2.7 Selection of a suitable sexing technique

The CHD-based technique remains the first method of choice in molecular avian sexing. It is accurate, easy and relatively cheap. The processing of a sample including DNA extraction, PCR and gel electrophoresis may take about five hours. It is recommended to check the most suitable CHD-linked primer pairs by starting with analysing the primers

which were successfully applied in closely related species. If no information is available, then testing both P2/P8 and 2550F/2718R primers for their applicability in the specific species studied. The other techniques described may be recommended only when the CHD-based techniques do not work as they are more laborious, time consuming and costly (Dubeic and Zagalska-Meubauer, 2006).

The aim of establishing a sexing technique that is minimally invasive, fast, accurate, and cheap with high-throughput applicability has only been partially achieved because all these procedures still involve DNA extraction, PCR and gel electrophoresis. However, the aim of minimizing the intensiveness and stressful procedures that the birds are subjected to has been accomplished because only small amount of tissue is needed to conduct the procedure and is preserved in some ways, like blood blotted on a filter paper as source of DNA. However, these techniques are costly and time-consuming with limited applicability as routine methodology (Morinha *et al.*, 2011). Advances in real-time PCR based techniques overcome some limitations of the more classical molecular analysis methodologies.

2.8 Real-time PCR methods for sex determination

Over the past few years, new advanced methodologies have been proposed for high-throughput avian molecular sexing, such as real-time PCR using TaqMan probes (Chou *et al.*, 2010; Rosenthal *et al.*, 2010) and real-time PCR with melting curve analysis (Chang *et al.*, 2008, Brubaker *et al.*, 2011). Recently, the high-resolution melt (HRM) analysis started to be utilized in combination with real-time PCR technology.

2.8.1 Real-time PCR using TaqMan probes

The real-time quantitative TaqMan assay requires a fluorogenic probe with a target sequence localized within the amplicon defined by a gene-specific PCR primer pair. The oligonucleotide probe has a fluorescent reporter dye covalently attached to its 5’ end and a quencher dye attached at the 3’end. The probe anneals downstream from one of the primer sites when the target sequence is present. While the probe is intact, the quencher dye absorbs the fluorescence emission of the reporter dye. The cleavage of the probe by Taq polymerase during the extension phase of PCR separates the reporter and quencher dyes, which increases the fluorescent emission of the reporter dye. This step removes the probe from the target strand, allowing the primer extension until the end of the template strand. The fluorescence intensity released by reporter dye molecules cleaved from the probes during the PCR is proportional to the amount of specific amplified products (Morinha *et al*., 2012). Gender identification based on qPCR using TaqMan assay of this study was developed based on CHD1 gene using different strategies. The high sensitivity, efficiency and specificity of this technology allow a high-throughput sex identification of birds. The shortfalls that come with the TaqMan assays are associated with cost and the necessity of species-specific probes (Morinha *et al*., 2012). In Figure 2.4, the function of Taqman probes is illustrated.

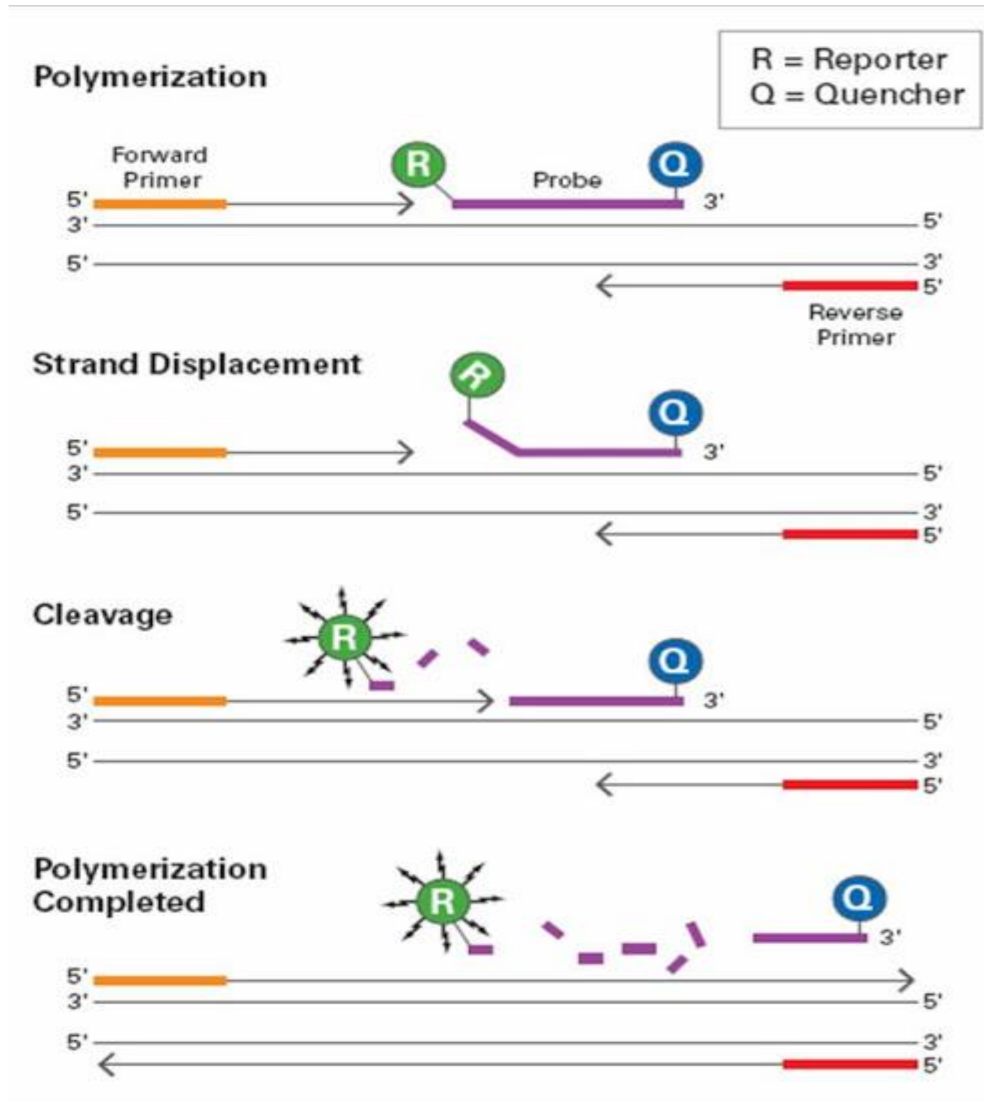

Figure 2.4: Diagrammatic representation of the function of Taqman probes.

2.8.2 Real-time PCR combined with melting curve analysis (MCA)

The real-time PCR combined with melting curve analysis is based on dsDNA binding dye that intercalates with dsDNA, allowing fluorescent detection of the PCR product. The fluorescence intensity increases as more dsDNA is formed. The saturating dye has high fluorescence in the bound state. The amplicons are identified by analysis of their specific

melting temperature. The varying melting temperature of the amplicons allow the discrimination of different products. This technique was proposed for high-throughput avian molecular sexing. The studies which used this technique were based on PCR amplification of the CHD1 gene with primer pairs that allowed the detection of significant difference in the melting temperature between CHD1Z and CHD1W amplicons. The real-time instrument discriminated the melting peaks related to distinct melting temperature values. The sensitivity and high-throughput applicability are advantages of this method (Morinha *et al.*, 2012).

2.8.3 HRM analysis

HRM analysis is a post-PCR analysis method used for identifying genetic variation in nucleic acid sequences. The method is based on PCR melting curve techniques and is enabled by the recent availability of improved double-stranded DNA (dsDNA)-binding dyes along with next generation real-time PCR instrumentation and analysis software. HRM analysis can discriminate between DNA sequences based on their composition, length, GC content, or strand complementarity (Montgomery *et al.*, 2010).

HRM analysis starts with PCR amplification of the region of interest in the presence of a dsDNA-binding dye (e.g., EvaGreen). This dye intercalates into dsDNA and has high fluorescence in the bound state, and low fluorescence in the unbound state. Amplification is followed by a high-resolution melting step using instrumentation capable of capturing large fluorescent data points per change in temperature, with high precision. When

dsDNA melts into single strands, the dye is released, causing a change in fluorescence (Reed *et al.*, 2007).

The HRM analysis was introduced to avian molecular sexing. This technique allowed the rapid and accurate sex determination in Common Quail (*Coturnix coturnix*) and Japanese Quail (*Cortunix japonica*) using the universal primers P2/P8. The amplified sequences of CHD1Z and CHD1W differ only in 6bp. Specific melting curves were observed for males (ZZ) and females (ZW), which was impossible to observe using the protocols described before (Chang *et al.*, 2008, Brubaker *et al.*, 2011, Huang *et al.*, 2011) and primers P2/P8, because only one melting peak was detected in both males and females by the melting curve analysis. The small differences between the CHD1 amplicons amplified with P2/P8 primer pair allowed the study of a novel approach using the HRM approach for gender determination in these subspecies (Morinha *et al.*, 2011). The advantages and disadvantages of DNA based sexing techniques are tabulated in Table 2.2.

Table 2.2: Comparative description of the main advantages and limitations of sexing techniques based on their applicability in the field of molecular sexing of birds (Morinha *et al.*, 2012).

Methods	Ease of use and development	Labour intensiveness	Sensitivity	Reproducibility	High-throughput applicability	Cost	Diagnosis
PCR and agarose gel	Easy	Low	Moderate	High	Moderate	Low	++
PCR and acrylamide gel	Easy	Moderate	Moderate	High	Moderate	Low	+
SSCP	Easy	Moderate	High	Moderate	Low	Low	+
RFLP	Easy	Moderate	Moderate	Moderate	Low	Low	+
RAPD	Difficult	High	Low	Low	Low	Low	-
AFLP	Difficult	Moderate	Moderate	Moderate	Low	Moderate	-
Microsatellites	Moderate	Moderate	Moderate	Moderate	Low	Moderate	-
Capillary electrophoresis	Easy	Low	High	High	High	Moderate	++
qPCR using TaqMan probes	Moderate	Low	High	High	High	Moderate	++
Real-time PCR with MCA	Easy	Low	High	High	High	Low	+++
HRM	Easy	Low	High	High	High	Low	+++

- Fair
+ good
++ very good
+++ excellent

The molecular techniques for avian molecular sexing were clearly improved from previous invasive methods such as laparoscopy to more modern, less invasive, more specific and cost-effective methods such as real-time PCR-HRM. Several methods were presented as appropriate approaches to overcome the challenges with sex identification in a wide range of bird species. Advances in PCR-based technologies allowed the development of simple, rapid, high sensitive and cost effective protocols. The real-time PCR platforms offer all these advantages, making them extremely competitive, regarding the performance and requirements of classical methodologies as seen in Table 2.2.

The HRM assay overcomes the resolution limitation of the real-time PCR combined with the melting curve analysis. The two techniques will further be compared and discussed in detail in Chapter 3. The simplicity, high sensitivity/specificity, low cost relatively to traditional methods that require electrophoresis and techniques using labelled probes make HRM a suitable cost-effective approach for avian molecular sexing (Morinha *et al.*, 2012).

2.9 Rational, guidelines from the industrial partner, aim and objectives as well as experimental strategy

2.9.1 Rational

Many avian species are sexually monomorphic with no easily visible differences between males and females. For bird breeders, this presents a problem. Numerous methods was developed for bird sex identification, including the observation of the bird's behaviour, laparoscopy and surgical sexing of birds. However, all of these traditional methods have their limitations. Therefore the introduction of molecular sexing techniques constituted a breakthrough in the reliability and rapidity of sex identification in birds. DNA sexing has quickly risen to become the method of choice for determining the sex of avian monomorphic species.

Lumegen Laboratories (Pty) Ltd, a molecular diagnostics company based in Potchefstroom, South Africa, focuses on veterinary DNA diagnostics services, including

DNA sexing of birds. Lumegen Laboratories (Pty) Ltd receives blood samples from bird breeders all over South Africa and utilizes a conventional PCR (and gel electrophoresis) approach to determine the sex of the samples. Unfortunately, the conventional PCR-based techniques currently used at Lumegen Laboratories (Pty) Ltd for DNA sexing of birds is time consuming and laborious, reducing their high-throughput applicability and financial feasibility. It was therefore not surprising when the North-West University was approached by Lumegen Laboratories (Pty) Ltd for help investigating and implementing an advanced DNA sexing technique for birds that would be faster, accurate and less prone to contamination than the conventional PCR technique they were using. THRIP (Technology and Human Resource for Industry Programme) funding was obtained to support this program and a contractual agreement was established between the North-West University and Lumegen Laboratories (Pty) Ltd, the industrial partner, regarding the proposed research. This MSc study formed part of the bigger research program.

2.9.2 Guidelines from the industrial partner

From the start of this study, clear guidelines were provided by the industrial partner Lumegen Laboratories (Pty) Ltd regarding the scope of the study. Firstly, the new method must be able to be performed on the equipment already available at Lumegen Laboratories (Pty) Ltd. This was essential since there was no budget for new equipment at the industrial partner. Secondly, it was critical to keep the cost of the new test below a specific price ceiling, in other words keep the test affordable and thus financially competitive. This was a huge constraint to the study as the cost implication of any

analytical approach first had to be considered before it could be applied. Furthermore, any analytical approach considered too expensive (not financially feasible for diagnostic implementation by the industrial partner) had to be abandoned and alternatives considered.

2.9.3 Aim and objectives

2.9.3.1 Aim

The **aim** of this study is to develop a polymerase chain reaction with high resolution melt (PCR-HRM) analysis for avian sex determination.

2.9.3.2 Objectives

The mentioned aim will be accomplished by completing the following objectives:

1. Obtain samples of specific avian species bred in South Africa [and thus frequently analysed by Lumegen Laboratories (Pty) Ltd, the industrial partner] to conduct the study

2. Evaluate the existing PCR protocol for avian molecular sexing, and apply it to determine the sex of the chosen samples.

3. Design and optimize a PCR-HRM protocol for avian molecular sexing.

4. Compare the accuracy of the PCR-HRM assay to the conventional PCR using different avian species.

2.9.4 Experimental strategy

Figure 2.4 illustrates the experimental strategy that was followed in this study. In order to fulfil the aim and objectives set for the study, genetic information was gathered on the Lumegen Laboratories (Pty) Ltd database for seven target bird species bred in South Africa and specific samples chosen for inclusion in the study. The existing DNA sexing PCR protocol was evaluated and optimized using a few samples of known sex. The PCR-HRM approach workflow was then designed and technical equipment was set up. DNA was isolated from the target species to perform the PCR-HRM analysis. The PCR-HRM assay was optimized using a few samples of known sex and the assay was then performed, along with conventional PCR approach, on male and female samples chosen from the seven species. The results from the PCR-HRM test were compared with conventional PCR and conclusions were made regarding the applicability of the PCR-HRM assay in a diagnostic setup.

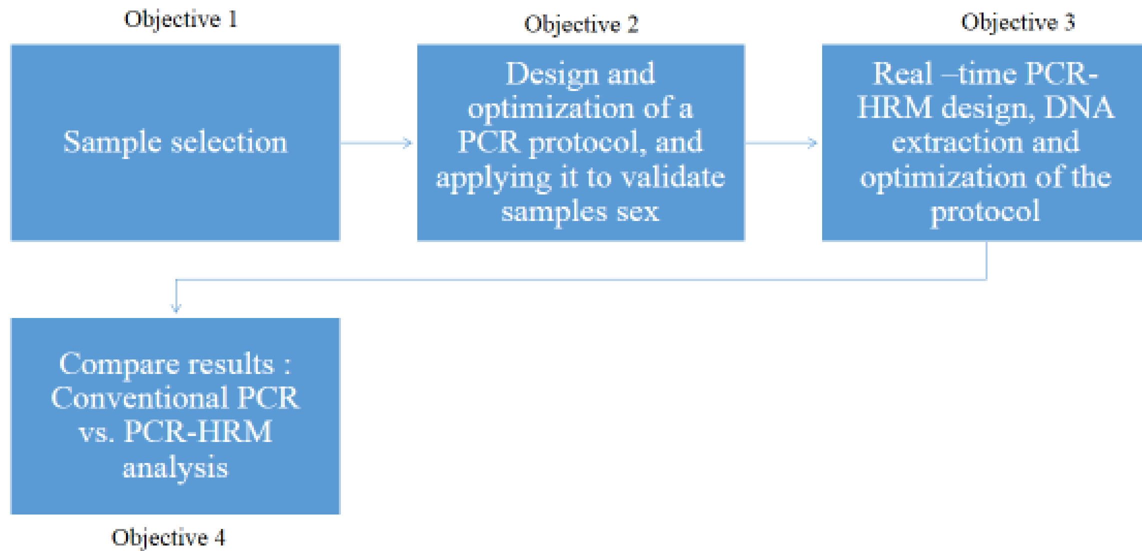

Figure 2.5: Experimental strategy workflow. The experimental strategy of this study consisted of identifying relevant samples to be included in the study, evaluation and optimization of conventional PCR, as well as design and optimization of the PCR-HRM assay. The results from both sexing techniques, namely, conventional PCR and PCR-HRM were compared

CHAPTER 3: Materials and methods

3.1 Materials

3.1.1 Sample collection

The blood samples utilized in this study were obtained from Lumegen Laboratories (Pty) Ltd, the industrial partner. Lumegen Laboratories (Pty) Ltd daily receives blood samples from bird breeders all over South Africa for diagnostic work. The blood samples are spotted onto Guthrie cards by the bird breeders, dried, sealed in a microcentrifuge tube (to prevent cross-contamination) and then sent to Lumegen Laboratories (Pty) Ltd for analysis. At Lumegen Laboratories (Pty) Ltd, each sample is given a unique laboratory number and the information on the sample is captured in a database before analyses. Lumegen Laboratories (Pty) Ltd also obtained written consent from the participating bird breeders for the samples they submitted for diagnostic testing to be included in this research project. Furthermore, the samples used in this study was only linked to the unique laboratory numbers, thus no confidential information (like the name of the breeder) was given to anyone involved in this study, thus all samples were considered anonymized.

3.1.2 List of species selected for inclusion in this study

The following species were selected for inclusion in the method optimization and/or validation parts of this study: *Agapornis fischeri* (Fischer's Lovebird), *Agapornis lilianae*

(Nyasa Lovebird), *Agapornis nigrigenis* (Black-cheeked Lovebird), *Agapornis roseicollis* (Peach-faced Lovebird), *Eolophus roseicapilla* (Galah Cockatoo), *Pyrrhura molinae* (Green-cheeked conure), *Myiopsitta monachus* (Quaker parrot), *Bolborhynchus lineola* (Lineolated Parakeet).

3.1.3 Reagents and buffers

Gene Ruler DNA ladder mix #SM0331 was purchased from Thermo Scientific, South Africa. KAPA blood PCR mix B (2X) was obtained from KAPA Biosystems purchased from Lasec, SA (Cape Town, SA). The oligonucleotides (2550F and 2718R) primers for the CHD1Z & CHD1W fragments, as originally described by Fridolfsson and Ellegren (1999) were obtained from Whitehead Scientific (Pty) Ltd, purchased from Integrated DNA Technologies (sequences shown in Table 2.1). The oligonucleotides P2/P8 for the CHD1Z & CHD1W fragments, as originally described by Griffiths *et al*. (1998) were obtained from Inqaba Biotec. The KAPA HRM Fast Master mix (2x) was purchased from KAPA Biosystems purchased from Lasec, SA (Cape Town, SA). EvaGreen dye, (20x in water) was obtained from Biotium, purchased from Anatech (Johannesburg, South Africa).

3.2 Methods

3.2.1 DNA Isolation

Genomic DNA was isolated from the Guthrie cards using the Zymogen extraction kit from Zymo Research according to the manufacturer's protocol for purification of total DNA from

animal blood (spin column protocol), with some modifications. Approximately 2 mm^2 of the Guthrie card with blood was used for DNA extraction, and the remaining blood on the card was stored for later use in the diagnostic validation method, the conventional PCR. All samples were incubated in 400 µl lysis buffer [10 mM Tris (pH 7.5), 400 mM NaCl, and 2 mM EDTA (pH 8.0)] at 55 °C on the heating block for about 1 - 2 hours with vortexing every 15 minutes. When the tissue was completely lysed, the solution was transferred into Zymo spin column followed by centrifugation at 10 000 x *g* for one minute to trap the DNA onto the spin column. The Zymo spin column was placed in a new collection tube, and the tube with the flow through was discarded. The DNA trapped on the column was washed by adding 200 µl of DNA pre-washing buffer and centrifuged at 10 000 x *g* for one minute. Another subsequent washing step of genomic DNA was done by adding 500 µl of gDNA wash buffer to the spin column and centrifuging at 10 000 x *g*. The DNA was eluted by adding 60 µl of elution buffer and centrifuging at 10 000 x *g* for one minute. The DNA was quantified and the purity calculated using the Nano Drop® ND100 spectrophotometer (Nano Drop Technologies, Thermo Fisher Scientific, USA).

3.2.2 PCR-HRM using KAPA HRM Master-mix

The PCR-HRM analysis was carried out on the Step One Plus™ real-time PCR system and the HRM software v2.2.3 (Applied Biosystems). Following DNA isolation, the reaction set up was as follows: each 20 µl reaction consisted of 10 µl KAPA HRM Master-mix, 1 µl (2550F/2718R) primers (10 µM each) to a final concentration of 500 nM, 2.4 µl (25 mM) $MgCl_2$, 2 µl DNA and 4.6 µl H_2O (molecular grade). The amplification protocol was

composed of the following steps: initial denaturation at 95°C for 5 minutes followed by 40 cycles of denaturation at 95 °C for 30 seconds, 49 °C annealing temperature for 30 seconds, and extension at 72 °C for 30 seconds. The melting curves of the PCR amplicons were then generated with temperatures as follows: 95 °C for 15 seconds, 60 °C for 1 minute and 95 °C for 15 seconds taking the fluorescent measurement after every 0.1 °C from 60 °C to 95 °C.

3.2.3 Conventional PCR

The conventional PCR method (currently employed at Lumegen Laboratories (Pty) Ltd, the industrial partner, served as a validation method for the PCR-HRM approach. A small piece of Guthrie card with blotted blood, approximately 1 – 2 mm^2 was clipped from each sample using sterilized scissors and tweezers. The small pieces of Guthrie cards with blood were then placed into their respective PCR tubes. To prevent cross contamination, the scissors were rinsed in 96% ethanol and the ethanol excess was burned off on a gas burner, and were let to cool down before starting with the next sample.

For a 20 µl reaction, the following reagents were used: 10 µl KAPA Blood PCR mix B (2X), 9 µl H_2O (molecular grade), 1 µl forward (2550F) and reverse primers (2718R) (10 µM each) to a final concentration of 500 nM, as well as 1 – 2 mm^2 piece of Guthrie card with blood blotted onto it as template DNA. The PCR amplifications were carried out in the T100™ Thermal cycler (Bio-Rad) with the following cycling conditions: activation at 95 °C for 5 minutes, 40 cycles of strand denaturation at 95 °C for 30 seconds, primer

annealing at 49 °C for 30 seconds, and extension at 72 °C for 30 seconds with a final 72 °C extension for 5 minutes and hold at 4 °C.

To test whether or not PCR fragments were successfully produced, the PCR mixtures were loaded along with 1x Loading Dye Solution [6x: 10 mM Tris-HCl (pH 7.6), 0.03% bromophenol blue, 0.03% xylene cyanol FF, 60% glycerol and 60mM EDTA] into a 2% (w/v) agarose gel in 2x TAE, stained with 1x ethidium bromide with the concentration of 0.5 µg/ml. The samples were then run at 60 V for 60 minutes. The gel was photographed under UV exposure with a G: Box gel documentation system from Syngene™ using GeneSys™ version 1.1.2.

3.2.4 Post-PCR melting curve analysis (MCA)

After the conventional PCR (Section 3.2.3) was complete, the MCA reaction was performed and consisted of 5 µl of post-PCR mixture, 1 µl EvaGreen dye (Biotium) and 14 µl H_2O (molecular grade) mixed in a real-time PCR tube. The MCA was performed on the Step One plus real-time PCR instrument and software v.2.3 (Applied Biosystems), and the melt curves were generated by the pre-melt conditions at 60 °C to 95 °C.

3.2.5 Post-PCR high resolution melt (HRM) analysis

HRM analysis was carried out on the Step One Plus™ real-time PCR system and the HRM software v2.2.3 (Applied Biosystems). The Step One plus real-time PCR system

was programmed to skip the real-time PCR amplification step as the amplification step was performed using the conventional PCR as discussed in Section 3.2.3. Only the HRM analysis was performed on the instrument. A 20 µl reaction was prepared for each sample and consisted of 1 µl of EvaGreen dye (Biotium), 5 µl of post-PCR mixture and 14 µl of H_2O (molecular grade) mixed in a real-time PCR tube. The melting curves of the PCR amplicons were then generated with temperatures as follows: 95 °C for 15 seconds, 60 °C for 1 minute and 95 °C for 15 seconds taking the fluorescent measurement after every 0.1 °C / s from 60 °C to 95 °C. The melting curve data was analysed by the HRM software (ver.2.2.3, Applied Biosystems). The melting profiles obtained were normalized by adjusting the pre- and post- denaturation transition regions, which were defined as 100% fluorescence, where the amplicons were double stranded and 0 % fluorescence where the amplicons were single stranded. The normalization of the curves was important to eliminate the differences in background fluorescence. The classification of the clusters is influenced by the melt region selected. The software automatically clustered the data with similar melting characteristics and attributed a confidence score to each sample. The software calculated the difference plot by subtracting the other curves from the baseline, which was a reference variant (sample) selected, and all samples were compared to the reference variant at the largest difference between the temperatures. Clustered samples of all species were displayed as fluorescence vs. temperature plots. These settings were used in this study to analyse the melt curves obtained for all species.

CHAPTER 4: Standardization of the analytical methods

4.1 Introduction

Although the industrial partner to this study [Lumegen Laboratories (Pty) Ltd] already had a standardized conventional PCR assay for avian sex determination, the applicability of this method was first evaluated for utilization in this study to determine the sex of the chosen blood samples. A PCR-HRM assay was then standardized as an alternative to the conventional PCR assay. Comparison of these two methods are illustrated in Chapter 5.

4.2 Evaluation and optimization of the conventional PCR protocol for avian molecular sexing

The PCR process is widely used in a tremendous variety of experimental applications to produce high yields of specific nucleotide sequences. It is imperative to optimize the PCR protocol since no single set of conditions can be applicable to all PCR amplifications without species-specific tests (Thanou *et al*., 2013). A number of reaction components like reagent concentrations, time and temperature parameters must be adjusted within suggested ranges for optimal amplification of target DNA regions, thus maximizing the DNA yield during PCR. An annealing temperature gradient was performed to select the optimum annealing temperature for the CHD1Z and CHD1W allele amplification in a

Fischer's Lovebird sample using the 2550F/2718R primers. One sample from a previously identified female bird was used to perform the annealing temperature gradient. The same procedure was used for cutting the sample as explained in details in Section 3.2.3, taking into consideration the size of the sample cut. The size of the card was kept as uniform as possible in all respective wells, that is, approximately 2 mm^2 piece of Guthrie card as template DNA.

The thermal cycler was programmed to perform an annealing temperature gradient from 50.7 °C– 60 °C while keeping all conditions the same as explained in Section 3.2.3. An annealing temperature gradient PCR for the primer sets 2550F/2718R was performed in order to obtain a single temperature that provided efficient, specific amplification of the CHD1 gene sequence. The PCR products were analysed on a 2% agarose gel.

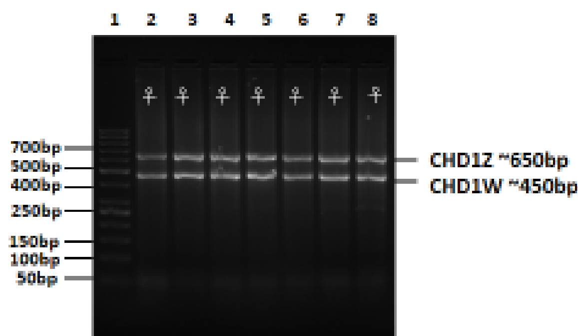

Figure 4.1: Annealing temperature gradient (50.7 °C - 60 °C) gel image of a female (♀) Fischer's Lovebird sample. A 2% agarose gel, stained with ethidium bromide, was used for separation of amplified DNA. Lane 1 represents a 50bp Gene Ruler DNA ladder. Lanes 2 to 8 shows the sample amplified at different annealing temperatures, i.e. Lane 2 = 60 °C, Lane 3 = 59.2 °C, Lane 4 = 58 °C, Lane 5 = 56.1 °C, Lane 6 = 53.8 °C, Lane 7 = 51.9 °C and Lane 8 = 50.7 °C.

In Figure 4.1, the primers 2550F/2718R from Fridolfsson & Ellegren (1999) annealed to the target CHD1 gene and amplified the CHD1Z (~650bps) & CHD1W (~450bps) alleles successfully without non-specific product formation. From the lowest temperature which was 50.7 °C to the highest, 60 °C, the target CHD1 gene was successfully amplified. This was clear from the gel as all the lanes showed almost the same intensity of the PCR product, thus it can be deduced that the PCR works with the same efficiency with any annealing temperature between 50.7 °C and 59.2 °C. Although the specific CHD1Z and CHD1W alleles were amplified at an annealing temperature of 60 °C, the amplified products were less intense and thus less in concentration compared to the rest of the lanes on the gel. As such, the specific product formation at the highest annealing temperature (60 °C) was not considered optimal with regards to high amplified DNA yields, which indicates that 60 °C was too high as can be seen in Figure 4.1. This step, after all, was done to find the highest possible annealing temperature where the specific PCR products were still obtained with good yield. Consequently, the highest annealing temperature that showed good results as seen on the gel is 59.2 °C. However, for subsequent testing of other species, 59 °C was used for PCR amplification of different species.

Next, four Galah Cockatoo samples were tested along with a Fischer's Lovebird control sample using the same analytical conditions as explained previously (Section 3.2.3) with only a modified annealing temperature of 59 °C.

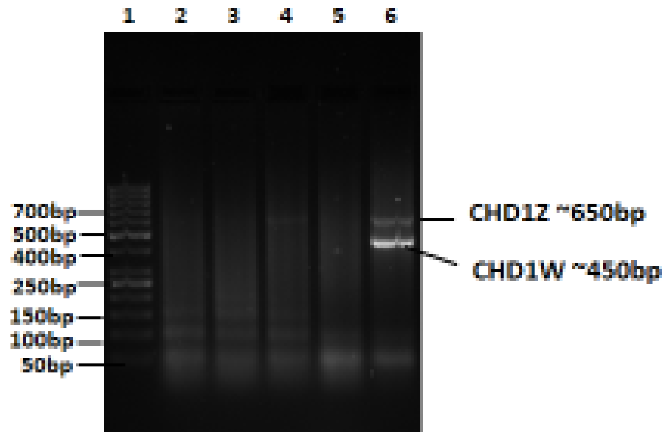

Figure 4.2: PCR amplification of Galah Cockatoo and Fischer's Lovebird gel image. A 2% agarose gel, stained with ethidium bromide, was used for separation of amplified DNA. Lane 1 represents a 50bp Gene Ruler DNA Ladder, Lanes 2 to 5 = Galah Cockatoo samples, Lane 6 = Fischer's Lovebird female (♀) sample detected by CHD1Z at ~650bp and CHD1W at ~450bp.

In Figure 4.2, no amplification was observed in lanes 2 to 5, which represents the Galah Cockatoo samples on a 2% agarose gel. However, the Fischer's Lovebird sample in lane 6 of Figure 4.2 showed relative good amplification. The results in lanes 2 to 5 demonstrates the inability of the primers to amplify the target CHD gene sequence at an annealing temperature of 59 °C. A possible explanation for this was attributed to the nucleotide sequence diversity among different bird species, possibly influencing the binding efficiency of the primers and thus the amplification efficiency. The CHD1Z PCR product (~650bp) and CHD1W PCR product (~450bp) were formed on a Fischer's Lovebird sample (lane 6), although the intensity of the CHD1Z and CHD1W fragments was not uniform, that is, the CHD1W amplicon was amplified preferentially. As a result, the CHD1W amplicons at ~450bp appeared brighter on the gel compared to the longer

CHD1Z amplicons at ~650bp. However, the sex of the bird could still be identified on the basis of product formation of two CHD1Z and CHD1W amplicons at ~650bp and ~450bp respectively, identifying the sample as a female. Thus an annealing temperature of 59 °C was considered inadequate for amplification of Galah Cockatoo samples.

Further optimization was done to enhance the amplification efficiency of the CHD1 gene on the species in question, particularly focusing on the annealing temperature. The annealing temperature plays a decisive role in the determination of a successful PCR amplification. Too high annealing temperature leads to no product formation at all, whereas too low annealing temperature causes the primers to bind non-specifically, resulting in non-specific amplification. From the data presented in Figure 4.2, the annealing temperature of 59 °C used could not be utilized to analyse Galah Cockatoo samples. Thus, reducing the annealing temperature could possibly lead to high throughput applicability of the protocol for sexing a number of species.

Following the unsuccessful amplification using a high annealing temperature, five Fischer's Lovebird samples of known sex were tested (Figure 4.3) with a slightly modified protocol, particularly the annealing temperature which was reduced to 49 °C [the annealing temperature used by the industrial partner, Lumegen Laboratories (Pty) Ltd]. The samples were cut and genetic sexing was performed using the described PCR protocol (Section 3.2.3). The PCR products were then analysed on a 2% agarose gel for product visualization.

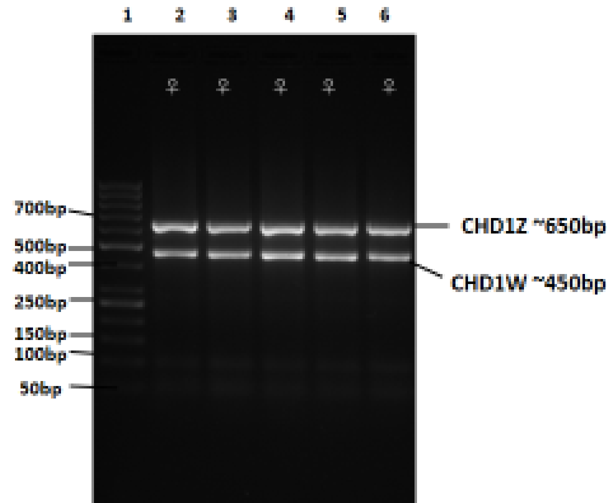

Figure 4.3: PCR amplification of Fischer's Lovebird samples with reduced annealing temperature. A 2% agarose gel, stained with ethidium bromide, was used for separation of amplified DNA**.** Lane 1 represents a 50bp Gene Ruler DNA ladder, Lanes 2 to 6 = Fischer's Lovebird female (♀) samples identified by CHD1Z and CHD1W bands at ~650bp and ~450bp respectively.

The results in Figure 4.3 clearly demonstrate high quality CHD1 gene amplification when the primer annealing temperature was reduced from 59 °C to 49 °C. The PCR amplicon yields were very high and efficient, indicating that an annealing temperature of 49 °C was rather favourable with regards to amplification efficiency and PCR quality. No other non-specific PCR products were observed, thus the annealing temperature was not considered too low either. Next, the applicability of the reduced annealing temperature of 49 °C was tested on the Galah Cockatoo samples, which previously could not be amplified at an annealing temperature of 59 °C (Figure 4.2). The same four samples (and in the same run order) were subsequently re-tested under the same conditions (Section

3.2.3) but with reduced annealing temperature of 49 °C. The products were analysed and interpreted on a 2% agarose gel (Figure 4.4).

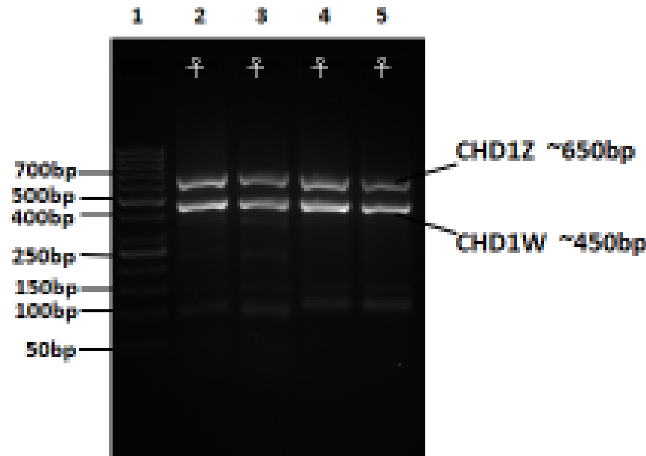

Figure 4.4: PCR amplification of Galah Cockatoo samples gel image. A 2% agarose gel, stained with ethidium bromide, was used for separation of amplified DNA. Lane 1 represents a 50bp Gene Ruler DNA ladder, Lanes 2 to 5 = four female (♀) samples detected by CHD1Z at ~650bp and CHD1W at ~450bp.

The CHD1 fragments in four Galah Cockatoo samples amplified with the 2550F/2718R primer set revealed two bands for all samples which were well separated on a 2% agarose gel as illustrated in Figure 4.4. The annealing temperature reduction from high 59 °C to 49 °C favoured the PCR amplification of the CHD1 gene in both Fischer's Lovebird and Galah Cockatoo species, as both species showed good amplification (Figures 4.3 and 4.4). The reduced annealing temperature of 49 °C was then used to investigate its applicability and to ensure veracity on a wider range of species. Nyasa Lovebird species was tested while keeping the conditions the same.

Since there is no universally applicable sex identification technique that can be considered accurate and reliable without thorough species-specific testing (Thanou *et al.*,

2013), more samples from different species were tested to ensure the accuracy, reliability and reproducibility of the current protocol. The standard method was tested on Nyasa Lovebirds to establish it's applicability to the species. Five samples of known sex were selected, and amplified in the same manner as described (Section 3.2.3), albeit with an annealing temperature of 49 °C (Figure 4.5).

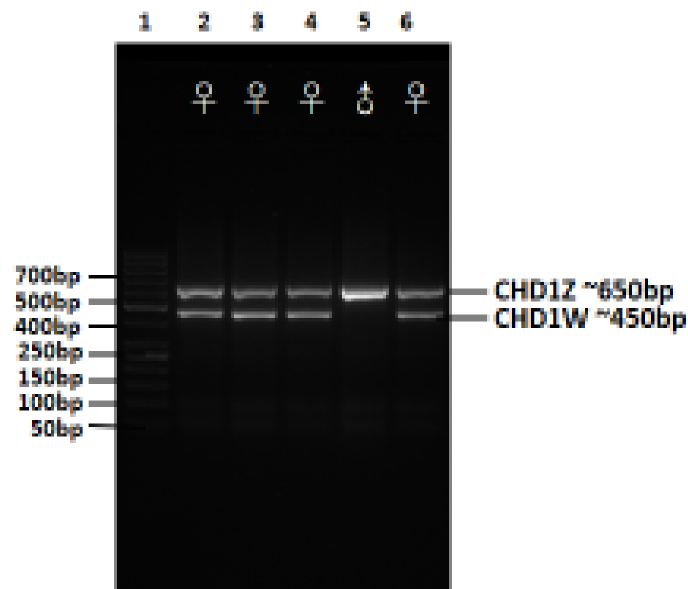

Figure 4.5: PCR amplification of Nyasa Lovebird samples gel image. A 2% agarose gel, stained with ethidium bromide, was used for separation of amplified DNA. Lane 1 represents a 50bp Gene Ruler DNA ladder, Lanes 2, 3, 4 & 6 = Female samples (♀) identified by CHD1Z and CHD1W at ~650bp and ~450bp respectively, Lane 5 = Male identified by a single CHD1Z band at 650bp (♂).

The CHD1 fragments amplified revealed one band for a male sample indicated by the presence of a single CHD1Z at ~650bp and two bands for females identified by two bands CHD1Z at ~650bp and CHD1W ~450bp respectively as presented in Figure 4.5. The results confirmed that the conventional PCR method using 2550F/2718R primers was used successfully to identify the sex of few different bird species tested. The reproducibility of the PCR test to correctly sex individuals using relatively few known-sex individuals was successfully accomplished.

4.3 Optimisation of the PCR-HRM assay

High resolution melting (HRM) analysis is an advanced post-PCR analysis technique applied for identifying genetic variation in DNA sequences. This method is based on PCR melting curve techniques with advanced dsDNA binding dye, real-time PCR instrument and software. In HRM analysis, the region of interest is amplified by PCR in the presence of a fluorescent dsDNA-binding dye. After PCR amplification, the product is gradually melted, and the emitted fluorescence is measured on a specialized instrument to generate a characteristic curve. Factors such as GC content, length, sequence and heterozygosity influence the melt curve characteristics for each amplicon. The HRM analysis basically applies the same fundamental principles of DNA melt curve analysis but has some further refinements. The major difference lies in the complex alignment algorithms and new plot views introduced in HRM, which enables one to identify small differences in melt curve data (Montgomery *et al.*, 2010). The Step One Plus real-time

PCR system (Applied Biosystems) comes with the HRM software v.2.2.3 for data acquisition and data alignment.

The use of the HRM analysis is increasing in several fields of research such as reproductive biology, conservation of wild populations and microbiology (Morinha *et al.*, 2013). Here, a HRM protocol for avian molecular sexing was developed based on the amplification of the CHD1 gene and HRM analysis of PCR amplicons. The HRM analysis has advantages over classic molecular sexing and traditional sexing. However, the high sensitivity, performance and accuracy of the technique lies in the optimization process.

For successful HRM analysis, the optimization of the protocol for avian molecular genotyping must be considered carefully. In more technical terms, the high fidelity, sensitivity and accuracy of the technique in discriminating small variations in the DNA sequences based on their melting behaviour is dependent upon the assay design and optimization. The HRM analysis, recently introduced for molecular sex discrimination in birds was optimized in the next section. The HRM analysis work-flow was as illustrated in Figure 4.6

Figure 4.6: The HRM analysis workflow illustrated in logical steps.

4.3.1 Utilization of the KAPA HRM Fast master-mix for the PCR-HRM assay

Due to the industrial partner [Lumegen Laboratories (Pty) Ltd] already using the Kapa HRM Fast master-mix for other applications, it was suggested by them to use this same reagent in the planned avian molecular sexing PCR-HRM assay. The approach illustrated in Figure 4.6 was followed in an attempt to optimize the assay.

4.3.1.1 Calibration of the Step-one plus real-time PCR instrument

The Step One Plus real-time PCR system (Applied Biosystems) was calibrated both for the HRM dye and background. This step included running a background, custom dye, and HRM calibration plate which was successfully done.

4.3.1.2 KAPA HRM FAST Master mix assay optimization

DNA was isolated from six Fischer's Lovebird samples (of known sex) and the isolated DNA was quantified as explained in Section 3.2.1. The isolated DNA was used for the PCR-HRM assay as discussed Section 3.2.2. The PCR products were analysed on a 2% agarose gel is illustrated in Figure 4.7.

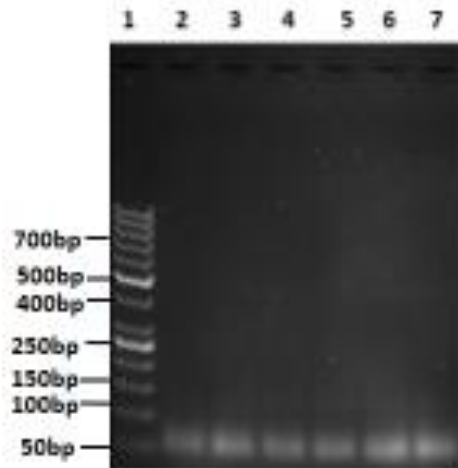

Figure 4.7: KAPA HRM FAST master-mix optimization gel image of Fischer's Lovebird samples. A 2% agarose gel, stained with ethidium bromide, was used for separation Lane 1 represents a 50bp Gene Ruler DNA ladder, Lanes 2 to 7 = Fischer's Lovebird samples.

The agarose gel revealed no PCR products formation at all using KAPA HRM FAST master-mix (Figure 4.7). The PCR amplification was unsuccessful; only the DNA ladder was observed on the gel. The reason for unsuccessful PCR amplification of the CHD1 fragment using the KAPA HRM FAST master - mix was unknown. The question arose if enough DNA was utilized per reaction, a variable investigated in the next section.

4.3.1.3 KAPA HRM FAST Master-mix optimization with different DNA concentrations

In an attempt to amplify the CHD1 gene, different DNA concentrations were explored to identify the optimal DNA concentration for the reaction. One Fischer's Lovebird sample

of known sex was used at different DNA concentrations. Firstly, the sample DNA was isolated and the concentration was calculated to be 8.3 ng/µl as described in Section 3.2.1. A DNA gradient series was prepared with PCR-HRM reaction containing different amounts of DNA (1, 5, 10, 25, 50 ng of DNA per reaction, respectively). The reaction was performed as described in Section 4.3.1.2 and again visualized using a 2% agarose gel. (Figure 4.8).

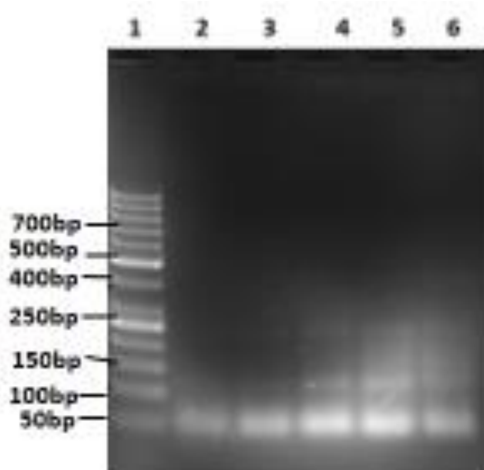

Figure 4.8: KAPA HRM FAST master-mix gel image of different DNA concentrations of a Fischer's Lovebird sample. A 2% agarose gel, stained with ethidium bromide, was used for separation. Lane1 = 50bp Gene Ruler DNA ladder, Lane 2 = 1 ng DNA, Lane 3 = 5 ng DNA, Lane 4 = 10 ng DNA, Lane 5 = 25 ng DNA, Lane 6 = 50 ng DNA.

Figure 4.8 demonstrates that no amplification of specific CHD1Z and CHD1W fragments was observed after different DNA concentrations were utilized in the PCR-HRM assay. At this point, it was clear that PCR amplification using the KAPA HRM master-mix failed as an approach for the real-time PCR-HRM assay for avian molecular sexing on the Step One Plus real-time PCR system due to failed PCR amplification observed in Figure 4.7

and Figure 4.8. Low quality DNA may interfere with PCR amplification. Also, PCR inhibitors such as proteases may have influenced the amplification efficiency with the HRM reagent.

Numerous attempts to amplify the CHD1Z and CHD1W fragments using the KAPA HRM master-mix failed as shown in Figure 4.7 and Figure 4.8. Initially it was thought that a specific sample used could be the problem for the failed amplification. Since the samples available to this study was not sterile blood in a blood collection tube (the norm for biological studies like this), but rather blood collected and dried on a Guthrie card (that could be contaminated by unknown contaminants), different blood samples were tested for amplification. However, no sample or DNA concentration resulted in the formation of any CHD1Z and CHD1W amplicons.

4.3.2 Establishing the EvaGreen dye PCR-HRM assay

The original motivation for using the KAPA HRM master-mix was to amplify the DNA in the presence of a fluorescent due, so that a post-PCR HRM analysis could be performed. However, the CHD1Z and CHD1W fragments could not be amplified using the KAPA HRM master-mix (Section 4.3.1). But the required fragments amplified very nicely using the conventional PCR (Section 4.2). Thus the thought arose if the conventional PCR could be utilized to amplify the CHD1Z and CHD1W fragments before a fluorescent dye is added (post-PCR) to perform the HRM reaction with.

EvaGreen dye is a green fluorescent nucleic acid dye with features that make the dye useful for several applications, including qPCR, melt curve analysis, capillary electrophoresis, etc. Compared with SYBR Green, EvaGreen dye is less inhibitory towards PCR and less likely to cause non-specific amplification. The dye is essentially non-fluorescent by itself, but becomes highly fluorescent upon binding to dsDNA (Wang *et al*., 2006). Thus melting curve analysis is based on the EvaGreen saturating dye intercalating into the dsDNA, allowing fluorescent detection of PCR product. Although the dye can be included during the PCR reaction, it was tested in this study as a post-PCR additive before HRM analysis.

4.3.2.1 Amplification of Fischer's Lovebird samples

In order to test the applicability of the EvaGreen dye and the melting curve analysis as a post-PCR approach, ten Fischer's Lovebird samples of known sex were prepared and the conventional PCR (Section 3.2.3) used to amplify the required fragments. Figures 4.9 illustrates the results of the amplified PCR products analysed on a 2% agarose gel. The results demonstrate good PCR amplification of the CHD fragment, with specific product formation showing uniform band intensity without non-specific products. Thus allowing easy differentiation of females and males using the described PCR protocol.

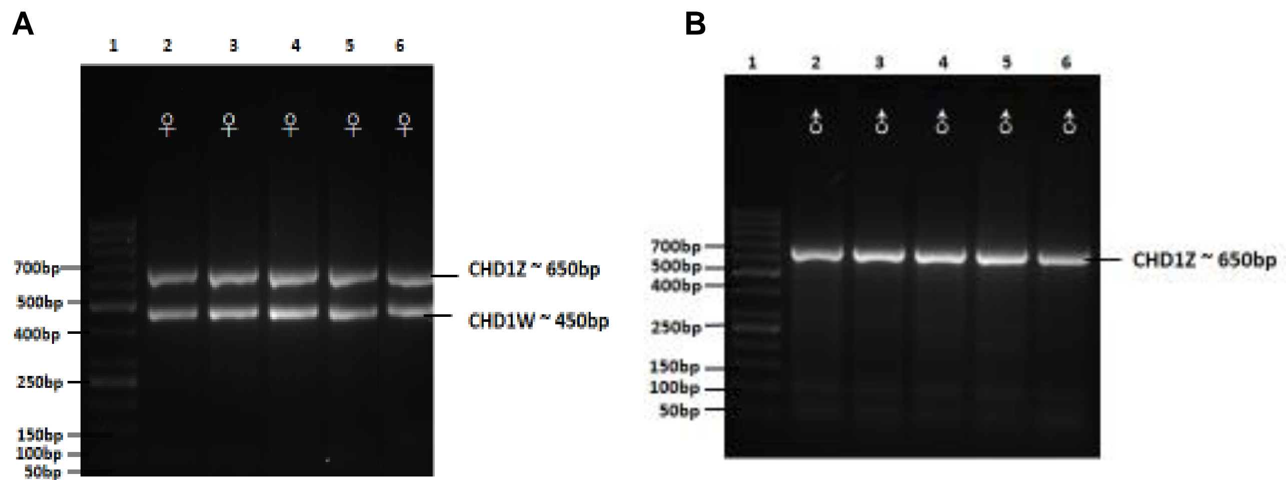

Figure 4.9: PCR amplification of Fischer's Lovebird samples gel image for EvaGreen melting curve analysis. A 2% agarose gel, stained with ethidium bromide, was used for separation of amplified DNA. **(A)** Lane 1 represents a 50bp Gene Ruler DNA ladder, Lanes 2 to 6 = Female samples (♀) identified by CHD1Z and CHD1W at 650bp and 450bp respectively. **(B)** Lane 1 represents a 50bp Gene Ruler DNA ladder, Lanes 2 to 6 = Male samples identified by a single CHD1Z band at 650bp (♂).

4.3.2.2 Melting curve analysis of Fischer's Lovebird samples

After the PCR amplification and gel electrophoresis (Figure 4.9), the PCR mixture that remained was used for the melting curve analysis (MCA) and high resolution melt (HRM) as described in details in Sections 3.2.4 and 3.2.5, respectively. In short, 5 µl of the post-PCR mixture was mixed with 1 µl EvaGreen dye and 14 µl H_2O in a real-time PCR tube.

The melting curve analysis was performed on the five female and five male samples and the results illustrated Figure 4.10.

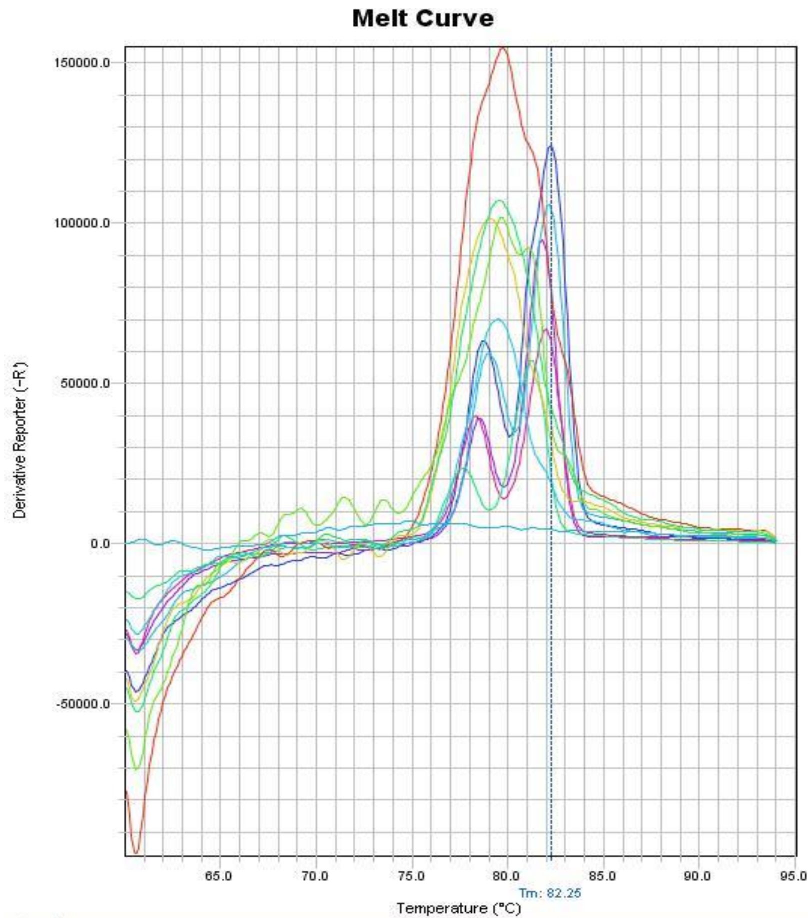

Figure 4.10: Melting curve analysis (MCA) using EvaGreen on Fischer's Lovebird samples. A derivative melt curve plot, Derivative Reporter (-R) vs. Temperature (°C) which shows the inflection point on the slope as a more easily visualized melt peak. Curve colours represent each sample. Males are represented by two peaks and females by one peak.

The melt curves as shown in Figure 4.10 were generated by slowly denaturing (melting) the DNA amplicons through a range of temperatures in the presence of a dsDNA-binding

dye, EvaGreen dye. During melting, the dye bound to dsDNA amplicons fluoresces, and that fluorescence is continuously detected by an optical system. At low temperature, the DNA will be double-stranded and the dye will strongly fluoresce. As the temperature increases, the dsDNA will denature and form ssDNA, releasing the EvaGreen dye, resulting in a 1000 fold decrease in fluorescence as the unbound dye is essentially non-fluorescent.

The melt curve analysis software defines the melting temperature (Tm) of a PCR product as the inflection point of the melt curve. To visualize the Tm more clearly, the negative first derivatives were plotted, making the Tm of the products appear as peaks, as illustrated in Figure 4.10. Non-specific products may also appear in the derivative curve. These peaks are typically of lower intensity and represent products that are shorter in length, thus appearing at a lower temperature than the primary product. However, no prominent peaks were observed as a result of non-specific amplification in Figure 4.10. The male samples were identified in Figure 4.10 by their double Tm peaks at approximately 79 °C and 82.25 °C, and females were identified by a broad, wide single peak with a Tm of approximately 79 °C. The results obtained were consistent with the results reported in a study for determining the sex of Japanese quail, where females exhibited one broad peak, and males had two peaks in the MCA (Brubaker *et al.,* 2011; Huang *et al*., 2011). It has been reported that 400bp-800bp PCR products have multiple melting domains, which results in two peaks for males (Wittwer *et al.,* 2003). Preferential amplification of multiple melting domains in fragments may mask diagnostic peaks in other species (Chen *et al*., 2012). Thus, the technique was not recommended for high throughput applicability of gender identification due to the possibility of incorrect

interpretation of the melting peaks, which may require the selection of sex-specific primers to improve the melting curve results.

4.3.2.3 High resolution melt (HRM) analysis of Fischer's Lovebird samples

The HRM analysis, which is a post-PCR method introduced for molecular sex determination of birds was investigated. This technique uses the same principles of DNA melt curve but with further refinements. The HRM differs from the standard MCA in that it usually uses brighter dyes at higher concentrations without inhibiting PCR and the advanced software which uses new fluorescent scaling algorithms and plots (Reed *et al.*, 2007). In this method, new plot views were applied to better visualize small differences in melt curve data. The HRM, as an extended analysis of a melt curve, required an additional analysis software to normalize melt curves, apply an optional temperature shift, to plot curves in a difference plot and cluster curves into groups representing different genotypes.

The remaining amplicons from each of the ten Fischer's Lovebird samples, after PCR and gel electrophoresis (Section 4.3.2.1) and after the melting curve analysis (Section 4.3.2.2) were then used to investigate and establish the HRM analysis. The samples were analysed by the HRM approach as explained in Section 3.2.5. In short, 5 µl of the post-PCR mixture was mixed with 1 µl EvaGreen dye and 14 µl H_2O in a real-time PCR tube. The HRM analysis results for ten Fischer's Lovebirds are shown in Figure 4.11, starting with the raw (unprocessed) melt curves.

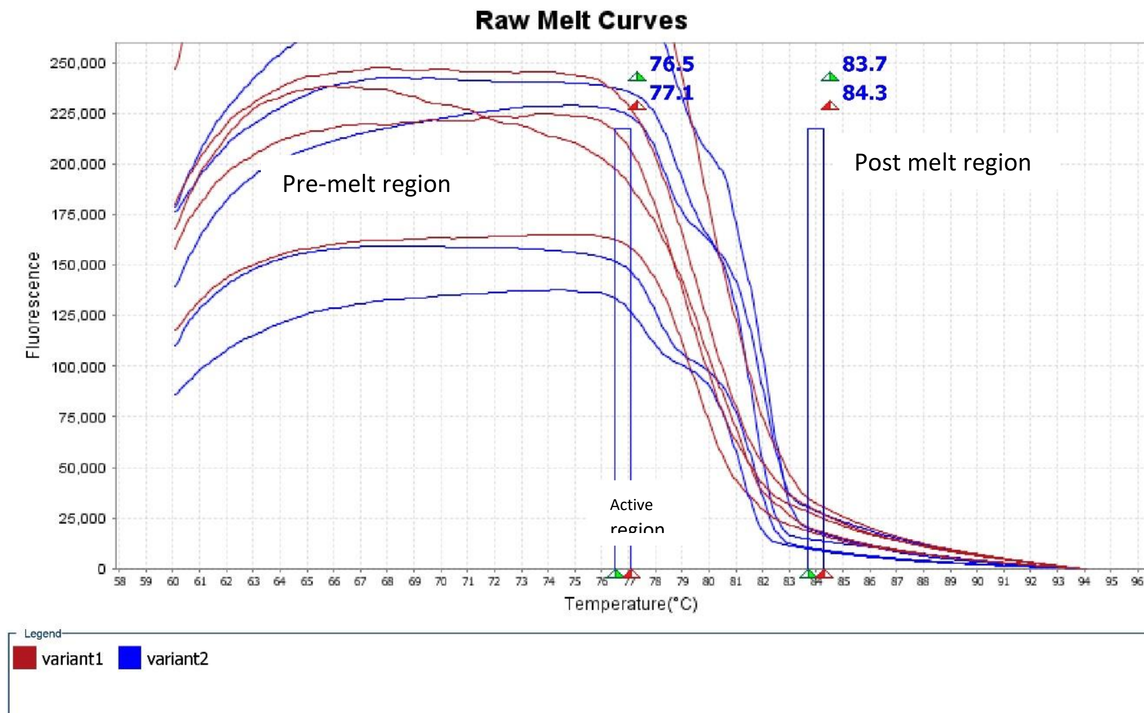

Figure 4.11: Raw data melt curve results for ten Fischer's Lovebird samples. Each curve represents an individual sample.

Figure 4.11 illustrates a raw melt curve plot, showing the high initial fluorescence when all products were double-stranded and the maximum amount of dye was bound. As the temperature increased, the dsDNA melted into ssDNA releasing the dye, decreasing the signal intensity. The data collected during the melt curve experiment displayed a variance of initial fluorescence readings as seen in Figure 4.11 between approximately 85 000 to 250 000 fluorescence units, making it difficult to discriminate differences based on the temperature shift of individual samples. The range of initial fluorescence variance called for the data normalization in order to eliminate the fluorescence variance observed in the

figure. The pre-melt region, was detected by the software as 100% fluorescence, where every amplicon was double-stranded. The change in fluorescence for each sample, on the active region, was scored as the active optimum change, and was used to plot the aligned melt curve (Figure 4.12). The post-melt region on the right of the active region was used by the software to determine the 0% fluorescence where all amplicons were single-stranded. The active melt region was designated by pre-and post- melt regions (double bars) that were used to align the data, thus producing a clearer view of the melt curve results of individual samples based on their melting profiles. The aligned melt curve is shown in Figure 4.12.

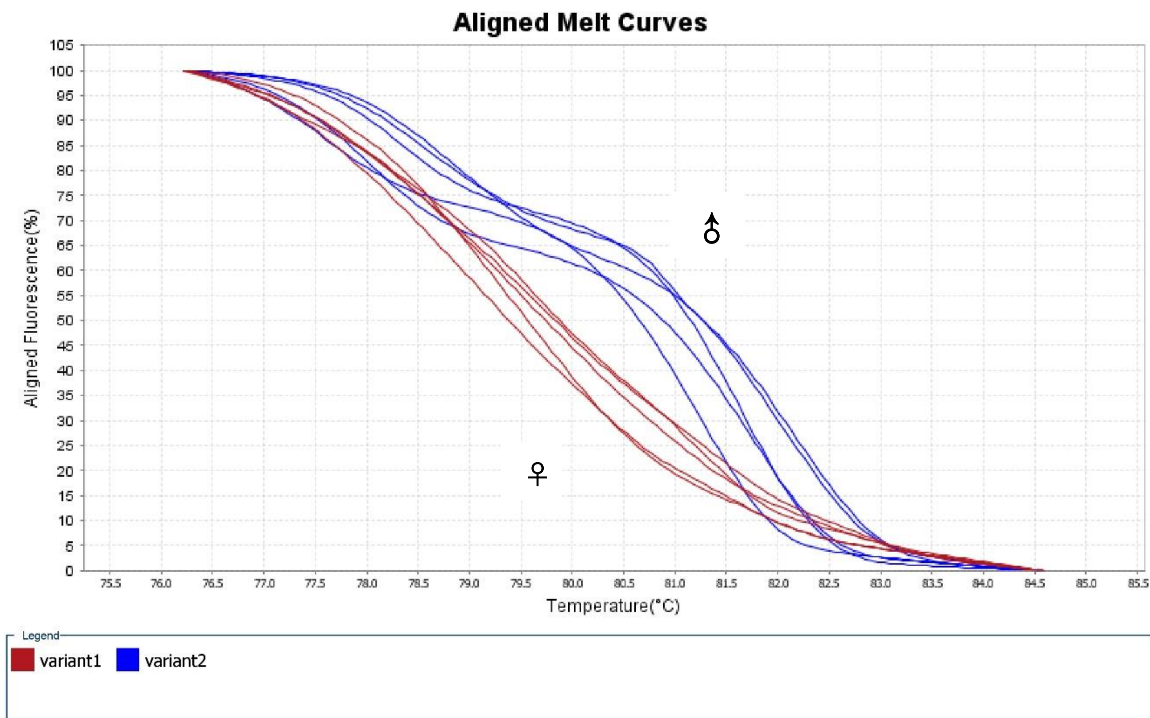

Figure 4.12: Aligned melt curve of ten Fischer's Lovebird samples. The pre-and post-melt regions were used to align data by positioning the parallel double bars as shown before.

Figure 4.12 illustrates the aligned plot, which provided a better scaled view of the data, thus enabling easy discrimination of the variants that displayed visible differences in the temperature shift of individuals. On the contrary, the data produced by the standard raw melt curves were inconclusive, as the melt curves were affected by the different intensity of fluorescence between each sample. As a result, the HRM software automatically normalized the data, particularly focusing on the active melt region which is flanked by parallel bars as shown in Figure 4.11. The data normalization of the active melt region from unprocessed to processed data eliminated the initial fluorescence variance in all samples such that the plateau was the same for each amplicon at 100% fluorescence and 0% fluorescence, thus causing a melting temperature shift and providing distinct melt curves for males and females. For more distinct differences between the variants, the software automatically calculated the difference plot (Figure 4.13).

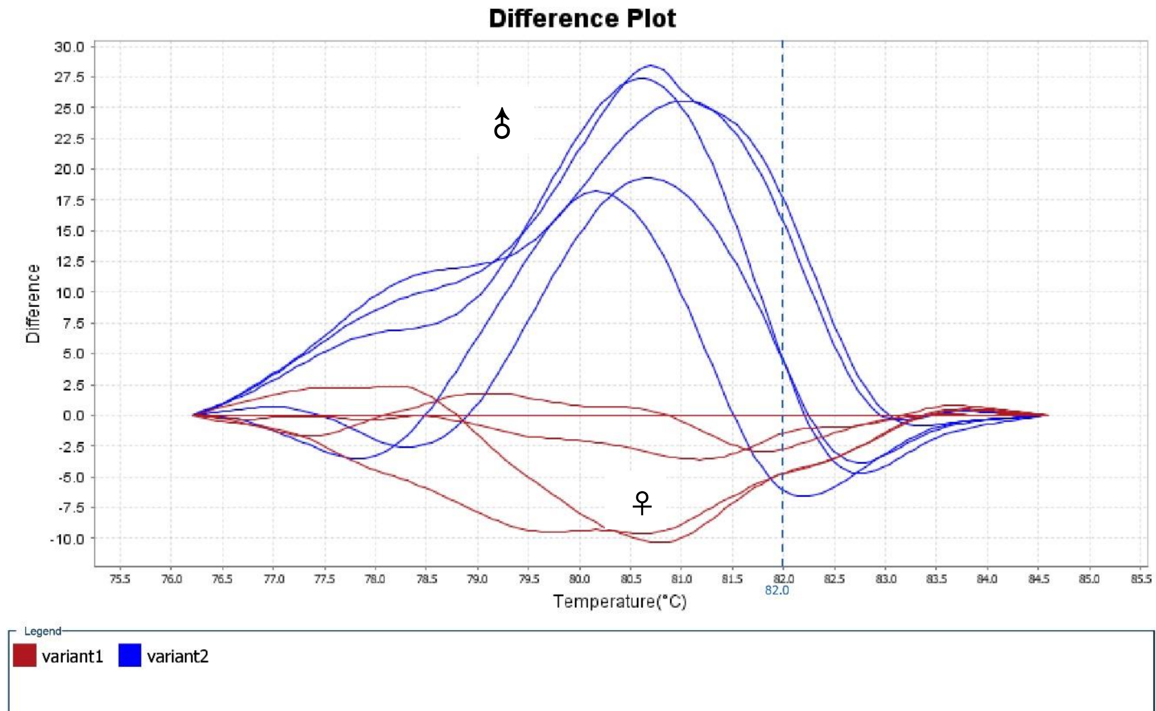

Figure 4.13: HRM analysis data difference plot of ten Fischer's Lovebird samples. The HRM software application allowed the calculation of the difference plot and plots the Difference vs. Temperature (°C). Two variants are distinct – female samples (marked red) and male samples (marked blue).

The HRM software automatically calculated the difference plot for more distinct differences between the genotypes and attributed a confidence score with 100% accuracy. The HRM software allowed the calculation of the difference plot by subtracting the normalized fluorescence data of a user-defined genotype from that of each of the other samples in the HRM analysis. One female sample was randomly chosen by the software and used as a baseline as shown in Figure 4.13. The position of each sample, relative to the baseline, was plotted against the temperature. The differences between the CHD1Z/CHD1W (female samples) and CHD1Z (male samples) alleles based on their

melting curves were best visualized on this plot which resulted in accurate sex identification by a PCR-HRM assay.

The 10 Fisher's Lovebird samples, identified as 5 females and 5 males using the conventional PCR approach (Figure 4.9) was accurately identified as 5 females and 5 males using the newly established EvaGreen dye PCR-HRM approach employing EvaGreen as a fluorescent dye (Figure 4.13).From this figure the sex of all the samples could easily be established. Thus the new EvaGreen dye PCR-HRM assay holds promise as a feasible approach for sexing of avian species.

4.3.3 The effect of different primers on the EvaGreen dye PCR-HRM assay

The CHD1 gene provides an improved basis for DNA sexing, and the amplification of this gene often produces conspicuous results when one of the commonly utilized primer pairs is used; that is, 2550F/2718R (Fridolfsson & Ellegren, 1999) and P2/P8 (Griffiths *et al.*, 1998). The length of the CHD gene amplicon is slightly longer in the W chromosome, compared to the Z chromosome, in many bird species when amplified with P2/P8.

P2/P8 primers (Griffiths *et al.*,1998) have been reported to produce only one fragment in both sexes of specific species as a result of DNA sequence being shorter on Z chromosome than that from the W chromosome and may be preferentially amplified leading to females being misidentified as males (Dubeic and Zagalska-Meubauer, 2006). The amplicon length difference between the CHD1W and CHD1Z are small in most avian species amplified with P2/P8, which could lead to incorrect sex identification (Dubeic and

Zagalska-Meubauer, 2006). However, one advantage of P2/P8 primers is that they have been shown to amplify the target regions in a large number of non-ratites species (Cerit and Avanus, 2007).

On the other hand, the 2550F/2718R primers (Fridolfsson & Ellegren, 1999) have been reported to produce only one band both in males and females in some species (Dubeic and Zagalska-Meubauer, 2006). However, the differences between the CHD1 fragments amplified with this set of primers is larger than that obtained when the P2/P8 primers are used. However, these primers have not been tested in as many non-ratites species as P2/P8, therefore it is recommended to test both primers to select the best suited for the study of species analysed (Thanou *et al.*, 2013).

4.3.3.1 Conventional PCR analysis of Peach-faced Lovebird samples amplified with P2/P8 and 2550F/2718R primer sets

The P2/P8 and 2550F/2718R primer sets for avian molecular sexing was used to analyse Peach-faced Lovebird samples with the conventional PCR analysis, as described in Section 3.2.3. Ten Peach-faced Lovebird samples were selected, amplified using both primer pairs and analysed on a 2% agarose gel. Figure 4.14 illustrates the results obtained with the P2/P8 primer pair while Figure 4.15 illustrates the results obtained by employing the 2550F/2718R primer set.

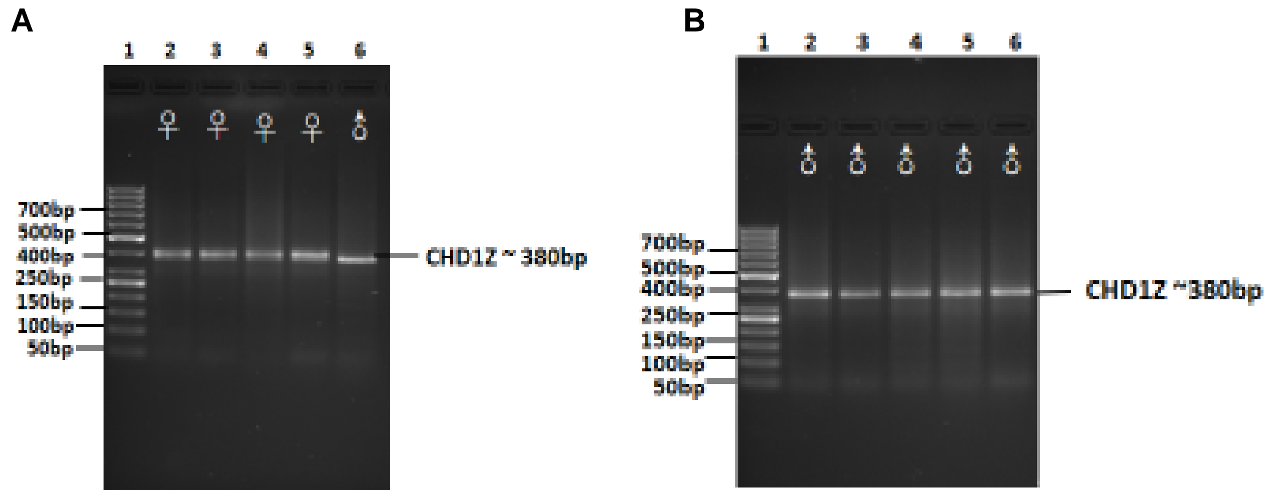

Figure 4.14: PCR amplification of Peach-faced Lovebird samples with the P2/P8 primer set. A 2% agarose gel, stained with ethidium bromide, was used for separation of amplified DNA**. (A)** Lane 1 represents a 50bp Gene Ruler DNA ladder, Lanes 2 to 5 = Female samples (♀) were identified by the CHD1Z and CHD1W at 380bp and 400bp respectively, Lane 6 = Male sample identified by CHD1Z at 380bp (♂). **(B)** Lane 1 represents a 50bp Gene Ruler DNA ladder, Lanes 2 to 6 = Male samples identified by a single band CHD1Z at 380bp (♂).

Figure 4.14A indicates four female samples and one male, which were successfully amplified as detected by the presence of the CHD1 fragments (CHD1Z and CHD1W) at ~380bp and ~400bp respectively. One male sample was detected by the presence of a single band in lane 6 at ~380bp. Figure 4.14B indicates all male samples detected by the presence of one band (CHD1Z) at ~380bp in all respective lanes 2 to 6. The results presented in Figures 4.14 were 100% consistent with the results reported on the Lumegen Laboratories (Pty) Ltd database for the samples of known sex used for this test, as they had previously been tested for sex [by Lumegen Laboratories (Pty) Ltd diagnostic

service]. Next the results were compared to those obtained using the 2550F/2718R primer set (Figure 4.15).

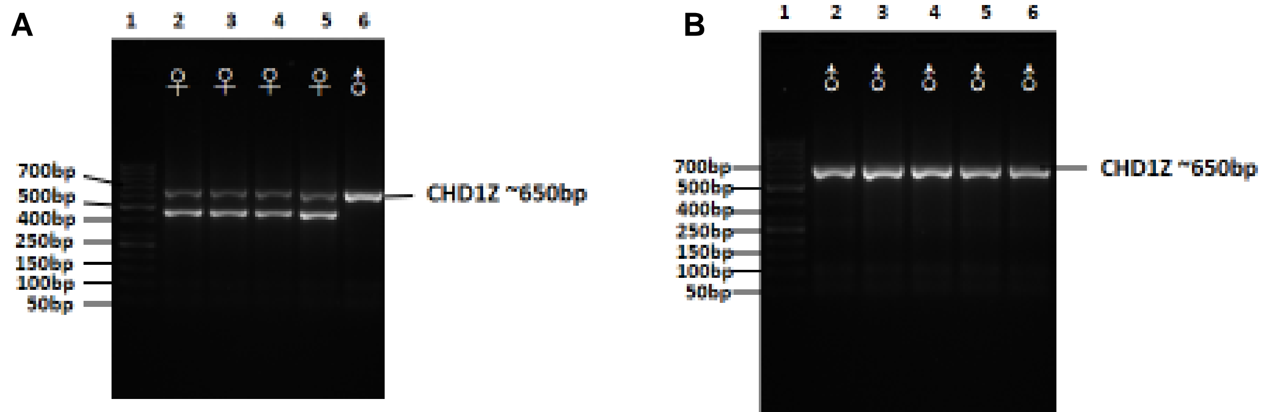

Figure 4.15: PCR amplification of Peach-faced Lovebird, four females and one male amplified with 2550F/2718R primers gel image. A 2% agarose gel, stained with ethidium bromide, was used for separation of amplified DNA. **(A)** Lane 1 represents 50bp Gene Ruler DNA ladder, Lanes 2 to 5 = Female samples (♀) detected by CHD1Z & CHD1W at ~650bp and ~450bp respectively, Lane 6 = Male sample CHD1Z at ~650bp (♂). **(B)** Lane 1 represents a 50bp Gene Ruler DNA ladder, Lanes 2 to 6 = Male samples identified by the presence of CHD1Z at ~650bp (♂).

Figure 4.15A indicates four female samples (lanes 2 – 5) and one male sample (lane 6), which were successfully amplified. The female samples show PCR fragments at ~650bp and ~450bp, while the male sample show a single amplicon at ~450bp. Figure 4.15B show five male samples. The results obtained with the 2550F/2718R primers (Figure 4.15) were thus consistent with the results obtained with P2/P8 primer pair (Figure 4.15. However, the fragments amplified with 2550F/2718R are larger (with ~200bp difference)

between the CHD1 amplicons as illustrated in Figure 4.15 compared to Figure 4.14, where the differences between the CHD1 fragments were relatively small (about ~20bp). Thus although the same nett result was obtained using the different primer sets (avian samples were correctly sexed), the PCR amplicons had to be tested using the post-PCR EvaGreen MCA (Section 3.2.4) and HRM (Section 3.2.5) assays.

4.3.3.2 EvaGreen HRM analyses of Peach-faced Lovebird samples amplified with P2/P8 and 2550F/2718R primer sets

The EvaGreen HRM analysis of fragments amplified with P2/P8 and 2550F/2718R primer sets were prepared as explained in Section 3.2.5. The aligned melt curves (for both primer sets) were generated by the Step One plus software and the results illustrated Figure 4.16.

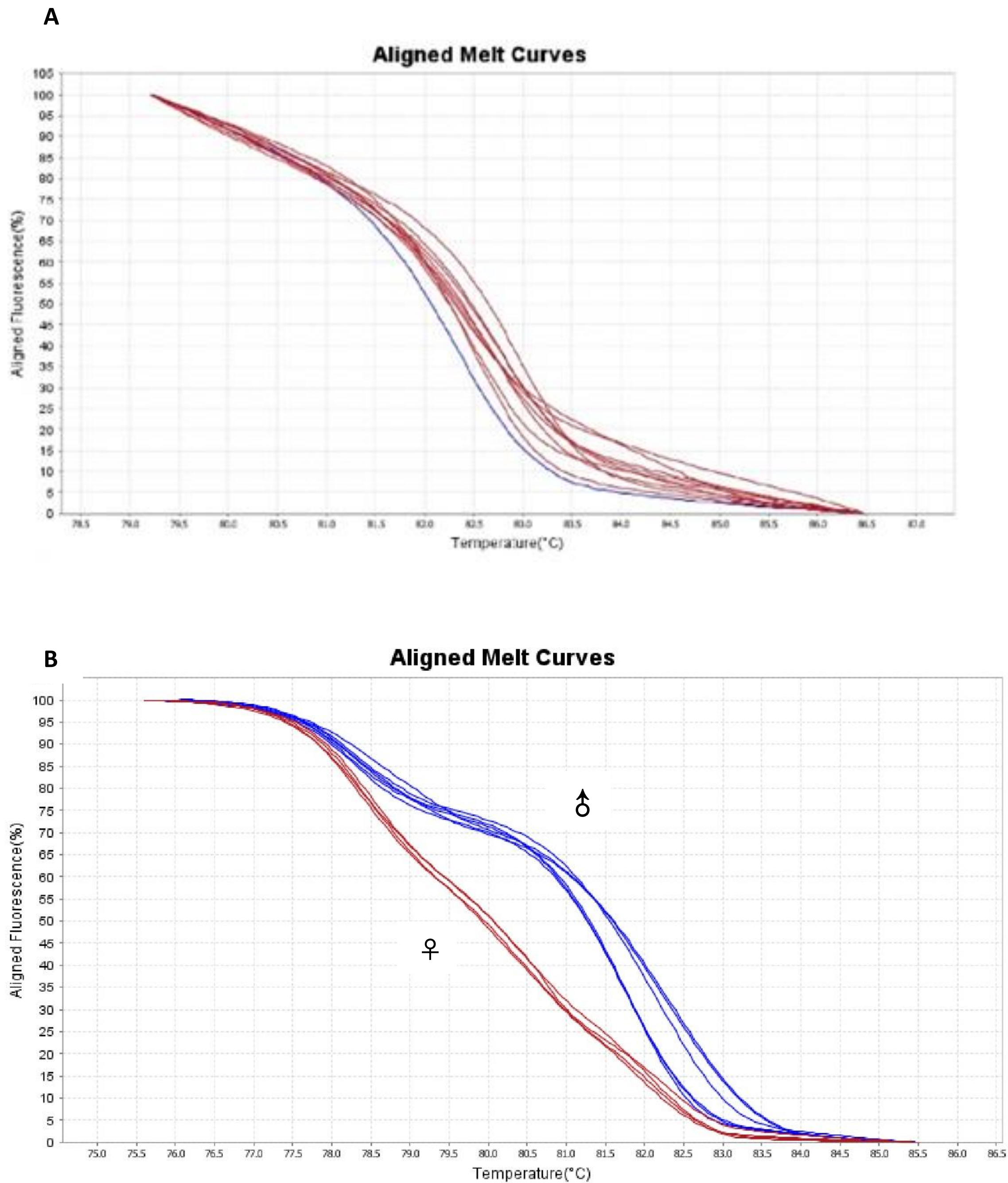

Figure 4.16: Aligned melt curves of ten Peach-faced Lovebird samples amplified with P2/P8 and 2550F/2718R primer sets. **(A)** P2/P8 primer set. **(B)** 2550F/2718R primer set. The pre-and post-melt regions were used to align data by positioning the parallel double bars as shown. Data is shown as processed (normalized) fluorescence unit.

The melting curves presented in Figure 4.16A could not be used for diagnostics of gender as the curves were all very similar, showing no difference between males and females. In essence, the aligned melt plot should enable easy visualization of the differences between the two genotypes based on their melt curve behaviour. However, the discrimination between CHD1 amplicons amplified with P2/P8 could not be observed in Peach-faced Lovebird samples using the post-PCR EvaGreen HRM approach. A possible explanation to the inapplicability of the HRM approach to correctly identify the CHD1 amplicons based on their melting profiles was linked to the small length difference between the CHD1 amplicons obtained for males and females. The melting curve in Figure 4.16A demonstrates the eliminated initial fluorescence variance between the two genotypes. As such, the plateau was the same for each amplicon at 100% fluorescence and 0% fluorescence, which usually creates a temperature shift between the two variants. However, the melting temperature shift between males and females was not detected. Only one sample (shown in blue) was identified differently, in other words as another variant (compared to the rest of the samples).

Figure 4.16 B illustrates the aligned melt curves of the ten Peach-faced Lovebird samples amplified with the 2550F/2718R primer set. The male- and female samples produced distinct melting behaviour. A temperature shift was observed in the samples analysed, and the software grouped the samples based on their melting characteristics, providing much clearer differences between males and females using the 2550F/2718R primer set (Figure 4.16B) compared to that obtained with the P2/P8 primer set (Figure 4.16A). Next the difference plots were calculated for the samples amplified with the P2/P8 and 2550F/2718R primer sets (Figure 4.17).

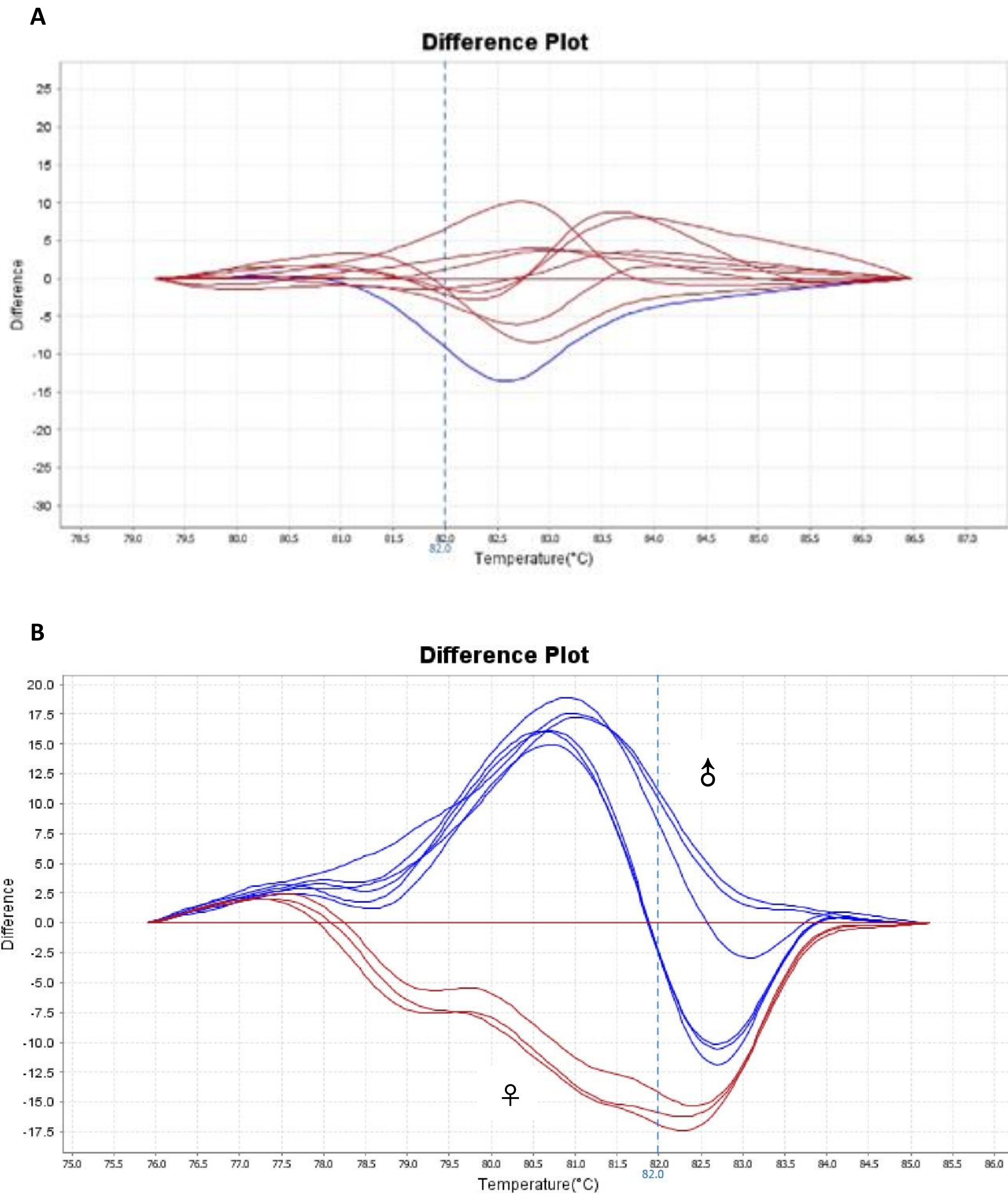

Figure 4.17: Difference plots of ten Peach-faced Lovebird samples amplified with P2/P8 and 2550F/2718R primer sets. **(A)** P2/P8 primer set. The sex of the samples could not be determined as only one sample was identified as variant 1 (Blue) compared to the rest of the samples that were identified as variant 2 (Red). **(B)** 2550F/2718R primer set. Two variants are indistinct, female samples (Red) and male samples (Blue).

Although the new approach, based on HRM curve analysis of the CHD1 gene for avian molecular sexing was successfully applied to differentiate sex based on melt curve behaviour in Common quail and Japanese quail with six nucleotide differences between the CHD1 amplicons (Morinha *et al*., 2011), on the contrary, Figure 4.17 demonstrates that the EvaGreen HRM approach for avian molecular sexing of Peach-faced Lovebird using the P2/P8 primers with intronic length differences of about ~20bp was unsuccessful. The difference plot (Figure 4.17A) could not reveal significant differences between the CHD1 amplicons based on their melting behaviour. Although Morinha *et al*. (2011) could detect a six nucleotide differences between CHD1 amplicons using their HRM setup, it was not possible to detect a 20 bp difference in this study using the real-time PCR instrument and EvaGreen approach.

When the same ten Peach-faced Lovebird samples were amplified using the 2550F/2718R primer set, the greater differences (~200bp) between the CHD1 amplicons allowed the distinct variation between the melting curve characteristic of male- and female samples. The samples were clearly grouped into two variants (genders), which corresponded 100% to the results obtained using the conventional PCR. This contrary to the CHD1 amplicons amplified with P2/P8 primer set which demonstrated an inability to distinguish the two genotypes based on their melting shapes by the HRM approach. Thus the difference plot revealed more distinct, diagnostic results based on the amplicon melting behaviour on the samples amplified with 2550F/2718R primer pair, compared to that amplified using the P2/P8 primers.

Based on these results, it was decided to only use the 2550F/2718R primer set (and not the P2/P8 primer set) from here onwards in the study. Next, the applicability and

reproducibility of the newly established EvaGreen dye PCR-HRM assay (using the 2550F/2718R primer set) was tested on different avian species.

CHAPTER 5: Comparison of the conventional PCR and EvaGreen dye PCR-HRM assays in different avian species

5.1 Introduction

In this chapter, the conventional PCR is compared to the newly established EvaGreen dye PCR-HRM assay using seven different avian species. Since the conventional PCR is currently used for DNA sexing of avian samples by the industrial partner to this study, Lumegen Laboratories Pty (Ltd), this assay was considered the standard in this study and thus the results obtained with the EvaGreen PCR-HMR assay was compared to that obtained with the conventional PCR. For each of the seven species included here, the conventional PCR is used to determine the sex of the chosen samples, visualized using agarose gel electrophoreses. The gel images are followed by the EvaGreen HRM results for each species. Also, the statistical analysis on the accuracy of the methods are discussed at the end of each species analysed. Concluding this chapter is a tabulated summary of all the results obtained on the seven species, summarizing the performance of the EvaGreen PCR-HRM assay compared to the conventional PCR.

5.2 Analysing different avian species samples using the standardized methods

5.2.1 Fischer's Lovebird

Fischer’s Lovebird samples of known sex were tested, ten females and ten males. The CHD1Z and CHD1W alleles were amplified by PCR using the optimized protocol in Section 3.2.3. The PCR products were loaded on a 2% agarose gel as seen in Figure 5.1.

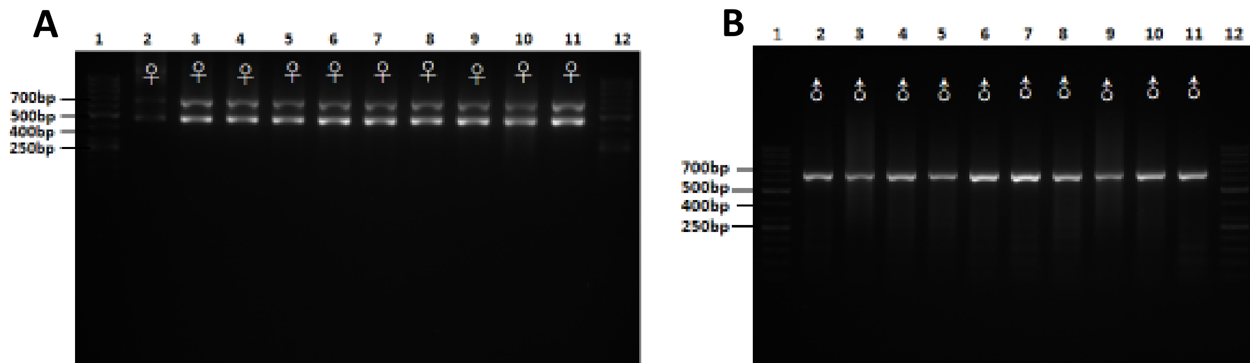

Figure 5.1: Agarose gel image of Fischer’s Lovebird samples. A 2% agarose gel, stained with ethidium bromide, was used for separation of amplified DNA. **(A)** Lane 1 represents a 50bp Gene Ruler DNA ladder, Lane 2 to 11 = Fischer’s Lovebird female samples (♀) identified by CHD1Z and CHD1W at ~650bp and ~450bp respectively, Lane 12 represents a 50bp Gene Ruler DNA ladder. **(B)** Lane 1 represents a 50bp Gene Ruler DNA ladder, Lane 2 to 11 = Fischer’s Lovebird male samples (♂), Lane 12 represents a 50bp Gene Ruler DNA ladder.

The amplicons of the CHD1 fragments using the optimized PCR protocol for sex determination of twenty individual samples were 100% correctly identified compared to the Lumegen Laboratories (Pty) Ltd database as seen in Figure 5.1. Therefore, the sex

identification of Fischer's Lovebird samples was successful since females are heterogametic, they presented two alleles (ZW) and males are homogametic, which presented two copies of the same allele (ZZ). As a result, double bands were observed in females at ~650bp and ~450bp, whereas a single band was observed in males at ~650bp. The results obtained were consistent with the results reported by (Cerit and Avanus, 2007). Following conventional PCR, the HRM assay was performed for the twenty samples using the protocol described in detail in Section 3.2.5. The HRM derivative melt curves are shown in Figure 5.2.

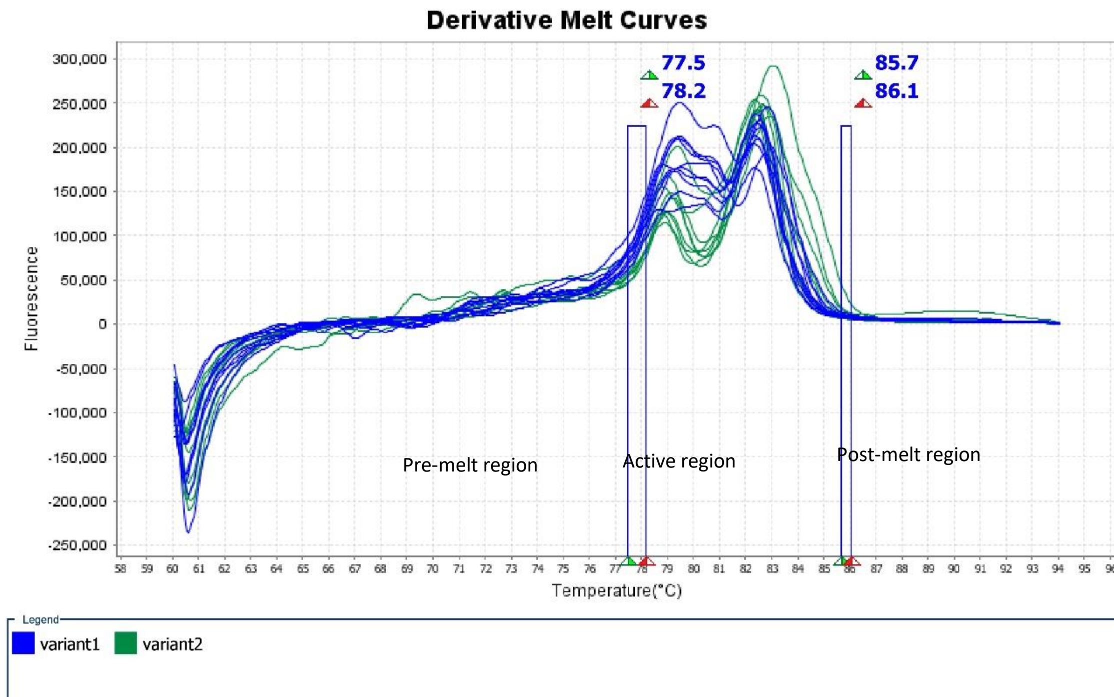

Figure 5.2: Derivative melt curves of Fischer's Lovebird. This view helped to ensure that the set pre- and post-melt regions do not encroach on the active region of the melt data. Each curve represents different samples. Males are represented by two peaks, females with three peaks.

Figure 5.2 illustrates the melt curve data which was imported to the HRM software and viewed using the derivative view. The melt curve data presented in this figure was useful in selecting the appropriate region for data alignment. Placement of the pre-and post-melt regions can affect resulting clusters and classifications of the HRM analysis results. For that reason, the positioning of the two bars, was the most critical, as correct placement of these bars maximized differences between the variants, males and females. Usually, the positioning of the two parallel bars is quite close to the start of the active melt region, without actually encroaching on the melt curve itself. Males and females displayed different melting curve shapes and temperature shift between the two variants was observed as shown in Figure 5.2. Males were identified by two peaks at ~78.5 °C and ~82.5 °C, whereas females were identified by three peaks at about ~78.5°, ~80.5°C and lastly at ~82.5 °C. In Figure 5.3, the active melt region, flanked by the two parallel bars was aligned by the HRM software for more diagnostic differences between males and females.

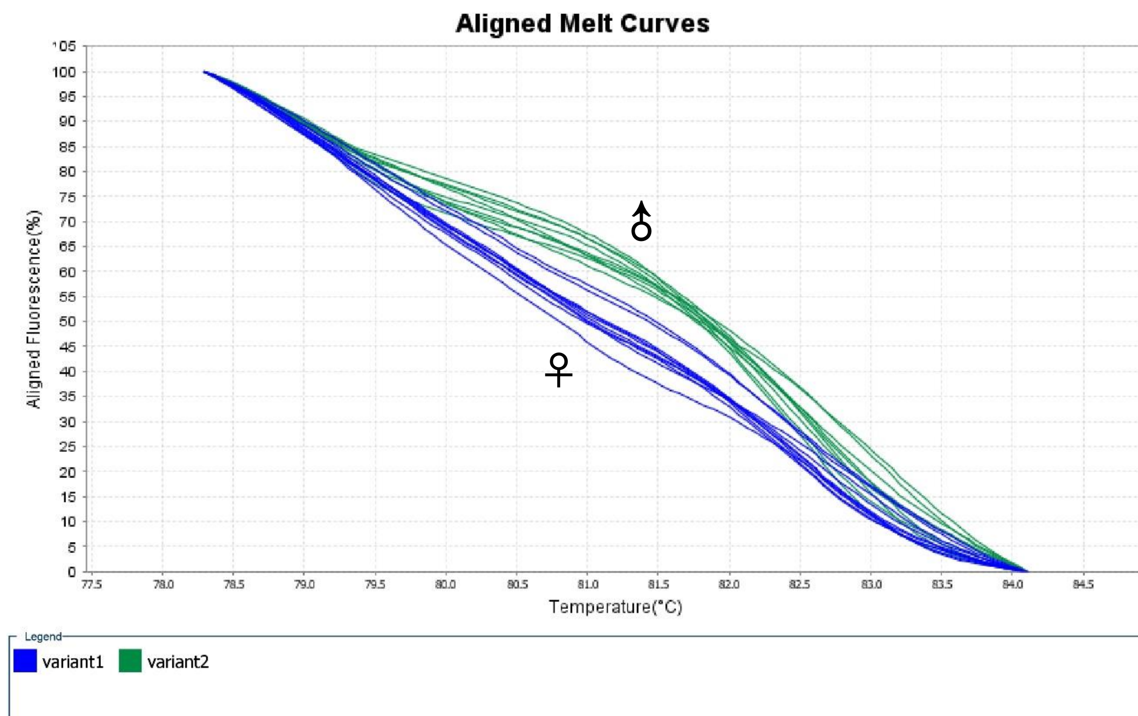

Figure 5.3: Aligned melt curves of Fischer's Lovebird samples. The pre-and post-melt regions were used to align data by positioning the parallel double bars as shown before. This is processed (normalized) fluorescence unit.

In Figure 5.3, the temperature shift between the two genotypes was observed after the raw data was processed and normalized, thus enabling distinct melt profiles of the two variants. The HRM analysis focused on both the shape of the melt curve and temperature shift between the two variants and the samples were successfully grouped according to sex. In Figure 5.4, the software calculated the difference plot between males and females.

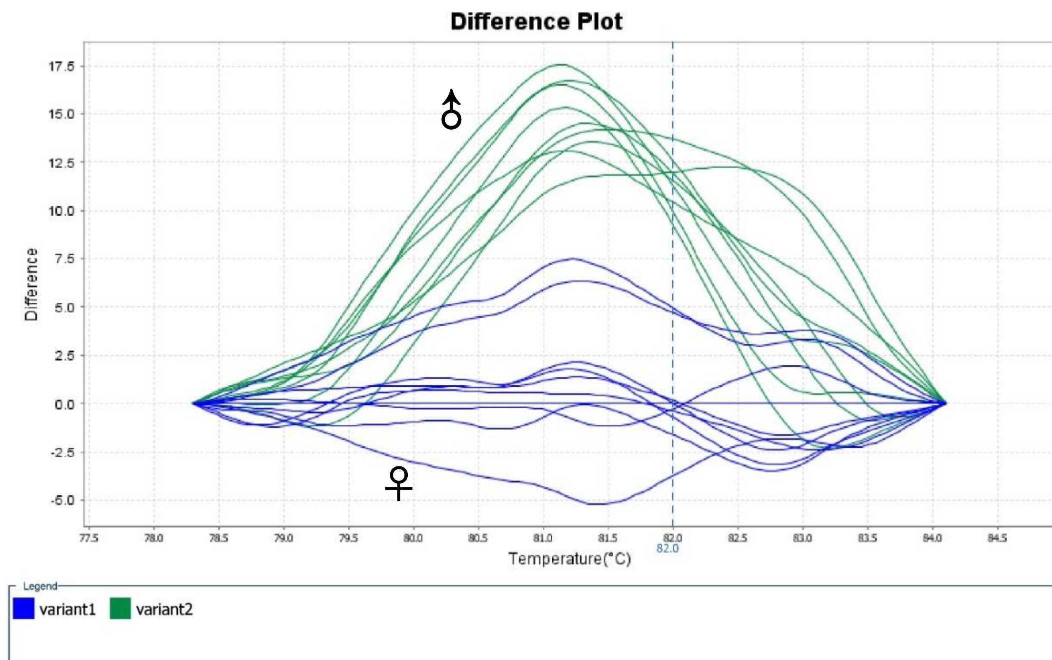

Figure 5.4: HRM analysis data difference plot of Fischer's Lovebird samples. The HRM software application allowed the calculation of the difference plot and plotted the Difference vs. Temperature (°C).

The HRM software automatically plotted the difference plot relative to the baseline as illustrated in Figure 5.4. From the result presented in this figure, it was observed that 19/20 unknown genotypes were classified as related to known genotype, while one sample was identified as a variation. This was evident by the observed discrepancy between the conventional PCR and EvaGreen PCR-HRM analysis. The sample in lane 3 of Figure 5.1B was identified as a third variant by the HRM software, which then led to the exclusion of the sample by the software. PCR amplification of this sample demonstrated a slight weaker CHD1Z PCR product formation with a visual smear observed on the agarose gel in lane 3 of Figure 5.1B. Although the sex of the sample could still be identified using the conventional PCR approach, EvaGreen PCR-HRM

approach misidentified this sample, resulting in a 19 out of 20 accuracy match, thus 95% accuracy (Table 5.1). The findings illustrated here demonstrate that high sensitivity and resolution of the EvaGreen PCR-HRM analysis is dependent upon the quality of PCR. A slight smear on the gel (thus non-specific product formation) led to one sample (from 20) being misidentified.

5.2.2 Nyasa Lovebird

The same procedures and approach was followed here as used in Section 5.2.1 for the Fischer's Lovebird samples. Nyasa Lovebird samples were used to test the applicability of the EvaGreen HRM assay. Figure 5.5 illustrates the results obtained with the conventional PCR.

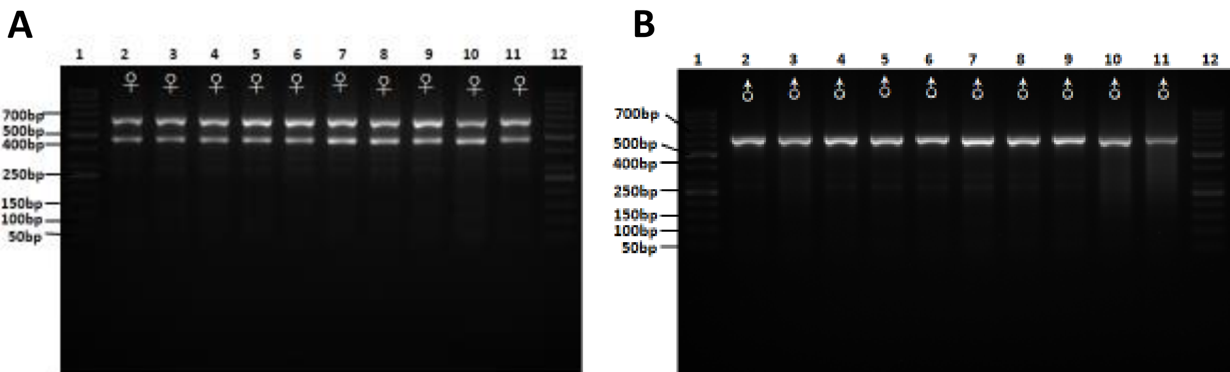

Figure 5.5: **Agarose gel image of Nyasa Lovebird samples.** A 2% agarose gel, stained with ethidium bromide, was used for separation of amplified DNA. **(A)** Lane1 represents a 50bp Gene Ruler DNA ladder, Lane 2 to 11 = Nyasa Lovebird female samples identified by CHD1Z and CHD1W at ~650bp and ~450bp, Lane 12 represents a 50bp Gene Ruler

DNA ladder. **(B)** Lane1 represents a 50bp Gene Ruler DNA ladder, Lane 2 - 11 = Nyasa Lovebird male samples, Lane 12 represents a 50bp Gene Ruler DNA ladder.

The results demonstrated that the optimized conventional PCR protocol was applied successfully for sexing Nyasa Lovebird without modifications. The gender of twenty individual samples was clearly identified. Next, the samples were analysed by the HRM analysis and the results from both techniques were compared. The derivative melt curves are shown in Figure 5.6.

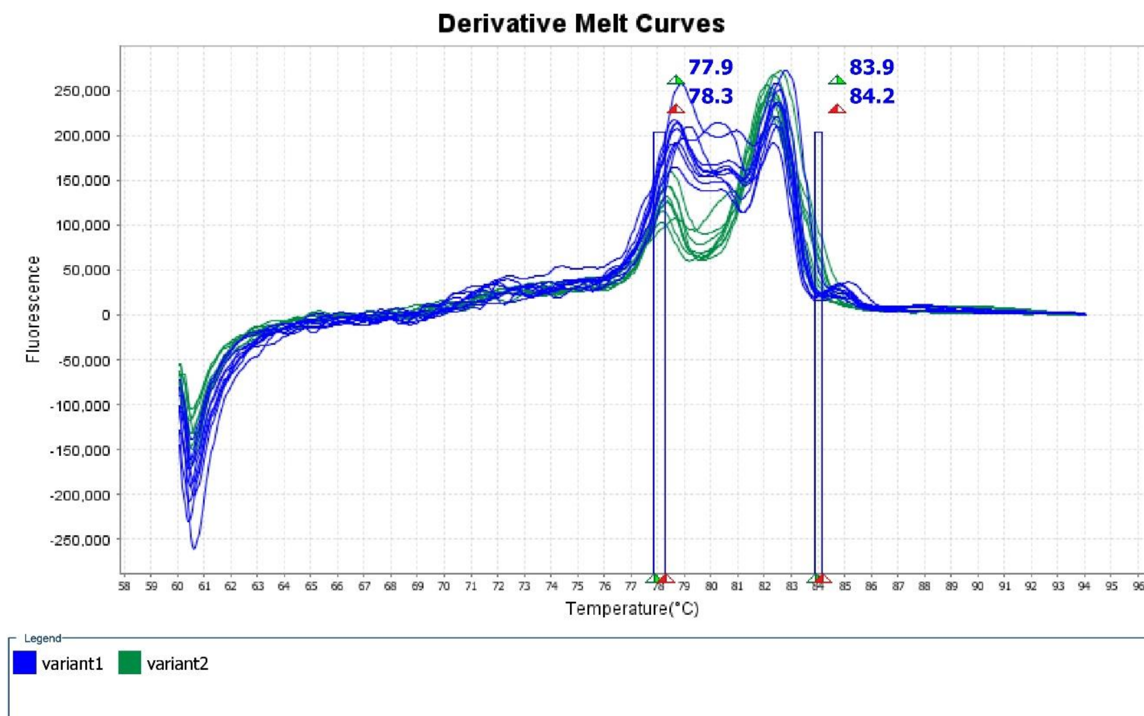

Figure 5.6: Derivative melt curves of Nyasa Lovebird. This view helped to identify the pre- and post-melt regions for data alignment. Each curve represents individual sample. Males are represented by two peaks, Females are represented by three peaks.

Figure 5.6 shows the melt curve data which was imported to the HRM software. The designated melt curves for normalization were fixed at temperatures ranging from 77.9 - 78.3 °C and 83.9 - 84.2 °C for twenty Fischer's Lovebird samples. Females were identified by three peaks at the following melting temperatures: One peak at ~78.8 °C, second small peak at ~80.5 °C and the last peak was observed at about ~82.5 °C. Males were identified by two peaks at ~78.3°C and ~82.5°C respectively. Males and females displayed different melting profiles with regards to the shape and melting temperature when the bars were shifted as observed in Figure 5.6. The active region was selected and the melt data was aligned (Figure 5.7).

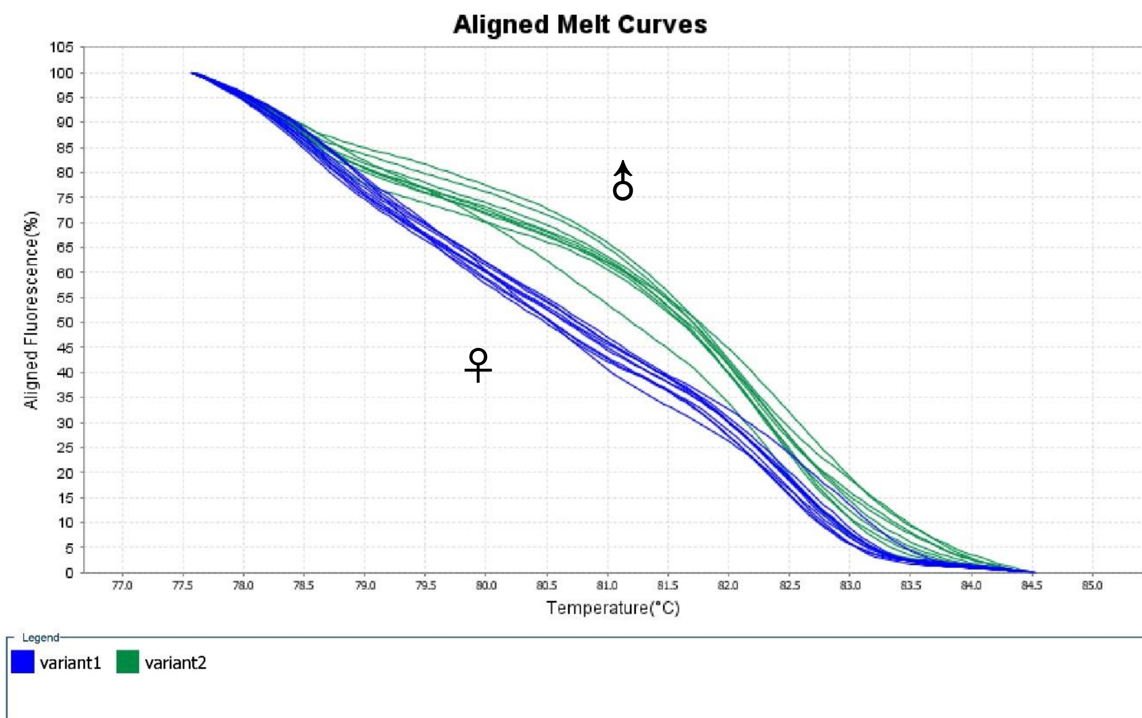

Figure 5.7: Aligned melt curves of Nyasa Lovebird. The pre-and post-melt regions were used to align data by positioning the parallel double bars. This is processed (normalized) fluorescence unit.

The HRM assay, as an extended analysis of a melt curve using a software, automatically processed the raw data (not shown) to provide a conspicuous view of the melt data with distinguished melting profiles between the two genotypes, males and females as seen in Figure 5.7. The difference in the melting profiles and shapes between males and females were better visualized on the difference plot (Figure 5.8).

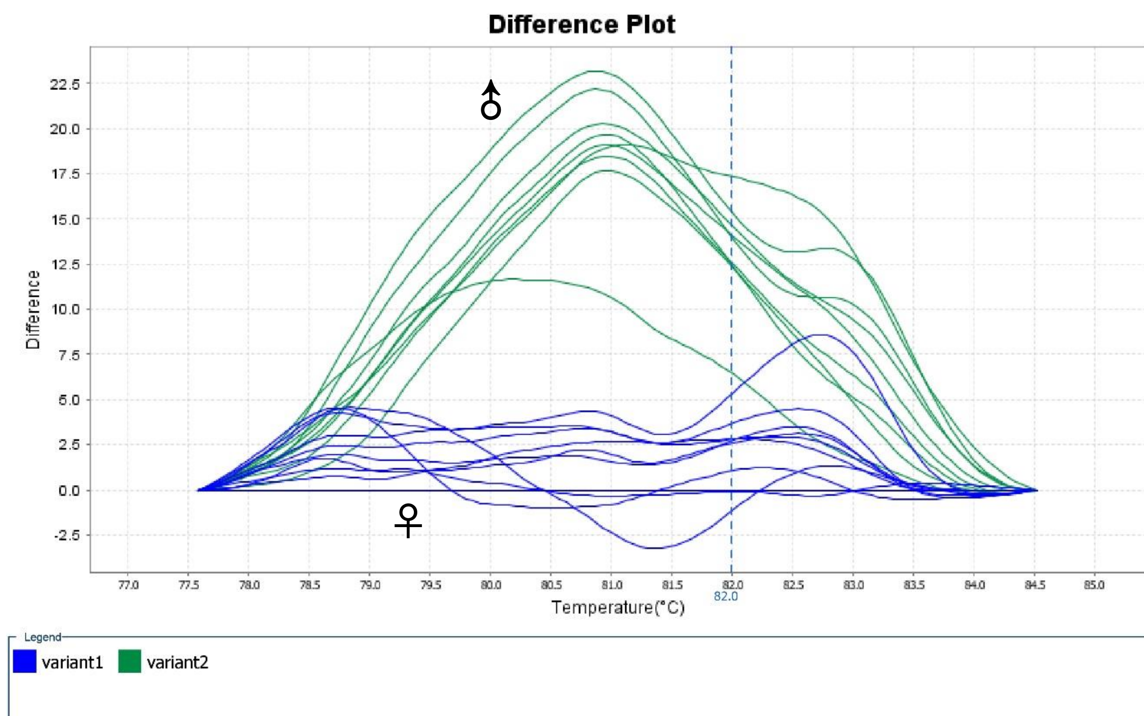

Figure 5.8: HRM analysis data difference plot of Nyasa Lovebird. The HRM software application allowed the calculation of the difference plot and plotted the Difference vs. Temperature (°C). Two variants are distinct. Each curve represents a different sample.

Unknown genotypes were categorized based on comparison with the defined genotype in Figure 5.8. The difference plot profiles were obtained and clustered into two groups

representing different genotypes (males and females), which then resulted in sex differentiation of 19/20 samples by the HRM analysis. One sample, corresponding to lane 11 on the gel image in Figure 5.5B, was identified as a variant. Despite the weak PCR product observed for this specific sample on the agarose gel in Figure 5.5 (and relative prominent smear in the lane), the sex of the bird could still be identified by the presence of the CHD1Z amplicon at ~650bp, identifying the sample as a male. Thus the EvaGreen HRM analysis matched the PCR and gel electrophoresis results with 95% accuracy as illustrated in Table 5.1.

5.2.3 Black-cheeked Lovebird

The applicability of the new approach for sex determination of ten females and ten males of Black-Cheeked Lovebird was tested as discussed in Section 5.2.1. The PCR products were separated on a 2% agarose gel as can be seen Figure 5.9.

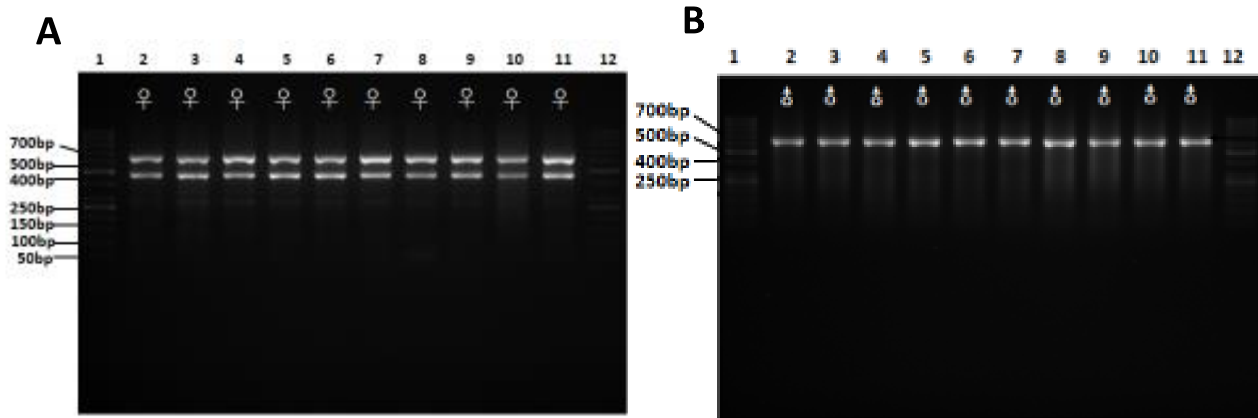

Figure 5.9: **Agarose gel image of Black-Cheeked Lovebird samples**. A 2% agarose gel, stained with ethidium bromide, was used for separation of amplified DNA. **(A)** Lane1 represents a 50bp Gene Ruler DNA ladder, Lane 2 to 11 = Black-Cheeked Lovebird female samples, Lane 12 represents a 50bp Gene Ruler DNA ladder. **(B)** Lane1 represents a 50bp Gene Ruler DNA ladder, Lane 2-11 Black-cheeked Lovebird male samples, Lane 12 represents a 50bp Gene Ruler DNA ladder.

Figures 5.9 demonstrate that the CHD1 gene amplification of all samples produced conspicuous results. Technical problems such as PCR competition and non-specific primer binding have been reported to interfere with the correct sex identification (Griffiths *et al*., 1998). PCR amplification of the CHD1 gene has been reported to produce one PCR product in both males and females (Dubeic and Zagalska-Meubauer, 2006; Ong and Vellayan, 2008), some products that could not be distinguished by agarose gel (Dawson *et al*., 2001). Despite all the technical problems reported, a standard protocol for conventional PCR-based on CHD1Z and CHD1W intron polymorphism was successfully drawn up and evaluated for its accuracy in sexing Black cheeked Lovebird. The difference between the band pattern of male and female individuals was easily visualized on a 2%

agarose gel. All twenty samples amplified were correctly sexed, compared to the Lumegen Laboratories (Pty) Ltd database.

The PCR products generated by conventional PCR and loaded on gels as indicated in Figure 5.9 were then analysed by the HRM assay explained in detail in Section 3.2.5 and the result is shown in Figure 5.10.

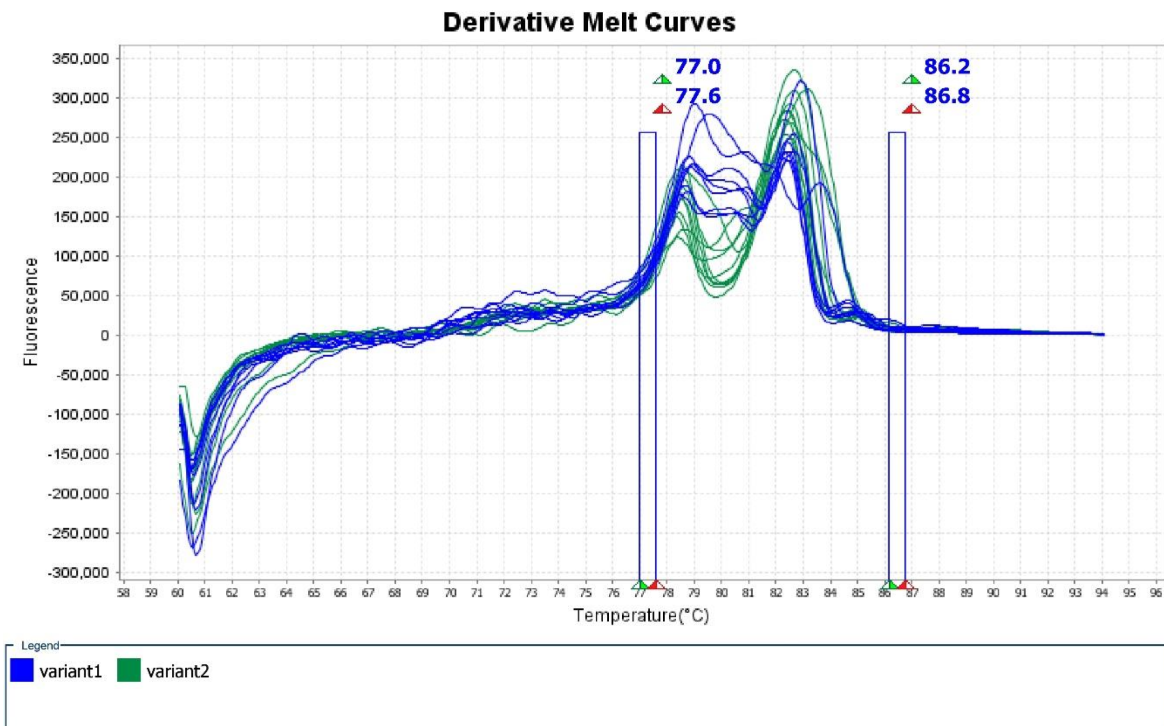

Figure 5.10: Derivative melt curves of Black-cheeked Lovebird. This view helped to place pre- and post-melt settings. Each curve represents a different samples. Males are represented by two curves, females by three peaks.

Figure 5.10 illustrates the melt derivative curve data generated and imported to the HRM software, which then enabled the melt data to be viewed in the derivative plot in order to select the most appropriate region for aligning the data. The derivative melt curve allowed the identification of males by two peaks at about ~78.5 °C and ~82.5 °C respectively, while females were identified by three peaks at ~79 °C, ~80.5 °C and ~82.5 °C respectively. The melt curve data was aligned by the HRM software as illustrated in Figure 5.11.

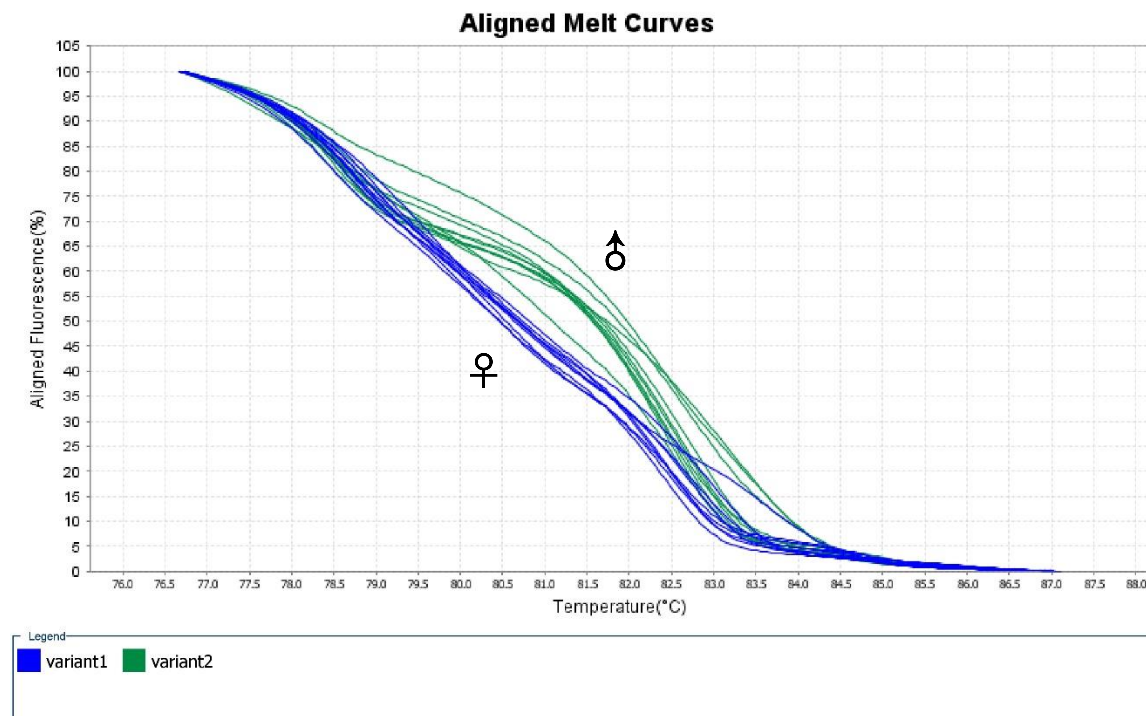

Figure 5.11: Aligned melt curve of Black-cheeked Lovebird. The pre-and post-melt regions were used to align data by positioning the parallel double bars**.** This is processed (normalized) fluorescence unit.

Figure 5.11 demonstrates the processed data which was automatically aligned by the HRM software to provide unambiguous view of the data with distinguished melting profiles between the two variants. The data was successfully normalized, and samples were clustered into groups representing different genotypes, males and females. The software calculated the difference plot between the two variants at the largest difference between the temperatures to determine allelic variance between the two variants as seen in Figure 5.12.

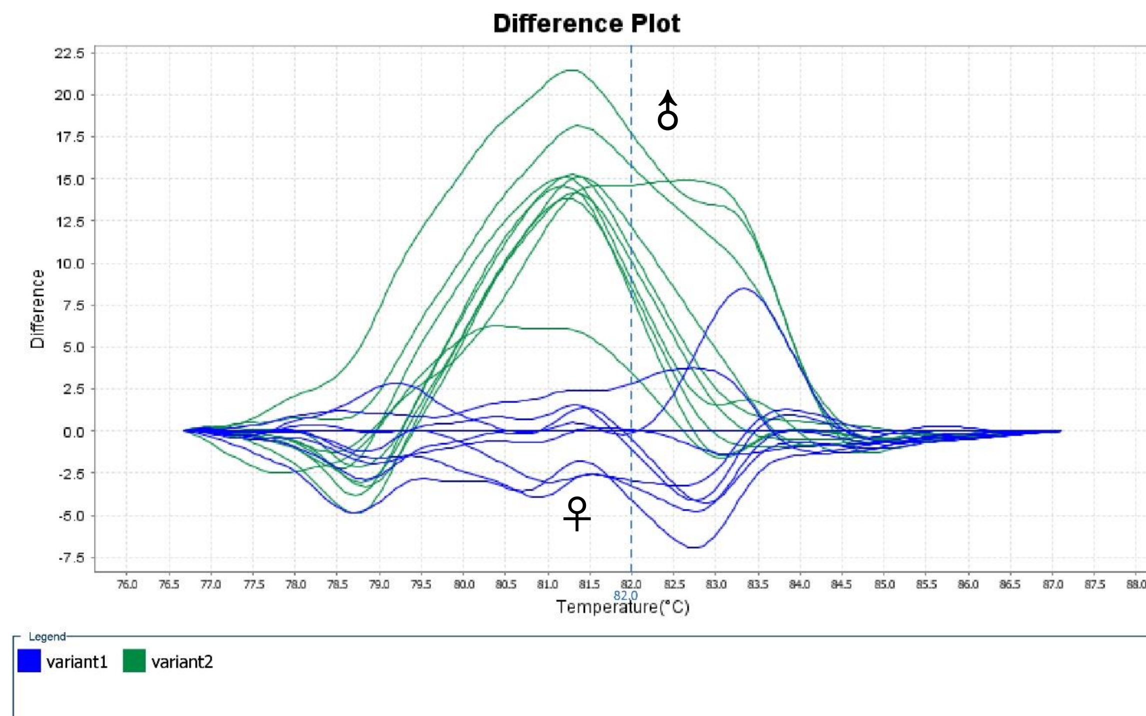

Figure 5.12: HRM analysis data difference plot of Black-Cheeked Lovebird samples. The HRM software application allowed the calculation of the difference plot and plotted the Difference vs. Temperature (°C). Two variants are distinct.

The melting curve profiles showed the expected differences in normalized temperature-data between males and females, allowing accurate gender distinction of 19/20 samples, compared to the conventional PCR. One female sample (in lane 10 of Figure 5.9A) was identified as a third variation, and thus excluded by the software. This specific sample showed a slight smear, compared to the other nine samples on the gel. Although the sample could easily be identified as female using the conventional PCR, the EvaGreen HMR approach could not identify the sex of the sample, again illustrating that the quality of the PCR amplification is essential for effective post-PCR HMR sex identification. Nevertheless, the EvaGreen HRM analysis for sexing Black-cheeked Lovebird was achieved with high success rate of 95% accuracy as can be seen in Table 5.1.

5.2.4 Peach-faced Lovebird

The applicability of the PCR protocol was tested for avian molecular sexing of ten females and ten males of Peach-faced Lovebird species. The PCR amplicons were loaded on a 2% agarose gel using the protocol described in Section 3.2.3 as the result depicted in Figure 5.13, followed by the HRM analysis.

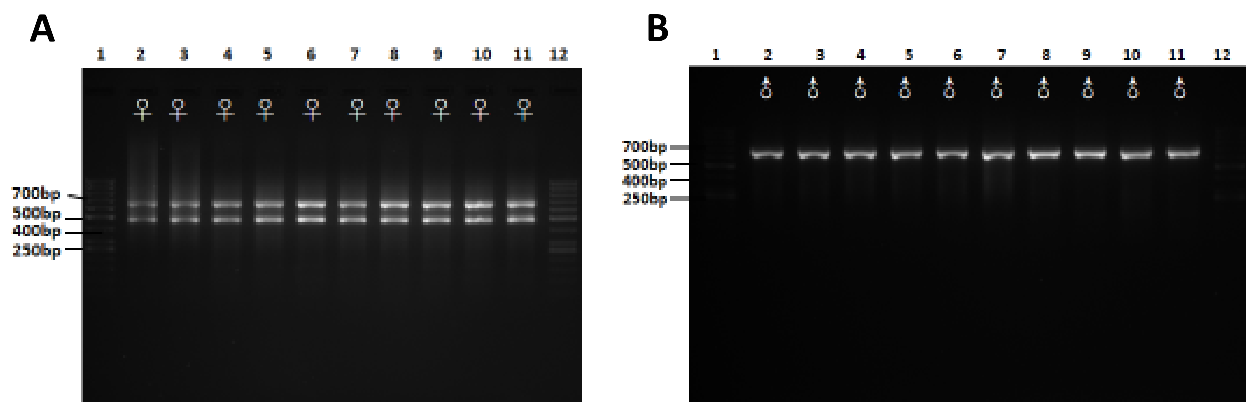

Figure 5.13: Agarose gel image of Peach-faced Lovebird samples. A 2% agarose gel, stained with ethidium bromide, was used for separation of amplified DNA. **(A)** Lane1 represents a 50bp Gene Ruler DNA ladder, Lanes 2 to 11 Peach-faced Lovebird female samples, Lane 12 represents a 50bp Gene Ruler DNA ladder. **(B)** Lane1 represents a 50bp Gene Ruler DNA ladder, Lane 2 to 11 = Peach-faced Lovebird female samples, Lane 12 represents a 50bp Gene Ruler DNA ladder.

All twenty samples, ten males and ten females were correctly identified as seen on Figure 5.13, and the accuracy of the PCR test was 100% consistent with the results documented for those samples on the database from Lumegen Laboratories (Pty) Ltd. With the 2550F/2718R primers, used in the protocol, the W and Z products cannot be confused, making sex identification easier. The only possible error utilizing the optimized protocol with 2550F/27118R was reported as the misidentification of females as males due to allelic dropout in the CHD1W exon, preventing the amplification of W chromosome (Arnold *et al*., 2003; Robertson and Gemmell 2006). However, none of the 10 female samples tested here showed any allelic dropout due to a polymorphism. Even in the case of a polymorphism, it would still be possible to accurately identify the sex of the bird because 2% agarose gels provided adequate resolution to discriminate between the Z

and W product, since they differ by ~200bp. Males revealed one CHD1Z band at ~650bp, while females revealed two bands CHD1Z & CHD1W at ~650bp and ~450bp respectively. The application of the optimized PCR protocol led to the correct identification of sex in all cases with clear band patterns. The results obtained with conventional PCR were compared with the results obtained by the HRM analysis. The melt curve data is shown in Figure 5.14.

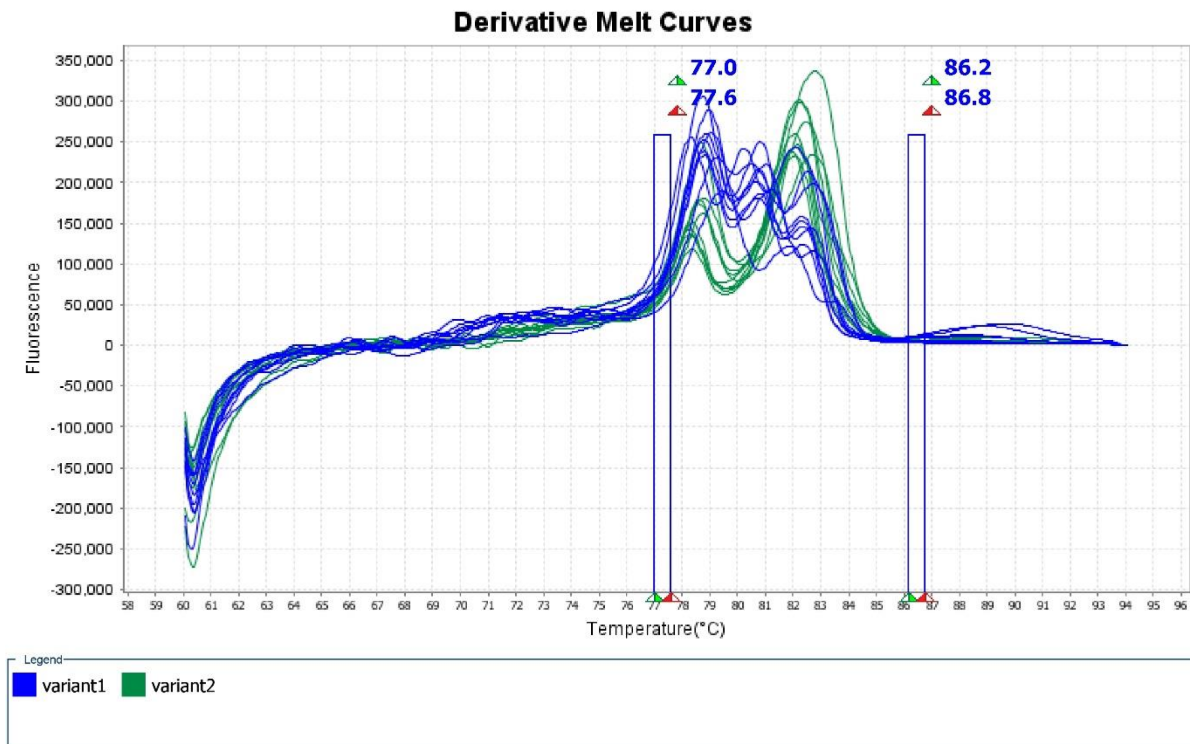

Figure 5.14: Derivative melt curve of Peach-faced Lovebird. This view helped to place pre- and post-melt settings. Each curve represent an individual sample. Males are represented by two peaks, Females are represented by three peaks.

The HRM, as an extended analysis of melt curves, was performed and the melt curves were clustered into groups representing different genotypes as seen in Figure 5.14. The two bars were placed at 77.0 - 77.6 °C and 86.2 - 86.8 °C, respectively, as can be seen in Figure 5.14. Some samples which were incorrectly identified when viewed on the derivative melt curve, were correctly identified after the optional temperature shift of the bars was performed by narrowing and widening of the active region. Female samples were identified by three peaks at about 78.8 °C, second peak at about 80.8 °C and the last peak at about 82.5 °C. Male samples were identified by two peaks at about 78.5°C and 82.5 °C. The differences between the variants were further analysed by aligning the active region between the two parallel bars. The aligned melt curve is shown in Figure 5.15.

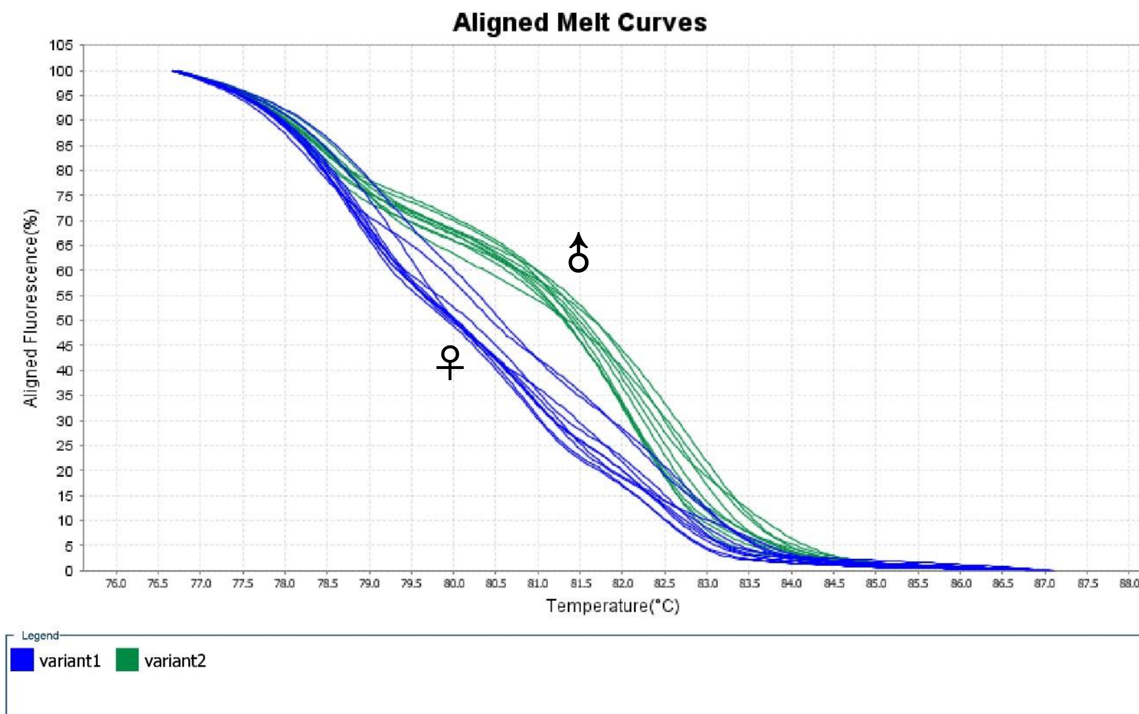

Figure 5.15: Aligned melt curves of Peach-faced Lovebird. The pre-and post-melt regions were used to align data by positioning the parallel double bars.

Figure 5.15 illustrates the aligned melt plot, which was processed and aligned to eradicate the fluorescence variations of individual samples. A temperature shift between the two variants was observed, which illustrated significant melting patterns for males grouped as one and females were also grouped as one based on the melting behaviour. Furthermore, the HRM software calculated the difference in Figure 5.16.

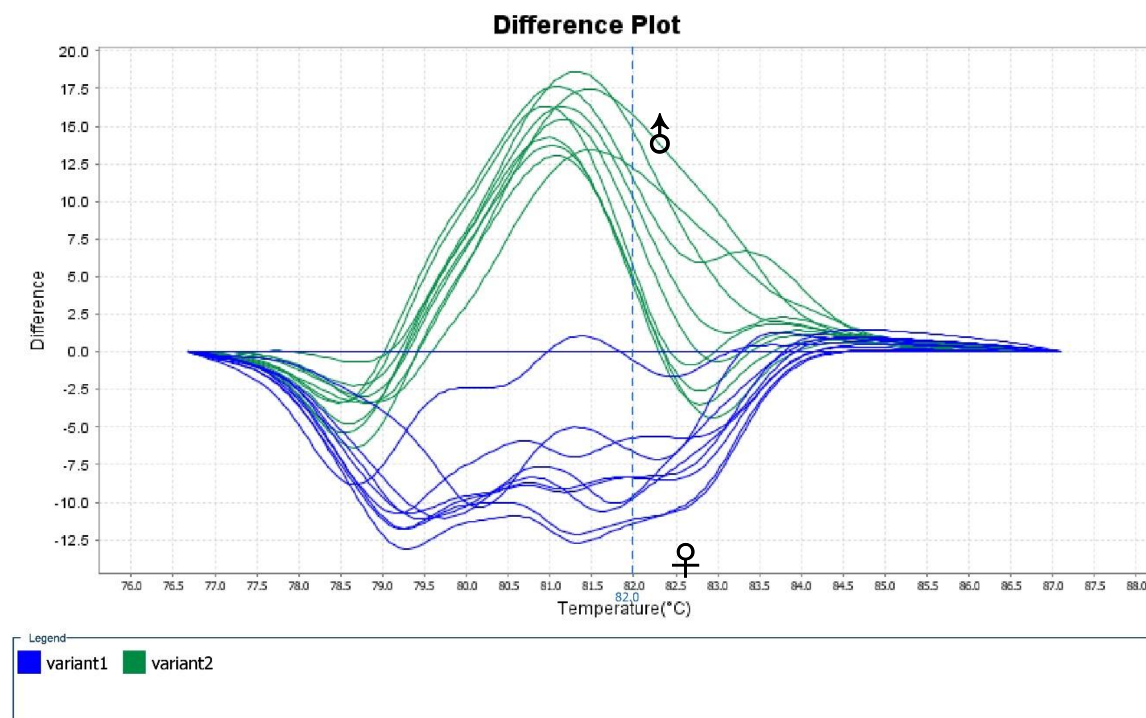

Figure 5.16: HRM analysis data difference plot of Peach-faced Lovebird. The HRM software application allowed the calculation of the difference plot and plotted the Difference vs. Temperature (°C). Two variants are distinct.

In Figure 5.16, the HRM analysis of the CHD1 gene for molecular sex identification of Peach-faced Lovebird samples are illustrated. This technique clearly demonstrated the

efficacy of the newly established EvaGreen HRM assay for a rapid and accurate gender discrimination of this species (avoiding the time consuming agarose gel electrophoresis step utilized in the conventional PCR). The HRM results were 100% consistent with the results obtained by conventional PCR and gel electrophoresis.

5.2.5 Green-cheeked Conure

Nineteen Green-cheeked Conure samples were amplified by PCR. The samples were loaded and separated on a 2% agarose gel as described in detail in Section 3.2.3. Figure 5.17 show the results obtained.

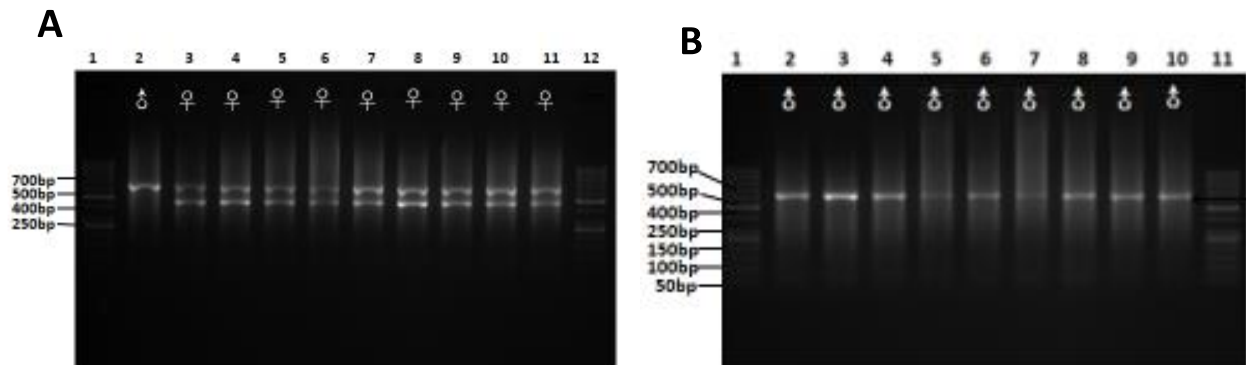

Figure 5.17: Agarose gel image of Green-cheeked Conure samples. A 2% agarose gel, stained with ethidium bromide, was used for separation of amplified DNA. **(A)** Lane1 represents a 50bp Gene Ruler DNA ladder, Lane 2 = Green-cheeked Conure male sample, Lanes 3 to 11 = Green-Cheeked female samples, Lane 12 represents a 50 bp Gene Ruler DNA ladder. **(B)** Lane1 represents a 50bp Gene Ruler DNA ladder, Lanes 2 to 10 = Green-cheeked Conure male samples, Lane 11 represents a 50bp Gene Ruler DNA ladder.

Figure 5.17 demonstrate agarose gel images indicating PCR products amplified by the optimized PCR protocol (Section 3.2.3). The gel electrophoresis revealed two bands of PCR products (CHD1Z) at ~650bp and (CHD1W) at ~450bp for females, whereas male birds were represented by a single band (CHD1Z) at ~650bp. Although the sex of all the samples tested could be identified on a 2% agarose gel on the basis of the specific PCR products formed, all the Green-cheeked Conure amplicons contained smears compared to the Fischer's Lovebird (Section 5.2.1), Nyasa Lovebird (Section 5.2.2), Black-cheeked Lovebird (Section 5.2.3) and Peach-faced Lovebird (Section 5.2.4). With the previously analysed species (Sections 5.2.1 – 5.2.4), individual samples that contained smears on the gel resulted in misidentification of sex and exclusion of those specific samples by the HRM software. With this in mind, the HMR EvaGreen HRM approach was applied to the amplified Green-cheeked Conure samples. The derivative melt curves are presented in Figure 5.18.

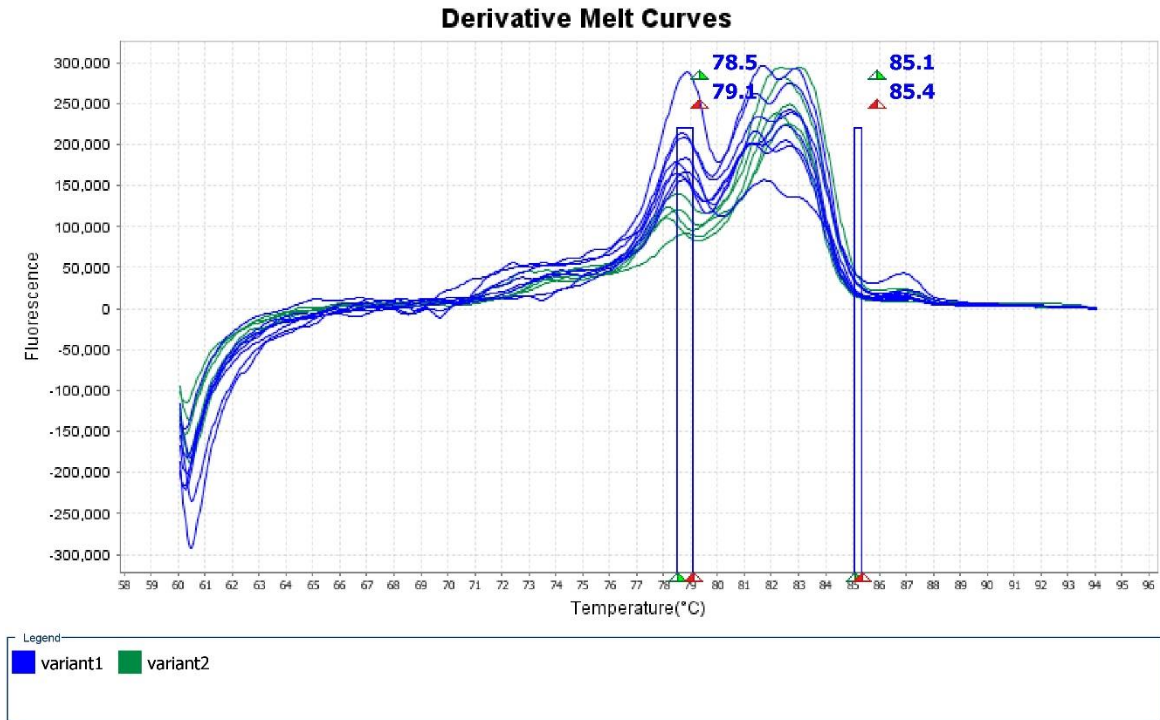

Figure 5.18: Derivative melt curves of Green-cheeked Conure. This view helped to place pre- and post-melt settings. Each curve represents different sample.

Figure 5.18 demonstrates the derivative melt curves. The placement of the parallel bars, as applied on Green cheeked Conure, could not increase the differences between the two variants. A possible explanation was linked to the poor PCR amplification characterized by smears observed in Figure 5.17. Males and females exhibited similar melting curves (Figure 5.18), identified by double peaks at ~72 °C and ~82.5 °C. The melt curve data was aligned and is illustrated Figure 5.19.

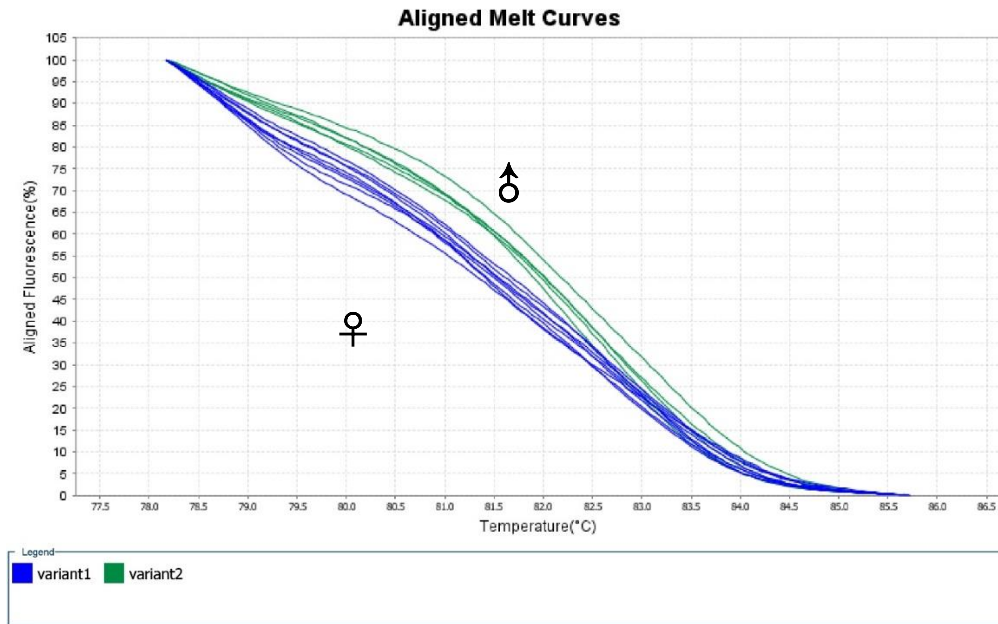

Figure 5.19: Aligned melt curves of Green-cheeked Conure. The pre-and post-melt regions were used to align data by positioning the parallel double bars. This is processed (normalized) fluorescence unit.

Figure 5.19 shows the aligned melt curve, which allowed the visualization between males and females based on temperature-shift between two variants characterized by their specific melting behaviour between the CHD1 amplicons. Furthermore, the software calculated the difference plot between two variants and grouped them according to sex in Figure 5.20.

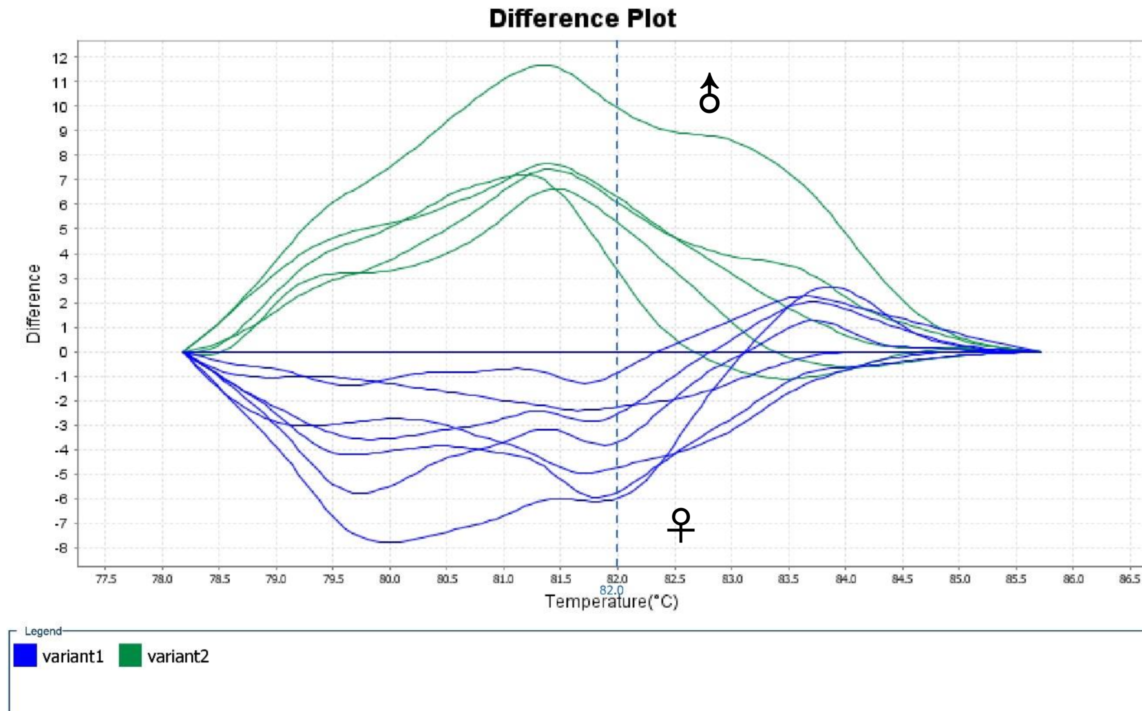

Figure 5.20: HRM analysis data difference plot of Green-cheeked Conure. The HRM software application allowed the calculation of the difference plot and plotted the Difference vs. Temperature (°C). Two variants are distinct. Each curve represents a different sample.

The melt curve profiles obtained were clustered into groups representing different genotypes on the difference plot, allowing sex identification of 13/19 samples as seen in Figure 5.20. The PCR vs. HRM analysis of Green cheeked Conure revealed 68% matching results as seen in Table 5.1. The sample in lane 6 of Figure 5.17A (female) was misidentified by the software, marked as a variant. The samples in lanes 5, 6, 7, 8 and 9 of Figure 5.17B (all males) were misidentified by the software as variants, thus automatically excluded by the HRM software.

Morinha *et al.*, (2013) reported unsuccessful HRM analysis using degraded biological samples collected from birds in advanced stages of decomposition, which did not allow the achievement of reproducible results. For that reason, they recommended using the same extraction protocol for all samples under analysis, ensure that all DNA are of the same quality and concentration in each HRM assay. Variations in DNA purity and integrity can interfere with the sensitivity and reproducibility of the HRM assays. However, it was impossible to ensure the same quality and concentration of all DNA samples due to the fact that the PCR amplifications were carried out directly from the Guthrie card with blood, without further purification steps. As such, the quality of PCR amplicons differs for each sample, possibly based on the quality of the sample. Another possibility is that the DNA sequence of the Green Cheek Conure slightly differs from that of the Lovebirds on the target region(s) of the primers, leading to slight mispriming and subsequent poor amplification. Although this is only an hypothesis, the idea was strengthened by the industrial partner, Lumegen Laboratories (Pty) Ltd, who confirmed that Green–cheeked Conures are notorious in DNA sexing, often leading to poor results/samples that cannot be sexed accurately, due to an unknown reason. Thus, whatever the reason for the poor amplification of the Green-cheeked Conure samples, it had a dramatic influence on the accuracy of the EvaGreen HRM approach in this species.

5.2.6 Quaker parrot

The twenty Quaker parrot samples (males and females) were tested with the conventional PCR protocol (Section 3.2.3). The PCR products were loaded and separated on a 2% agarose gel (Figure 5.21), followed by the HRM analysis.

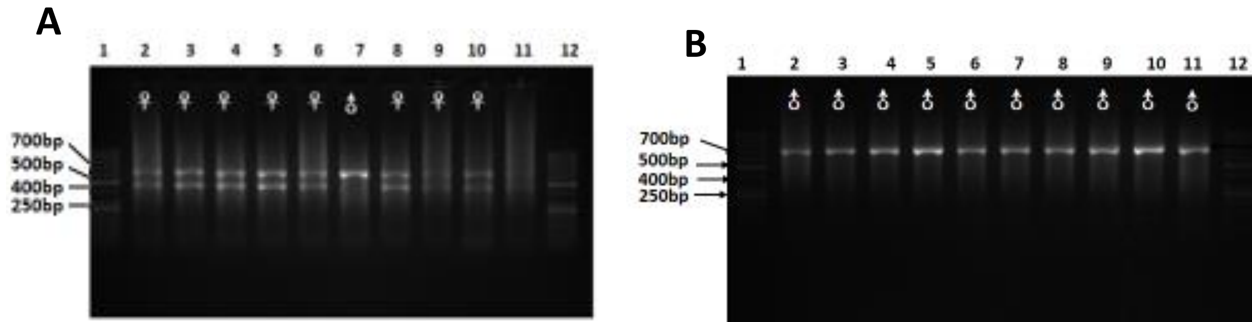

Figure 5.21: Agarose gel image of Quaker parrot samples. A 2% agarose gel, stained with ethidium bromide, was used for separation of amplified DNA. **(A)** Lane1 represents a 50bp Gene Ruler DNA ladder, Lanes 2 to 11 = Quaker parrot samples, Lane 12 represents a 50bp Gene Ruler DNA ladder. **(B)** Lane1 represents a 50bp Gene Ruler DNA ladder, Lanes 2 to 11 = Quaker parrot male samples, Lane 12 represents a 50bp Gene Ruler DNA ladder

In Figures 5.21, the female samples were identified by two bands, a CHD1Z fragment at ~650bp and a CHD1W fragment at ~450bp, whereas males were identified by one CHD1Z band at ~650bp. Due to the poor PCR amplification, the sex of some samples were difficult to identify on a 2% agarose gel. The two samples in lanes 9 and 11 of Figure 5.21A for instance, were difficult to identify as female due to poor amplification of the target sequences and results that are very smeary. Although all the previously analysed species, including the Green-cheeked Conure could be sexed with confidence using the

conventional PCR (Figures 5.1; 5.5; 5.9; 5.13; 5.17), the conventional PCR proofed problematic sexing the Quaker parrot samples. Next the samples were analysed by the HRM approach (Section 3.2.5) and the results illustrated in Figure 5.22.

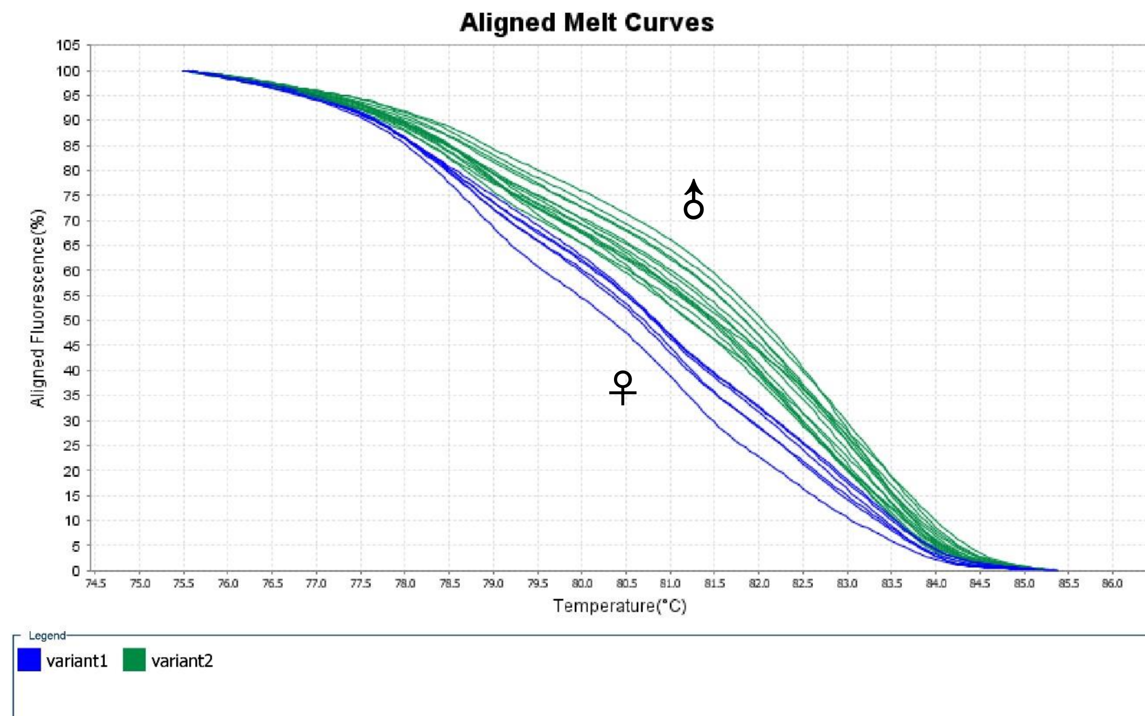

Figure 5.22: Aligned melt curves of Quaker parrot. The pre-and post-melt regions were used to align data by positioning the parallel double bars. This is processed (normalized) fluorescence unit.

Figure 5.22 demonstrates the processed aligned melt curves by the HRM software which provided a clearer view of the data between male and female samples. The HRM software clustered curves into groups relative to the baseline and calculated the difference plot (Figure 5.23).

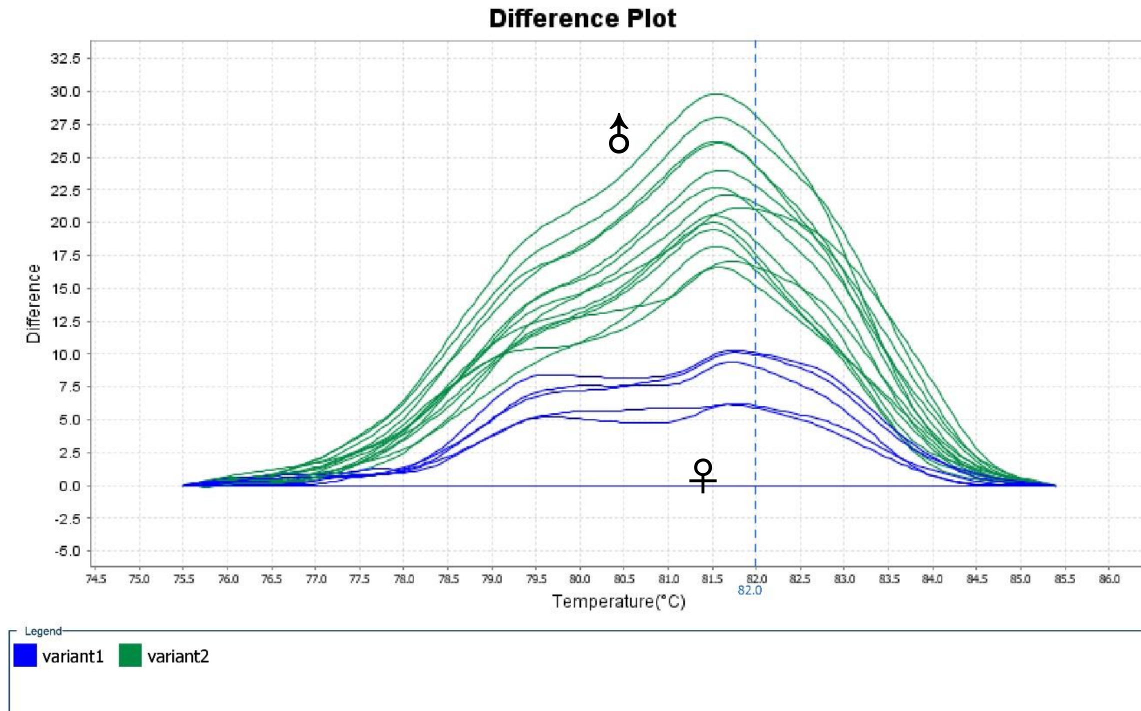

Figure 5.23: HRM analysis data difference plot of Quaker parrot. The HRM software application allowed the calculation of the difference plot and plotted the Difference vs. Temperature (°C). Two variants are distinct. Each curve represents a different sample.

The EvaGreen HRM assay applicability for avian molecular sexing of Quaker parrot was investigated as seen in Figure 5.23. Earlier, the HRM approach for avian molecular sexing was used for sexing Peach-faced Lovebird (Section 5.2.4) with a 100% success rate. The capability of the approach with regards to sensitivity/specificity was attributed to the good PCR amplification. In contrast, the EvaGreen HRM approach used here for sexing Quaker parrot samples led to the misidentification of three females as males. The incorrectly identified samples were found in: Lane 9, 10 & 11 of Figure 5.21. Contrary to previous species, where misidentified samples were grouped as a third variant, and thus

automatically excluded from the results by the software, here three female samples were misidentified as males, something highly problematic in a diagnostic setup.

5.2.7 Lineolated parakeet

The Lineolated parakeet samples tested by the conventional PCR. The PCR and gel electrophoresis as demonstrated in Figure 5.24 was carried out as described in Section 3.2.3 followed by the HRM analysis as described in Section 3.2.5.

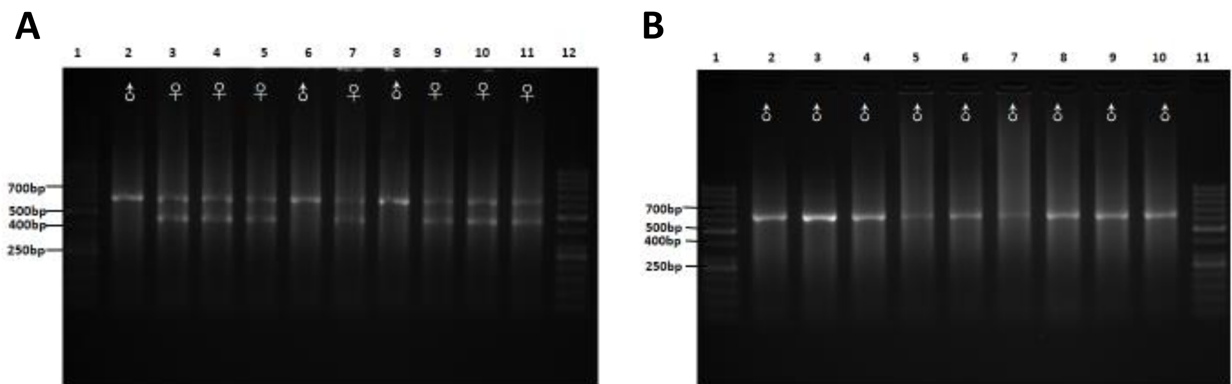

Figure 5.24: Agarose gel image of Lineolated parakeet samples. A 2% agarose gel, stained with ethidium bromide, was used for separation of amplified DNA. **(A)** Lane 1 represents a 50bp Gene Ruler DNA ladder, Lane 2-11 = Lineolated parakeet samples, Lane 12 represents a 50bp Gene Ruler DNA ladder. **(B)** Lane 1 represents a 50bp Gene Ruler DNA ladder, Lane 2-10 = Lineolated parakeet male samples, Lane 11 represents a 50bp Gene Ruler DNA ladder.

The female samples were identified by the presence of CHD1Z and CHD1W bands at ~650bp and ~450bp respectively, while males were identified by a single CHD1Z band at

~650bp as seen in Figure 5.24. Although all the samples contained smears and weak amplification of the target sequences, the sex of all the samples could be identified on the agarose gel. The PCR amplicons were then analysed by the HRM analysis in Figure 5.25 to Figure 5.27.

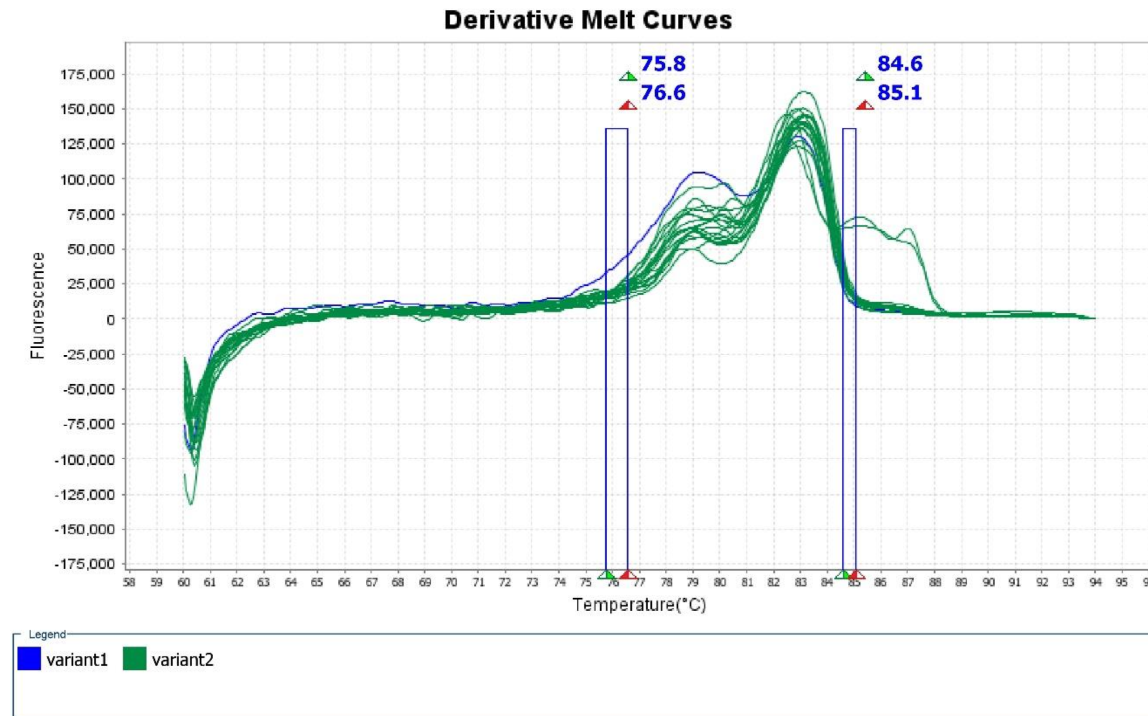

Figure 5.25: Derivative melt curves of Lineolated parakeet. This view helped to place pre- and post-melt settings. Each curve represents different sample. Males and females displayed similar melt curves.

Figure 5.25 demonstrates the derivative melt view of Lineolated parakeet samples which were analysed by the HRM protocol. Here, unsuccessful application of the HRM protocol was demonstrated due to failed sex differentiation between males and females. Males and females displayed similar melt profiles. Even with the adjustment of the parallel

temperature limit bars to increase the differences between variants, males and females still displayed similar melt profiles. Thus sex determination failed even after the optional temperature shift was applied, leading to misidentification of melt curve grouping between males and females. In Figure 5.26, the HRM software aligned melt curves is shown.

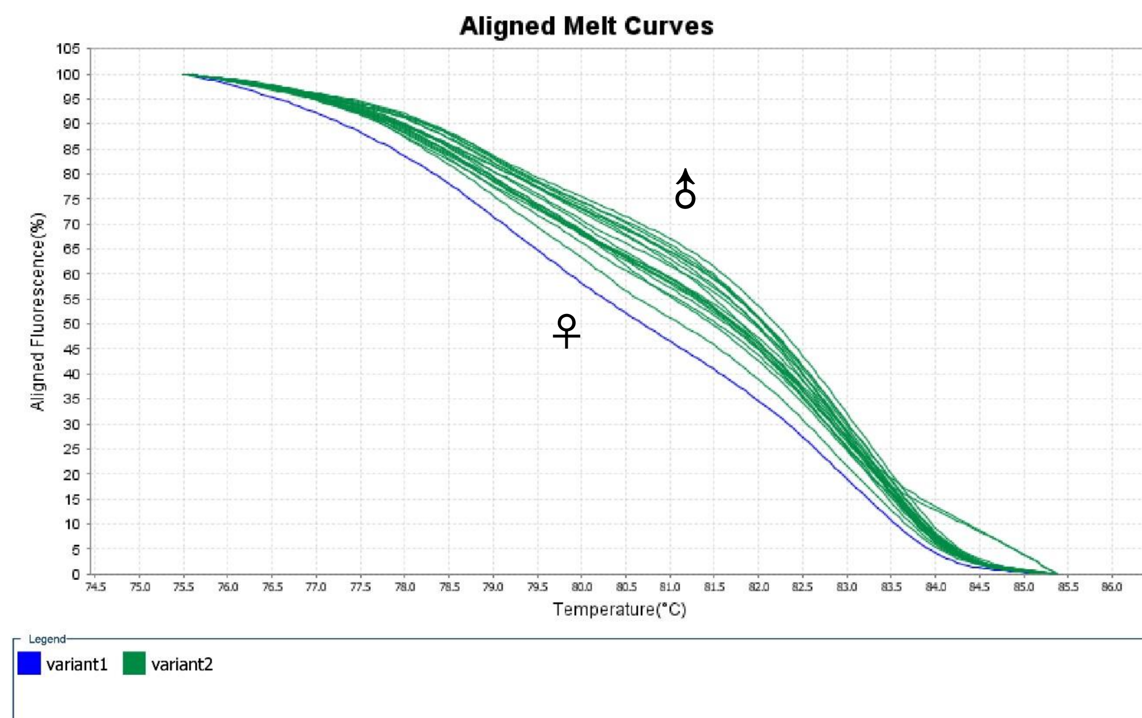

Figure 5.26: Aligned melt curves of Lineolated parakeet. The pre-and post-melt regions were used to align data by positioning the parallel double bars as shown in the figure above. This is processed (normalized) fluorescence unit.

Figure 5.26 demonstrates the aligned melt plot, which could not allow for the clear temperature-shift distinction between the female and male samples. As such, the aligned melt plot was not diagnostic for avian gender.

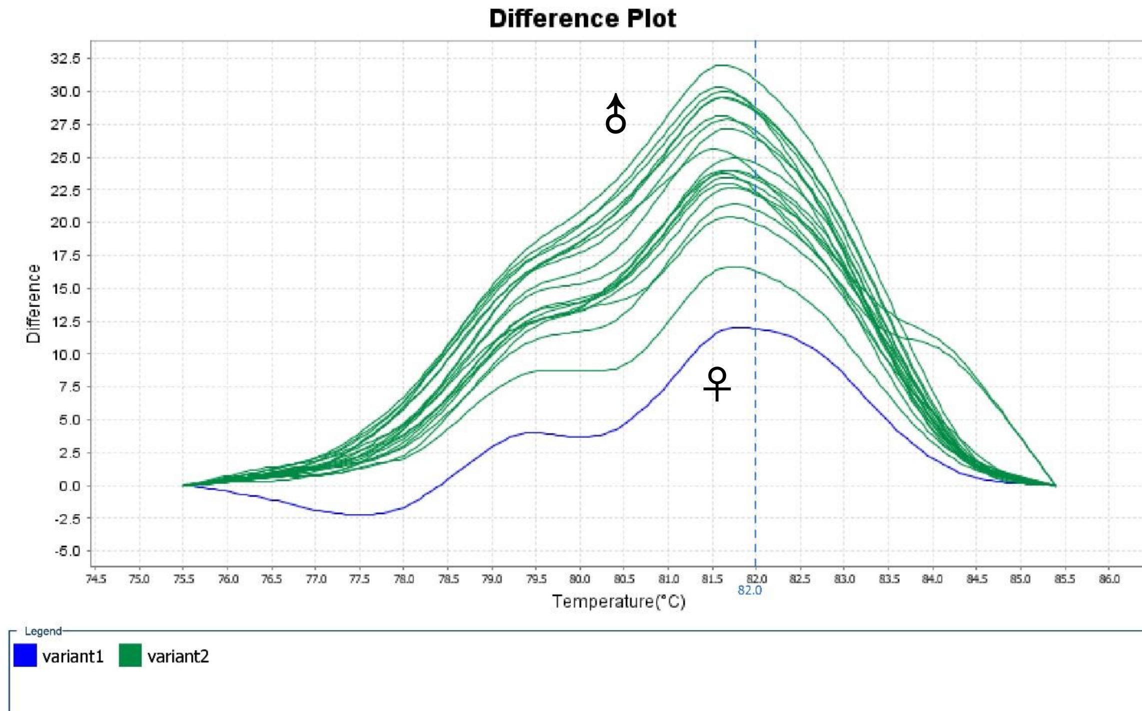

Figure 5.27: HRM analysis data difference plot of Lineolated parakeet. The HRM software application allowed the calculation of the difference plot and plotted the Difference vs. Temperature (°C). Two variants are distinct.

The outcome of EvaGreen HRM approach (Figure 5.27) clearly demonstrates the inapplicability of this protocol for avian molecular sexing of Lineolated parakeet samples. All samples showed smears and weak product formation as a result of poor PCR amplification (Figure 5.24), although the sex of all the samples could still be identified. With the EvaGreen HRM assay, from the seven female samples, only one sample was identified as a female, while the remaining six samples (lanes 4, 5, 7, 9, 10 and 11 of Figure 5.24A) were misidentified as males.

5.3 Comparing the analytical methods using the results obtained

All the results obtained on the seven different avian species employed to test and compare the newly established EvaGreen dye PCR-HRM assay to the conventional PCR, are summarized in Table 5.1.

Table 5.1: Evaluation of the PCR and HRM assay accuracy on different species

Species	Gender	Conventional PCR	HRM	Misclassified as	Accuracy per gender	Overall accuracy
Fischer's Lovebird (n=20)	Female	10	10	-	100%	95 %
	Male	10	9	Unknown	90%	
Nyasa Lovebird (n=20)	Female	10	10	-	100%	95 %
	Male	10	9	Unknown	90%	
Black-cheeked Lovebird (n=20)	Female	10	9	Unknown	90%	95 %
	Male	10	10	-	100%	
Peach-faced Lovebird (n=20)	Female	10	10	-	100%	100 %
	Male	10	10	-	100%	
Green-cheeked Conure (n=19)	Female	9	8	Unknown	89%	68 %
	Male	10	5	Unknown	50%	
Quaker parrot (n=20)	Female	9	6	Other gender	67%	85 %
	Male	11	14	Other gender	100%*	
Lineolated parakeet (n=19)	Female	7	1	Other gender	14%	68 %
	Male	12	18	Other gender	100%*	

N = Number of samples, * = where the specific gender was correctly identified but the actual number of individuals identified was influenced by the other gender being misidentified as this gender.

The newly established EvaGreen dye PCR-HRM assay compared relatively well to the conventional PCR in the four Lovebird species tested (Fischer's Lovebird, Nyasa Lovebird, Black-cheeked Lovebird and Peach-faced Lovebird). In the Peach-faced Lovebird, the results from the HRM assay corresponded 100% to that obtained with the conventional PCR. In the other three Lovebird species tested (Fischer's Lovebird, Nyasa Lovebird and Black-cheeked Lovebird), only one form the 20 samples were misclassified by the HRM assay, thus resulting in an overall accuracy of 95% (per species) of the HRM assay compared to the conventional PCR. Another positive aspect that have to be mentioned here is that the single sample per species that was misclassified was classified as a third variant, thus excluded from the results by the software. This means that all samples classified as a third variant could be re-analysed in a follow up batch, thus no sample was misclassified as another gender, which would make the assay not viable for diagnostic use.

For the remaining three species tested (Green-cheeked Conure, Quaker parrot, Lineolated parakeet), the HMR assay gave unsatisfactory results. With the Green-cheeked Conure, one female sample was misclassified while five male samples were misclassified, all marked as a third variant, and thus excluded from the results by the software. For the Quaker parrot and Lineolated parakeet, female samples were classified as male, thus the EvaGreen HRM assay could not be considered for diagnostics in these species.

According to Morinha *et al.* (2013), low quality DNA or the use of degraded biological samples which was collected in late stages of decomposition, is unsuitable for HRM analysis. In this study, a possible setback was that the PCR was done directly from a Guthrie card, meaning that poor amplification might be due to low DNA quality of the sample. This could be the result of an incorrect procedure followed during blood collection from the bird, the use of disinfectants like chlorine that contaminated the sample, over-saturation of the Guthrie card with blood or even contamination of the sample on the Guthrie card with solids like soil or avian faeces. With no control over the quality of the blood samples (and hence the DNA quality), it seems like the EvaGreen HRM assay is not performing as well as the conventional PCR. An approach that might be considered is to isolate DNA from the Guthrie cards and use this purified DNA for PCR amplification and then EvaGreen HRM analysis. Unfortunately, due to the cost of DNA isolation, this approach was not financially feasible in this study. As mentioned in Section 2.9.2, it was critical to keep the cost of the new test below a specific price ceiling, in other words keep the test affordable and thus financially competitive. Isolation of DNA would have lifted the cost per sample above the price ceiling, thus making the test financially not viable.

CHAPTER 6: Conclusions

6.1 Introduction

In Chapter 2, a literature overview of the importance of bird sexing, general methods for gender determination and the DNA based sexing methods were presented. A problem statement, aim and experimental approach were also presented in Chapter 2. The analytical methods were discussed in Chapter 3, followed by the standardization of the analytical methods in Chapter 4. The accuracy of the newly established EvaGreen PCR-HRM assay was compared to the conventional PCR (using seven avian species) in Chapter 5. In this chapter, conclusions will be drawn, linked to the specific objectives set for the study, in order to achieve the aim of establishing a PCR-HRM molecular avian sexing technique.

6.2 Problem statement, aim and objectives

Many bird species are sexually monomorphic, which means that it is difficult to distinguish between a male and female based on external appearance. There are a number of general techniques which have been used for bird sexing, including surgical sexing, hormonal measurement, karyotyping, etc. However, all these traditional techniques have limitations. The birds may suffer from stress due to invasive procedures and, the methods are also laborious and time consuming with limited applicability as routine methodology. The sexing techniques were improved by the introduction of DNA based techniques,

which became the preferred method of sexing. However, these techniques still involved PCR and gel electrophoresis and sometimes the use of restriction enzymes.

The conventional PCR-based techniques currently used for DNA sexing of birds at Lumegen Laboratories (Pty) Ltd, the industrial partner in this study, are time consuming (it includes PCR and gel electrophoresis) and laborious, reducing their high-throughput applicability. These shortfalls prompted the need for improvement of the sex determination protocol based on PCR-HRM analysis, which is rapid and accurate, with increased sensitivity and reduction in development time, labour and cost for molecular sex identification in birds.

The ***aim*** *of this study was to develop a polymerase chain reaction with high resolution melt (PCR-HRM) analysis for avian sex determination.* In this section, the outcome of the study is discussed in relation to the objectives in order to accomplish the aim of developing a sex determination protocol based on a PCR-HRM approach for bird sexing.

6.3 Conclusions

6.3.1 Objective 1- Obtain samples of specific avian species to conduct the study

Sample selection was the first objective and in order to achieve this objective, the bird breeders had to be approached for collaboration in the study. The industry partner, Lumegen Laboratories (Pty) Ltd compiled a list of target species to conduct the investigation. The samples had to be collected from Lumegen Laboratories (Pty) Ltd to pursue this study. All of the birds used in the study were of known sex, as determined by

conventional PCR at Lumegen Laboratories (Pty) Ltd. All samples were successfully collected from different species with respect to the list of target species as noted in Section 3.1.2.

6.3.2 Objective 2 – Evaluate the existing PCR protocol for avian molecular sexing, and apply it to determine the sex of the chosen samples

The PCR protocol currently employed at Lumegen Laboratories (Pty) Ltd, the industrial partner, used in this study as a validation for the HRM approach, had to be evaluated first. The annealing temperature of the PCR was optimized for successful PCR amplification of different species. A conclusion was reached that a high annealing temperature was not suitable for PCR amplification of all species. For instance, an annealing temperature of 59 °C was used for the amplification of Fischer's Lovebird and resulted in very good amplification. However, when the 59 °C annealing temperature was used for PCR amplification of Galah Cockatoo samples, no products could be observed. A possible reason as to why good amplification was observed for some species at higher annealing temperature while not for others was linked to a possible nucleotide sequence diversity among different bird species; which then results to lower binding affinity of primers to the template, and thus reducing amplification. Consequently, the annealing temperature had to be reduced in order to find a suitable temperature for successful amplification of many different species. The reduction of the annealing temperature proved to be a suitable approach for amplification of Galah Cockatoo samples which could not be amplified at a higher annealing temperature. Some samples demonstrated weak amplification with

smears on the gels. A possible explanation was linked to the quality of the sample DNA, which differed from sample to sample as all the samples were amplified directly from the Guthrie cards without DNA purification. This posed a challenge to the study because the amplification efficiency was compromised on some samples. Nonetheless, the sex of the birds could still be identified using the conventional PCR assay, regardless of the smears and weak PCR products. In conclusion, the PCR protocol has proven to be a reliable, precise, sensitive and efficient DNA based sexing technique for avian molecular sexing of different bird species with only a drop of blood, and with minimal optimization. Despite the labour intensiveness of this technique, it was successfully used for amplifying DNA from different species, with different DNA quality, without a need for sample purification.

6.3.3 Objective 3 – Design and optimization of a PCR-HRM protocol for avian molecular sexing

The PCR-HRM amplification of the CHD1 fragments using KAPA HRM Fast master-mix posed a challenge in this study as no PCR amplification could be obtained. Due to financial limitations to this project (keeping the cost of the new test below a specific price ceiling to make the test financially viable), trying more expensive HRM reagents to enhance PCR amplification was not economically justifiable. Thus, as a result of financial constraints, another approach that was within financial constrains had to be implemented to overcome this challenge - this included performing the conventional PCR amplification and HRM analysis of the amplicons post-PCR by introducing the EvaGreen dye. The standard melt curves had to be performed, followed by the HRM analysis development

which is more sensitive. The PCR-HRM assay was introduced and performed very well using Fischer's Lovebird samples. In an attempt to better the performance of the assay, different primers were tested. It can be concluded that the P2/P8 primer set cannot be used with the EvaGreen HRM assay as it was not able to distinguish male from female samples. However, when using the 2550F/2718R primer set, the assay performed very well and could subsequently be tested on different avian species.

One disadvantage of the newly established PCR-HRM assay is that a closed-tube system is not used, as the EvaGreen dye had to be added separately after PCR amplification, possibly increasing the risk of cross-contamination.

6.3.4 Objective 4 – Compare the accuracy of the PCR-HRM assay to the conventional PCR using different avian species

The fourth objective was to compare the results obtained with conventional PCR and the HRM assay, which were tabulated in Table 5.1. From the results, it was conclusive that the conventional PCR and gel electrophoresis could reveal clear differences between males and females in all species as a validation approach. However, some samples were identified with brighter bands than others, while others had smears as a result of (possible) different DNA quality that differed from sample to sample, which then influenced the amplification efficiency. A conclusion could be reached that this approach is reliable as seen in all the species tested. The results confirmed that the conventional PCR method using 2550F/2718R primers was successfully used to identify the sex of Fischer's Lovebird, Nyasa Lovebird, Black-cheeked Lovebird, Peach-faced Lovebird,

Green-cheeked Conure, Quaker parrot and Lineolated parakeet. Although some of the samples had smears, the sex of all the birds could still be identified with this approach in all the avian species tested.

The EvaGreen dye PCR-HRM analysis results differed from species to species. A conclusion could be reached that the sensitivity of the technique was highly dependent upon the quality of PCR. The HRM analysis results for Lovebird species was achieved with a high success rate (> 95%) as a result of good PCR amplification in all the Lovebird species. On the contrary, Green cheeked Conure and Lineolated parakeet species were poorly amplified and quite smeary, which then led to less sensitivity of the HRM technique in distinguishing the samples based on sex. It was concluded that the HRM software could not identify the samples of lower DNA quality as a male or a female, but those samples were rather identified as variations and excluded by the software during the analysis. The sample quality and poor amplification remains a challenge in this study for high sensitivity/specificity of the HRM protocol.

6.4 Final conclusion and future recommendation

Methods to identify gender are essential management tools that provide insight into the population dynamics and structure of wild and captive birds. Sex identification is necessary for the management and conservation of avian species, animal ecology studies, behaviour, population structure and life history. Sex identification in avian species can be performed by many techniques, such as vent sexing, laparoscopy and DNA based techniques. Preference between these methods also depend on laboratory facilities. In

this study, the applicability of a PCR-HRM protocol for molecular sexing of different bird species was studied. A simple protocol for all species was optimized, while keeping the same conditions for the reaction mixture. The melting curve profiles obtained were clustered into groups representing different genotypes, allowing precise sex differentiation of samples. The characteristic melting profiles obtained were the result of species- and sex-specific melting behaviour. The high variability in the CHD1Z and CHD1W sequences were the basis of these differences.

Although the PCR-HRM analysis failed as an approach for a highly sensitive, closed system for bird sexing, this project demonstrated the potential of HRM analysis for the improvement of avian molecular sexing protocols. This approach enabled the sexing (with limited success) of seven bird species with significant variability in amplicons. However, the HRM analysis for sex identification of Lovebirds was accomplished with a high success rate (> 95%). On the contrary, Green-cheeked Conure, Quaker parrot and Lineolated parakeet samples showed poor PCR amplification which directly affected the accuracy of the HRM technique. The uncontrollable effect of poor amplification as a result of poor DNA quality rendered the HRM approach inappropriate for high-throughput application in sexing bird species.

In conclusion, the HRM approach application in a commercial laboratory like the industry partner, Lumegen Laboratories (Pty) Ltd, would not be recommended due to the uncontrollable influence of DNA quality. Despite the labour intensiveness of the conventional PCR currently used at Lumegen Laboratories (Pty) Ltd, it is a standard protocol which has been used for sexing huge batches of samples with good results; irrespective of the sample quality. It was thus not plausible to implement the HRM

approach for commercial purpose as this technique relies on good DNA quality for high performance in distinguishing between males and females, which would then compromise the accuracy of avian DNA determination in a diagnostic setup. However, the HRM analysis demonstrated high potential as an avian molecular sexing technique on the Lovebirds. But even in the Lovebirds, low-quality DNA may produce nonspecific PCR products (seen as smears on an agarose gel), ultimately resulting in incorrect scoring of results in HRM analysis, or failed reactions. Samples that failed or were weakly amplified in the PCR phase have resulted in inconclusive or low-resolution HRM data. Therefore, for optimal results, it is recommended to apply the same DNA quality in HRM assays, as variations in DNA purity and integrity greatly influence the sensitivity and reproducibility of the assay. If DNA can be isolated and the pure DNA utilized in the EvaGreen dye PCR-HRM assay, perhaps better results can be obtained. To conclude, the development and reproducibility of this methodology for molecular sexing of different bird species will have to take into account the high sequence variability of the CHD1Z and CHD1W introns for specific species, as well as specific factors such as DNA concentration and quality, real-time instrument performance and dsDNA-binding dyes.

References

Archawaranon, M. 2004. Rapid sexing Hill Mynah Gracula religiosa by sex chromosomes. *Biotechnology*, 3(2), pp.160-164.

Arnold, K.E., Orr, K.J. and Griffiths, R. 2003. Primary sex ratios in birds: problems with molecular sex identification of undeveloped eggs. *Molecular Ecology*, 12(12), pp.3451-3458.

Balkiz, Ö., Dano, S., Barbraud, C., Tekin, S., Özesmi, U., Dündar, M. and Béchet, A. 2007. Sexing greater flamingo chicks from feather bulb DNA. *Waterbirds*, 30(3), pp.450-453.

Bermúdez-Humarán, L.G., Chávez-Zamarripa, P., Guzmán-Velasco, A., Leal-Garza, C.H. and Montes de Oca-Luna, R., 2002. Loss of restriction site DdeI, used for avian molecular sexing, in Oreophasis derbianus. *Reproduction in domestic animals*, *37*(6), pp.321-323.

Brubaker, J.L., Karouna-Renier, N.K., Chen, Y., Jenko, K., Sprague, D.T. and Henry, P.F. 2011. A non-invasive, direct real-time PCR method for sex determination in multiple avian species. *Molecular Ecology Resources*, 11(2), pp.415-417.

Boersma, P.D. and Davies, E.M. 1987. Sexing monomorphic birds by vent measurements. *The Auk*, 104(4), pp.779-783.

Cerit, H. and Avanus, K. 2007. Sex identification in avian species using DNA typing methods. *World's Poultry Science Journal*, 63(01), pp.91-100.

Chang, H.W., Cheng, C.A., Gu, D.L., Chang, C.C., Su, S.H., Wen, C.H., Chou, Y.C., Chou, T.C., Yao, C.T., Tsai, C.L. and Cheng, C.C, 2008. High-throughput avian molecular sexing by SYBR green-based real-time PCR combined with melting curve analysis. *BMC Biotechnology*, 8(1), p.12.

Chen, C.C., Liu, Y.S., Cheng, C.C., Wang, C.L., Liao, M.H., Tseng, C.N. and Chang, H.W. 2012. High-throughput sex identification by melting curve analysis in blue-breasted quail and chicken. *Theriogenology*, 77(9), pp.1951-1958.

Chou, T.C., Yao, C.T., Su, S.H., Hung, Y.C., Chen, W.S., Cheng, C.C., Tseng, C.N., Wang, H.M., Chou, Y.C., Li, S.L. and Gu, D.L., 2010. Validation of Spilornis cheela hoya TaqMan probes for potential gender identification of many Accipitridae species. *Theriogenology*, 73(3), pp.404-411.

Dawson, D.A., Darby, S., Hunter, F.M., Krupa, A.P., Jones, I.L. and Burke, T. 2001. A critique of avian CHD-based molecular sexing protocols illustrated by a Z-chromosome polymorphism detected in auklets. *Molecular Ecology Notes*, 1(3), pp.201-204.

Donohue, K.C. and Dufty, A.M. 2006. Sex determination of Red-tailed Hawks (Buteo jamaicensis calurus) using DNA analysis and morphometrics. *Journal of Field Ornithology*, 77(1), pp.74-79.

Dubeic, A. and Zagalska-Neubauer, M. 2006. Molecular techniques for sex identification in birds. *Biological Letters*, 43(1), pp.3-12.

Ellegren, H. 1996. First gene on the avian W chromosome (CHD) provides a tag for universal sexing of non-ratite birds. *Proceedings of the Royal Society of London B: Biological Sciences*, 263(1377), pp.1635-1641.

Fridolfsson, A.K. and Ellegren, H. 1999. A simple and universal method for molecular sexing of non-ratite birds. *Journal of Avian Biology*, 30(1), pp.116-121.

Griffiths, R. and Tiwari, B. 1993. The isolation of molecular genetic markers for the identification of sex. *Proceedings of the National Academy of Sciences*, 90(18), pp.8324-8326.

Griffiths, R., Double, M.C., Orr, K. and Dawson, R.J. 1998. A DNA test to sex most birds. *Molecular Ecology*, 7(8), pp.1071-1075.

Griffiths, R. and Orr, K. 1999. The use of amplified fragment length polymorphism (AFLP) in the isolation of sex-specific markers. *Molecular Ecology*, 8(4), pp.671-674.

Huang, H.W., Su, Y.F., Yao, C.T., Hung, Y.C., Chen, C.C., Cheng, C.C., Li, S.S.L. and Chang, H.W. 2011. High-throughput gender identification of three Columbidae species using melting curve analysis. *Theriogenology*, 75(1), pp.73-79.

Jensen, T., Pernasetti, F.M. and 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 Biology*, 22(6), pp.561-571.

Jodice, P.G.R., Lanctot, R.B., Gill, V.A., Roby, D.D. and Hatch, S.A. 2000. Sexing adult black-legged kittiwakes by DNA, behavior, and morphology. *Waterbirds*, pp.405-415.

Jones, K.L., Glenn, T.C., Lacy, R.C., Pierce, J.R., Unruh, N., Mirande, C.M. and Chavez-Ramirez, F. 2002. Refining the Whooping Crane Studbook by Incorporating Microsatellite DNA and Leg-Banding Analyses. *Conservation Biology*, 16(3), pp.789-799.

Kahn, N.W., St. John, J. and Quinn, T.W. 1998. Chromosome-specific intron size differences in the avian CHD gene provide an efficient method for sex identification in birds. *The Auk*, 115(4), pp.1074-1078.

Montgomery, J.L., Sanford, L.N. and Wittwer, C.T. 2010. High-resolution DNA melting analysis in clinical research and diagnostics. *Expert Review of Molecular Diagnostics*, 10(2), pp.219-240.

Morinha, F., Magalhães, P., Ferro, A., Guedes-Pinto, H., Rodrigues, R. and Bastos, E. 2011. Advances in molecular sexing of birds: a high-resolution melting-curve analysis based on CHD1 gene applied to Coturnix spp. In *Annales Zoologici Fennici* (Vol. 48, No. 6, pp. 371-375). Finnish Zoological and Botanical Publishing.

Morinha, F., Cabral, J.A. and Bastos, E. 2012. Molecular sexing of birds: A comparative review of polymerase chain reaction (PCR)-based methods. *Theriogenology*, 78(4), pp.703-714.

Morinha, F., Carvalho, M., Ferro, A., Guedes-Pinto, H., Rodrigues, R. and Bastos, E. 2013. Molecular sexing and analysis of CHD1-Z and CHD1-W sequence variations in wild common quail (Coturnix c. coturnix) and domesticated Japanese quail (Coturnix c. japonica). *Journal of Genetics*, 92(2), pp.39-43.

Olah, G., Heinsohn, R.G., Brightsmith, D.J., Espinoza, J.R. and Peakall, R. 2016. Validation of non-invasive genetic tagging in two large macaw species (Ara macao and A. chloropterus) of the Peruvian Amazon. *Conservation Genetics Resources*, 8(4), pp.499-509.

Ong, A.H. and Vellayan, S. 2008. An evaluation of CHD-Specific primer sets for sex typing of birds from feathers. *Zoo Biology*, 27(1), pp.62-69.

Port, J. and Greeney, H.F. 2012. Comparison of three molecular sexing primers in a monomorphic tropical furnariid, the Spotted Barbtail (Premnoplex brunnescens). *Boletin SAO*, 21(1), pp.13-18.

Ramos, P.S., Bastos, E., Mannan, R.W. and Guedes-Pinto, H. 2009. Polymerase chain reaction-single strand conformation polymorphism applied to sex identification of Accipiter cooperii. *Molecular and cellular Probes*, 23(2), pp.115-118.

Reed, G.H., Kent, J.O. and Wittwer, C.T. 2007. High-resolution DNA melting analysis for simple and efficient molecular diagnostics. *Pharmacogenomics*, 8(6), pp.597-608.

Robertson, B.C. and Gemmell, N.J. 2006. PCR-based sexing in conservation biology: Wrong answers from an accurate methodology? *Conservation Genetics*, 7(2), pp.267-271.

Rosenthal, N.F., Ellis, H., Shioda, K., Mahoney, C., Coser, K.R. and Shioda, T. 2010. High-throughput applicable genomic sex typing of chicken by TaqMan real-time quantitative polymerase chain reaction. *Poultry Science*, 89(7), pp.1451-1456.

Ryman, N. and Laikre, L. 1991. Effects of supportive breeding on the genetically effective population size. *Conservation Biology*, 5(3), pp.325-329.

Studer-Thiersch, A. 1986. Tarsus length as an indication of sex in the flamingo genus. *International Zoo Yearbook*, 24(1), pp.240-243.

Tella, J. and Torre I. 1993. Sexual size dimorphism and determination of sex in the Chough (*Pyrrhocorax pyrrhocorax*), *Journal of Ornithology,* 134, 187-90.

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

Vucicevic, M., Stevanov-Pavlovic, M., Stevanovic, J., Bosnjak, J., Gajic, B., Aleksic, N. and Stanimirovic, Z. 2013. Sex determination in 58 bird species and evaluation of CHD gene as a universal molecular marker in bird sexing. *Zoo Biology*, 32(3), pp.269-276.

Wang, W., Chen, K. and Xu, C., 2006. DNA quantification using EvaGreen and a real-time PCR instrument. *Analytical Biochemistry*, *356*(2), pp.303-305.

Wang, N. and Zhang, Z.W. 2009. The novel primers for sex identification in the brown eared-pheasant and their application to other species. *Molecular Ecology Resources*, 9(1), pp.186-188.

Welsh, J. and McClelland, M. 1990. Fingerprinting genomes using PCR with arbitrary primers. *Nucleic acids research*, 18(24), pp.7213-7218.

Wittwer, C.T., Reed, G.H., Gundry, C.N., Vandersteen, J.G., Pryor, R.J. 2003. High-resolution genotyping by amplicon melting analysis using LC Green. *Clinical Chemistry,* 49, 853–860.