CHARACTERISING MICROSATELLITE LOCI IN THE BLUE CRANE (*Grus paradisea*)

By

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ABSTRACT

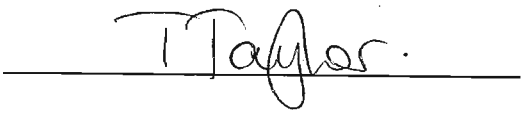
The blue crane (*Grus paradisea*) is endemic to southern Africa and has the smallest geographical range of the 15 crane species occurring world-wide. Although this species is still found throughout most of its historic range, it has experienced a significant and rapid decline in numbers over the last 20 years. One factor causing this decline is the illegal removal of chicks from the wild. Permits are required to keep, trade in and breed cranes in captivity. However, birds must be captive bred in order to obtain a permit. Therefore, chicks taken illegally from the wild are fraudulently incorporated into an existing captive population under the pretence that they offspring of a legal captive pair. This study describes the development of a set of microsatellite markers to assist the identification of illegal trade in the blue crane. These markers can ultimately be used to verify the relationship between the offspring and its claimed parents by performing parentage analyses. Forty microsatellite loci were obtained from genomic libraries previously developed in two other crane species and tested for cross-species utility in the blue crane. In addition, 42 loci were developed for this study from a blue crane species-specific genomic microsatellite library, of which 19 were tested for polymorphism in this species. The microsatellite markers characterised here were also tested for their utility in two other crane species: wattled crane (*G. carunculatus*) and grey-crowned crane (*Balearica regulorum*). One locus, *Gamu007*, was found to be sex-linked and therefore excluded from the set of markers. A total of 28 polymorphic loci were tested for the suitability in parentage analysis in the blue crane. Of these, a set of 16 loci were determined to be as suitable for this purpose. These loci were shown to be inherited in a Mendelian fashion in a single blue crane family. In addition, statistical analysis of the loci were identified as exhibiting linkage equilibrium, this was supported by their distant association on a predicted *Grus* microsatellite map based on the chicken genome. The selected loci were also identified as having a low frequency of null alleles as well as a total first and second parent exclusion power of 0.9999 and 1.0000, respectively. These loci provide a valuable tool for parentage testing in blue crane, and may also be valuable in population genetic studies to assist conservation strategies. In addition, this set may be used to assist legal cases involving the illegal trade in blue cranes upon completion of additional microsatellite marker validation procedures. Twenty-seven loci were polymorphic in the wattled and grey-crowned crane. These could provide a valuable source of microsatellite loci in these species, and could potentially eliminate the need for the development of a species-specific microsatellite library.

PREFACE

The results presented in this thesis follow from a study that was carried out at the School of Biological and Conservation Science, University of KwaZulu-Natal, Pietermaritzburg in collaboration with the Sheffield Molecular Genetics Facility, Department of Animal and Plant Sciences, University of Sheffield, United Kingdom under the supervision of Dr Tiawanna Taylor, Prof. Mike Perrin and Prof. Terry Burke.

This study represents original work by the author and has not otherwise been submitted in any form for any degree or diploma to any University, Where use has been made of the work of others it is duly acknowledged in the text.

Kathleen Frances Meares (candidate)



Dr Tiawanna A. Taylor (supervisor)

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This project would not have been possible without the guidance, advice and support of my supervisors at the University of KwaZulu-Natal, Dr Tee Taylor and Prof. Mike Perrin. Thanks also to my supervisor at the University of Sheffield, Prof. Terry A. Burke, as well Dr Debs Dawson for many hours of project discussions and problem solving.

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This dissertation is dedicated to my parents.

Kate Meares 2006

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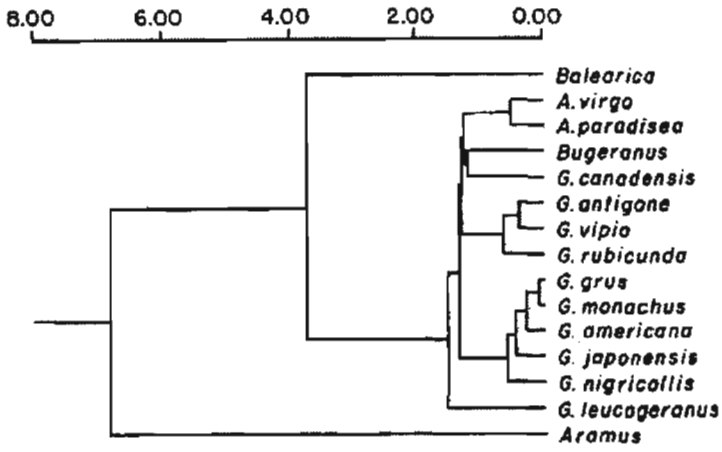
CHAPTER 1 INTRODUCTION

1.1 THE GRUIDAE

Species that fall under the taxonomic family Gruidae belong to one of the most ancient families of birds, with fossil records dating back more than 50 million years (Meine *et al.* 1997). Cranes often serve as flagship species in conserving wetlands and grasslands around the world (Meine *et al.* 1996a), and are an important measure of the health status of their habitats that are occupied by many other ecologically and economically important organisms. However, cranes are among the world's most threatened groups of birds and eleven of the fifteen species are globally threatened (Meine *et al.* 1996a). Anthropogenic forces such as habitat destruction, poisoning and disturbance are mostly causing the noted decline in global population numbers for many of the 15 species of crane (Meine *et al.* 1997).

The phylogenetic structuring of this family, although still mostly unresolved (Krajewski *et al.* 1994), shows the presence of two subfamilies: the Balearicinae (crowned cranes) comprising two species, and Gruinae comprising the remaining 13 species (Krajewski 1989). Although independently derived phylogenies agree with the placement of Balearicinae in a separate subfamily to Gruinae (Krajewski 1989; Sibley *et al.* 1990), the structure of the genera within the Gruinae subfamily remains unresolved. Two phylogenies created for this species using DNA-DNA hybridisation methods are provided below (Figure 1.1).



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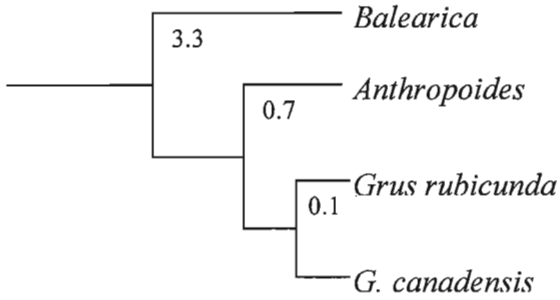

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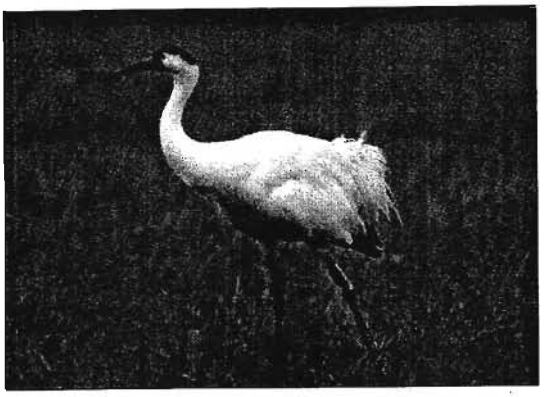
Figure 1.1 Phylogenies of the family Gruidea constructed using DNA-DNA hybridisation distances. Phylogeny (a) from Krajewski *et al.* (1989) and phylogeny (b) was adapted from Sibley *et al.* (1990).

These phylogenies aim to provide a graphical representation of the relationship between the crane species mentioned in this study. In addition to the blue crane (*G. paradisea* also known as *Anthropoides paradisea*) whose general biology shall be discussed in more detail (section 1.2), other species included in this study (Figure 1.2) are: the whooping crane (*G. americana*), occurring in North America; red-crowned crane (*G. japonensis*), occurring in East Asia; the wattled crane (*G. carunculatus*), occurring in Ethiopia, south-central and southern Africa; and grey-crowned crane (*Balearica regulorum*) occurring in east-central and southern Africa (Meine *et al.* 1996a). Of these, the grey-crowned crane and wattled are listed on the 2006 IUCN Red List as lower risk and vulnerable, respectively (IUCN 2006); the red-crowned crane and whooping crane are listed as endangered (IUCN 2006).



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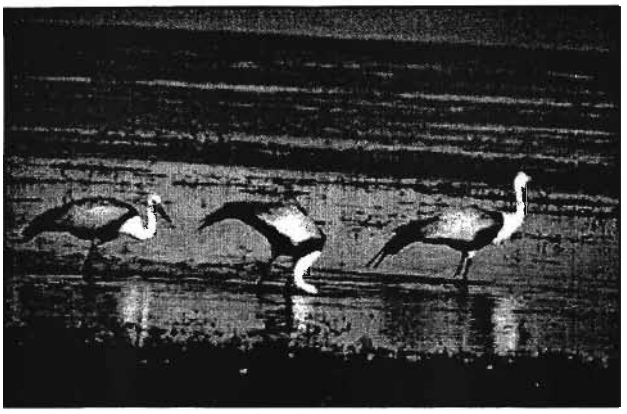

(e)

Figure 1.2. The five crane species that are mentioned in this study. (a) whooping crane (*Grus americana*); (b) red-crowned crane (*G. japonensis*); (c) blue crane (*G. paradisea*); (d) grey-crowned crane (*Balearica regulorum*); (e) wattled crane (*G. carunculatus*). Photos are from International Crane Foundation.

1.2 STUDY SPECIES

The blue crane is one of three species occurring in South Africa, and one of the 15 species occurring worldwide. This species is highly gregarious when not breeding, and can congregate in gatherings of up to one thousand birds (Allan 2001), but when breeding they separate in pairs or family groups (Maclean 1985). Blue cranes breed from October to February, the nest comprising a scrape on the ground thinly lined with plant material and typical clutch size of two eggs (Maclean 1985). Although they are ranked as one of the most conspicuous birds when breeding (Allan 2001), their alert nature decreases their vulnerability to predation.

1.2.1 Nomenclature

Whether the blue crane falls within the genus *Grus* or *Anthropoides* is an issue that has not yet been resolved among nomenclature authorities. Studies using DNA-DNA hybridisation and cytochrome-*b* analyses suggest this species should fall within the genus *Anthropoides,* together with the demoiselle crane (*A. virgo*) (Krajewski 1989; Krajewski *et al.* 1994; Sibley *et al.* 1990). This genus name has been adopted by the South African Crane Working Group (SACWG 2003), 'Robert's Birds of southern Africa' (Maclean 1985), and the International Crane Foundation (International Crane Foundation 2006). However, this species is known more commonly as *G. paradisea* by organisations such as CITES, the World Conservation Union (IUCN) Red List of Threatened Species (IUCN 2006) and the European Molecular Biology Laboratories - European Bioinformatics Institute (EMBL-EBI). Since this study involved the development of blue crane DNA sequences, which were submitted to EMBL, they were done so under the species name recognised by this database (*G. paradisea*). All subsequent references to this species in this thesis are done under this species name to provide consistency.

1.2.2 Distribution

The blue crane is endemic to southern Africa with most of its range occurring within South Africa (Harrison *et al.* 1997), but also occurring in an isolated breeding population in

Namibia centred on the Etosha Pan (Figure 1.3). In South Africa, its range comprises three regions regarded as strongholds within South Africa: Overberg/Swartland, Karoo and Eastern (Figure 1.3). The separation of the range into three strongholds within South Africa are the result of field observations where flocks have been observed to remain within each stronghold region areas (McCann *et al.* 2002). Microsatellite loci characterised in this species could be used to better understand the structure of the southern African blue crane population, and whether the three population strongholds identified by McCann *et al* (2002) are subpopulations.

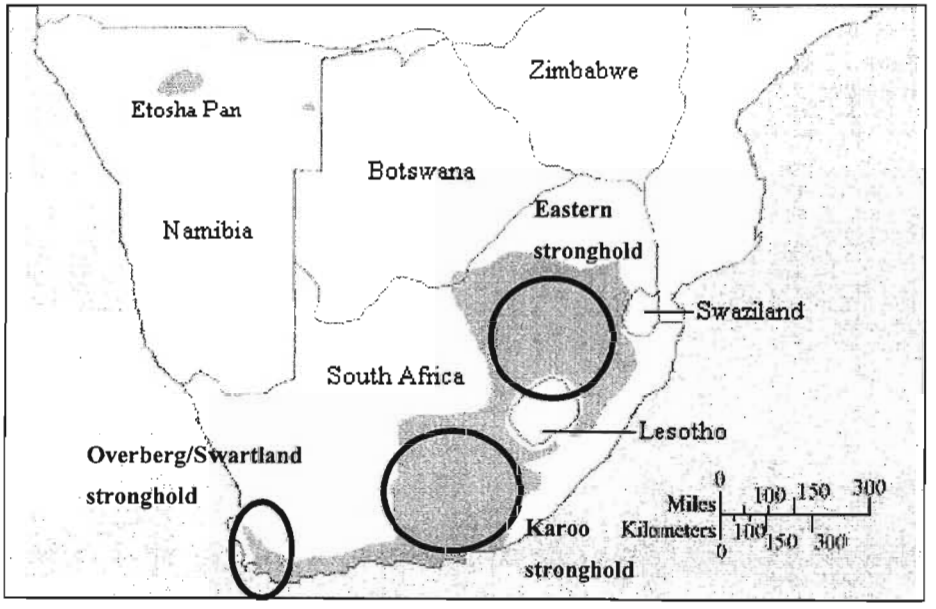

Figure 1.3 Map of southern Africa showing the distribution (shaded) of the blue crane (*Grus paradisea*). Picture courtesy of the International Crane Foundation. Population 'strongholds' are circled (from McCann *et al.* 2002).

Cranes are predominantly wetland birds (Allan 2001) but the blue crane is the least wetland-dependent of the three southern African crane species (McCann *et al.* 2002). Blue cranes prefer dry short grasslands (Maclean 1985) and are also known to feed and breed in cultivated habitats such as cereal crops and small livestock farms (SACWG 2000) where their main source of food is fallen grain (Allan 1995). For example, this species has become highly adapted to using artificial agricultural wheat-lands in the Western Cape province for both feeding and breeding grounds (McCann *et al.* 2002). Interestingly, more than 50 % of

South Africa's blue crane population inhabits transformed agricultural lands (McCann *et al.* 2002).

1.2.3 Status

Prior to 1980, there was little concern about the blue crane from a conservation standpoint. However a sudden and drastic decline in population numbers post 1980 lead to the blue crane having the most restricted distribution of the world's fifteen crane species (Meine *et al.* 1996b). This species has largely disappeared from the Transkei, Lesotho and Swaziland regions (Harrison *et al.* 1997). In addition, the Cape Province, KwaZulu-Natal and Gauteng Province flocks were estimated to have declined by 90% (Meine *et al.* 1996b). In recognition of this decline, this species was re-categorised from 'lower risk' in 1988 to 'vulnerable' in 1994 on the IUCN Red List of Endangered Species (IUCN 2006). Consequently, the South African Crane Working Group (SACWG) was formed in 1995 to address action plans to conserve this species. The most recent estimate of the South African blue crane population size is 20 000 - 21 000 individuals (McCann *et al.* 2002).

1.2.4 Anthropogenic threats

Habitat alteration

Blue cranes feed and breed predominantly in grassland (Maclean 1985) and cultivated habitats such as cereal-crop fields (SACWG 2000). Although grasslands were originally the primary stronghold of the blue crane, in the last 20 years more than 90% of the grassland population has disappeared (Allan 2001) with the main cause of the disappearance of this habitat being attributed to afforestation (conversion of open land to forested areas by planting trees or seeds). Before the year 2000, approximately 1.5 million hectares of grassland had been afforested in South Africa with *Eucalyptus* and pine plantations being the main cause (SACWG 2000). Consequently, these forestry-related agricultural practices put pressure on grasslands and the feeding and breeding grounds of the blue crane small shifted toward fairly shallow wetlands with short vegetation (McCann *et al.* 2002). In addition, blue cranes were seen to adapt to agricultural farmlands which are unstable in

nature due to frequent changes in land-uses in agriculture. Moreover, farmer-crane conflict either intentional or unintentional may lead to an increase in crane mortality through for example poisoning (see below). Therefore, although favourable conditions for this species on agricultural lands currently exist, economics and human pressures may drive the agriculture habitat to be unsuitable for blue cranes. Ultimately, future predictions of blue crane population sizes may be less stable than the figures currently show (McCann *et al.* 2002).

Poisoning

Poisoning and habitat alterations are the two key factors driving the decline of the South African blue crane population (SACWG 2000). Because cranes have been known to feed on and trample recently planted seedlings, poisonous substances are sometimes placed by the farmer or farm worker in an area where cranes are likely to ingest the chemicals, thereby directly targeting this species (Hudson 2002). In contrast, unintentional poisoning arises when pests such as insects or weeds are the target, but secondary poisoning might arise via the food chain when cranes accumulate these poisons within their system. Since afforestation has resulted in cranes moving on to agricultural lands, particularly cereal crop lands (Allan 2001), the number of incidents of direct and indirect crane poisonings might be expected to rise, and if so cause further declines in the blue crane population.

Illegal trade in cranes: removal of chicks from the wild

The blue crane is listed under CITES Appendix II (CITES 2006) under which species are defined as having a low current threat of extinction but could become threatened unless their trade is strictly regulated (Hemley 1994). The National Guideline for Trade and Keeping Cranes in Captivity developed by SACWG (SACWG 2003) recommends, among other requirements, that only organisations, facilities and individuals with the necessary permits, and who can prove that the cranes are captive bred, should be permitted to keep or trade in or obtain cranes. Despite these recommendations being in place, the removal of chicks from the wild has been identified as being a particular problem in the Eastern Cape and KwaZulu-Natal provinces where chicks are used as a food source, for medicinal

purposes and for cultural practices (McCann *et al.* 2002). In addition, chicks are removed from the wild to sell to bird breeders or to be kept as pets by private collectors (SACWG 2000). Consequently, the demand for captive birds by breeders and private collectors provided a market for the illegal removal from the wild and trading in birds. CapeNature (CapeNature 2003) have received reports of farmers and farm workers being offered money to illegally catch and sell crane chicks. It was further suggested that legally permitted cranes might be used either as a front for the movement of illegal birds or under the pretence that young birds removed from the wild are the offspring of a legal captive pair (SACWG 2003). Verifying information on the permits obtained by an organisation, facility or individual for the purpose of keeping, trading or obtaining cranes using genetic markers could identify illegal activity, thereby assisting the detection of the illegal trade in the blue crane.

In 2003, Cape Nature Conservation's Environmental Crime Investigations Unit (ECIS) won a court case where a bird breeder in the Western Cape Province, South Africa, was found to be guilty of illegally keeping a blue crane in captivity (CapeNature 2003). The court sentenced a R9000 fine to the breeder, or one year imprisonment, which was suspended for four years (CapeNature 2003).

The estimate, reported in 2002, of the number of blue cranes being kept in captivity without permits is approximately 2000 birds (Morrison 2002). However, SACWG has little actual data on the status of the illegal removal of chicks from the wild (Morrison 2002). A factor confounding the collections of such data is that parties involved in the illegal trade will act under the strictest codes of anonymity since their actions, if caught, could carry heavy penalties. Without real data however, it becomes difficult to monitor and control the illegal trade. Addressing these problems requires adequate enforcement of wildlife trade laws (section 1.3) to reduce the threats posed by the illegal trade on blue crane survival in the long term. This has been supported by its listing in CITES Appendix II.

1.3 ILLEGAL TRADE IN WILDLIFE

1.3.1 Its financial worth on an international scale

The legal trade in wildlife (animals, plants and by-products) has an annual estimated value of US$160 billion based on declared import values (Broad *et al.* 2003). The annual illegal trade in wildlife however has been estimated to be worth US$9.5-US$16 billion (Ministry of Agriculture and Forestry 2003). An earlier estimate was US$5-US$8 (UNEP 1998). Currently however, it has been realised that any attempt at estimating the value of this trade is unreliable (Broad *et al.* 2003; TRAFFIC 2006b). This is because so much of the illegal trade occurs without a trace and therefore, there is no way of obtaining estimates of figures such as costs involved, which species, and the current main routes taken for trading. However, there is consensus that the illegal trade in wildlife is worth billions annually (TRAFFIC 2006a).

1.3.2 CITES

Internationally, the convention at the forefront of controlling the trade in wildlife is the Convention on International Trade in Endangered Species of Fauna and Flora (CITES). A Secretariat (a board of CITES directors based in Geneva, Switzerland, CITES 2001) forms the CITES headquarters where a range of administrative tasks are performed. Although the Secretariat monitor implementation of CITES, they are not directly involved in implementing the convention in the signatory countries. Instead each member state is responsible for implementing and enforcing CITES (Hemley 1994) by delegating the responsibilities (administering and issuing permits) to a government department and one Scientific Authority (Burgener *et al.* 2001).

At the core of the convention are three Appendices that list species affected by the trade in wildlife. Each Appendix is differentiated by the level of threat the species is under as a result of trade. The trade in listed species is regulated by the countries that have ratified the convention. Species listed on Appendix I are those threatened with extinction and are, or may be, affected by international trade (Hemley 1994). Trade in these species is generally

banned, unless the trade is for scientific and conservation purposes and will not affect the species chance for survival (Hemley 1994). Appendix II species are not yet threatened with extinction by the trade, but may become so if the trade is not controlled (Hemley 1994). And Appendix III provides each party signed to the convention with the ability to list species already protected within their own borders thereby allowing each country to enforce its own trade regulations through cooperation with other countries (Hemley 1994). The implementation and enforcement of CITES is performed by each member state, each country has its own set of offences and penalties regarding the illegal trade in species under the above-mentioned CITES appendices that take place through their borders. The laws implemented in South Africa (section 1.3.3) therefore have direct consequences for CITES-listed species, such as the blue crane, entering and leaving its borders.

1.3.3 Control of the wildlife trade in South Africa

South Africa signed as a party to CITES in 1975 (Burgener *et al.* 2001) providing a legal framework to ensure its international trade does not threaten the survival of those species listed on the CITES appendices. CITES requires the appointment of a scientific and management authority at a national level for each party signed to the convention (Hemley 1994). In South Africa, these positions are both held by the Department of Environmental Affairs and Tourism (CITES 2005). These two authorities communicate with the CITES Secretariat to ensure the correct co-ordination of all CITES processes (e.g. issuing of import/export permits) are carried out effectively within the country at a national level. In addition to national authorities, each province within South Africa has a management authority competent to grant permits (CITES 2005). Assistance for the management authorities is provided by enforcement agencies such as the South African Police Services (SAPS) thereby ensuring compliance. The SAPS can open legal cases where individuals are suspected of violating the CITES rules governing the trade in wildlife through South African border and customs posts, both international and provincial.

The legislation used by the authorities to enforce wildlife trade rules in South Africa is The National Environmental Management Biodiversity Act (Act 10 of 2004). Its aim is: "To provide for the management and conservation of South Africa's biodiversity within the

framework of the National Environmental Management Act, 1998; the protection of species and ecosystems that warrant national protection; the sustainable use of indigenous biological resources; the fair and equitable sharing of benefits arising from bioprospecting involving indigenous biological resources; the establishment and functions of a South African National Biodiversity Institute; and for matters connected therewith" (DEAT 2004). This Act provides, among others, a description of the types of trade offences and suggested penalties which allow for the correct procedures to be carried out by all involved in the legal chain (enforcement agencies as well as management and scientific authorities). The restricted activities (Figure 1.4 & 1.5), offences (Figure 1.6) and penalties (Figure 1.7) as stated in the Biodiversity Act (DEAT 2004), are provided below. Since the trade in cranes is restricted by the issuing of permits, these sections have direct influences on the identification of the illegal trade in the blue crane.

Enforcing trade laws, with particular reference to the blue crane

At the enforcement agency level, those organisations and facilities involved in detecting wildlife crime, such as independent and accredited forensic laboratories, can be called upon to analyse evidence. It is here where molecular markers (section 1.4) have been identified as providing usable evidence in legal cases involving the illegal wildlife trade (DEFRA 2005). In blue cranes, illegal trade generally takes the form that young birds, taken illegally from the wild are being incorporated into existing South African captive populations under the fraudulent pretence that they are offspring of legal captive pairs (section 1.2.4). However, a permit is required to possess threatened species (DEAT 2004) such as the blue crane. But the chick must be captive bred in order to obtain a permit. Therefore, during the application of a permit, the chick is fraudulently claimed to be bred in captivity, thereby committing an offence as stated in section 101(3c) of the Biodiversity Act (DEAT 2004). A DNA parentage test would determine the relatedness of the chick to its claimed parents. Incriminating evidence would be parentage results that identify the adult individuals as being unrelated to the chick. The DNA parentage test however would not be able to identify the chick as being wild-caught, but that the claimed parents of the chick are not its parents, suggesting that the chick was either wild-caught or obtained through illegal sources

either from another existing captive population. Ultimately, this method of detecting illegal trade could provide the evidence required for use when enforcing existing trade laws.

(i) **hunting, catching, capturing or killing any living specimen of a listed threatened or protected species by any means**, method or device whatsoever, including searching, pursuing, driving, lying in wait, luring, alluring, discharging a missile or injuring with intent to hunt, catch, capture or kill any such specimen;

(ii) **gathering, collecting or plucking any specimen of a listed threatened or protected species;**

(iii) picking parts of, or cutting, chopping off, uprooting, damaging or destroying, any specimen of a listed threatened or protected species;

(iv) importing into the Republic, including introducing from the sea, any specimen of a listed threatened or protected species;

(v) **exporting from the Republic, including re-exporting from the Republic, any specimen of a listed threatened or protected species;**

(vi) having in possession or exercising physical control over any specimen of a listed threatened or protected species;

(vii) growing, breeding or in any other way propagating any specimen of a listed threatened or protected species, or causing it to multiply;

(viii) conveying, moving or otherwise translocating any specimen of a listed threatened or protected species;

(ix) **selling or otherwise trading in, buying, receiving, giving, donating or accepting as a gift, or in any way acquiring or disposing of any specimen of a listed threatened or protected species; or**

(x) any other prescribed activity which involves a specimen of a listed threatened or protected species;

Figure 1.4 Defining restricted activities in relation to a specimen of a listed threatened or protected species (DEAT 2004). Those restricted activities with particular reference to the blue crane are provided in bold.

(1) A person may not carry out a restricted activity involving a specimen of a listed threatened or protected species without a permit issued. This does not apply in respect of a specimen of a listed threatened or protected species conveyed from outside the Republic in transit through the Republic to a destination outside the Republic, provided that such transit through the Republic takes place under the control of an environmental management inspector.

(2) The Minister (the Cabinet member responsible for national environmental Management) may prohibit the carrying out of any activity *(a)* which is of a nature that may negatively impact on the survival of a listed threatened or protected species; and *(b)* which is specified in the notice, or prohibit the carrying out of such activity without a permit issued.

Figure 1.5 Restricted activities involving listed threatened or protected species (section 57 of the Biodiversity Act, DEAT 2004).

(1) A person is guilty of an offence if that person contravenes or fails to comply with a provision of—

(a) section 57(1);

(b) a notice published in terms of section 57(2);

(2) A person who is the holder of a permit is guilty of an offence if that person—

(a) contravenes or fails to comply with a provision of section 69(1) or 73(1);

(b) performs the activity for which the permit was issued otherwise than in accordance with any conditions subject to which the permit was issued; or

(c) permits or allows any other person to do, or to omit to do, anything which is an offence in terms of paragraph (a) or (b).

(3) A person is guilty of an offence if that person—

(a) fraudulently alters any permit;

(b) fabricates or forges any document for the purpose of passing it as a permit;

(c) passes, uses, alters or has in his or her possession any altered or false document purporting to be a permit; or

(d) knowingly makes any false statement or report for the purpose of obtaining a permit.

Figure 1.6 Offences that can be committed in terms of wildlife laws (section 101 of the Biodiversity Act, DEAT 2004).

(1) A person convicted of an offence in terms of section 101 is liable to a fine, or to imprisonment for a period not exceeding five years, or to both fine and such imprisonment.

(2) A fine in terms of subsection (1) may not exceed—
(a) an amount prescribed in terms of the Adjustment of Fines Act, 1991 (Act No. 101 of 1991); or
(b) if a person is convicted of an offence involving a specimen of a listed threatened or protected species, an amount determined in terms of paragraph (a) or which is equal to three times the commercial value of the specimen in respect of which the offence was committed, whichever is the greater.

Figure 1.7 Penalties awarded upon admission of guilt (section 102 of the Biodiversity Act, DEAT 2004)

1.4 MOLECULAR MARKERS

In the past decade, genetic approaches to answering ecological questions have become more efficient, powerful and flexible, and thus more widespread (Selkoe *et al.* 2006). Two of the most widely used molecular markers for studying population biology are minisatellites and microsatellites (Jones *et al.* 2003). Other markers have been used but without the same amount of success due to a number of disadvantageous attributes regarding their ease of use, level of variability and robustness in analysis. These markers include among others random amplified polymorphic DNA (RAPDs), amplified fragment length polymorphism (AFLPs), restriction fragment length polymorphism (RFLP), and single-nucleotide polymorphisms (SNPs). Although these markers have important applications in some areas of molecular genetics for example quantitative trait loci mapping (AFLPs and SNPs, Mueller *et al.* 1999; Vignal *et al.* 2002), and cultivar identification of micro-propagating plants (RFLPs, Congiu *et al.* 2000) their application toward parentage testing and population analyses is limited (reviewed in Parker *et al.* 1998; Sunnucks 2000). But because these analyses are frequently required to identify cases of illegal trade, only minisatellite and microsatellite markers which are capable of providing sound evidence in these areas are discussed here. Moreover, because the primary objective of this study is the development of a set of markers for parentage assignment to assist detecting illegal trade in the blue crane, the functionality of the markers discussed shall be in the context of parentage analysis.

1.4.1 Minisatellites

The term 'DNA fingerprinting' was coined in 1985 after the discovery that a core sequence used as a probe can detect many highly variable loci that differ between individuals (Jeffreys *et al.* 1985a). This has provided a tool with which one can distinguish human individuals by their DNA (Jeffreys *et al.* 1985a). The core sequences, known as minisatellites, comprise several hundred repeat units in tandem, each repeat unit being 15-60 bp long (Bruford *et al.* 1998). Briefly, the 'fingerprints', also known as DNA profiles, are created by cleaving the genomic DNA using a restriction enzyme. The DNA fragments created by the restriction process are then run an agarose gel, which separates the DNA fragments according to size. The minisatellite probes then hybridise to the complimentary DNA sites (Parker *et al.* 1998) and the final product is a banding pattern where each band represents a DNA fragment containing copies of the minisatellite repeat sequence. The number of repeats found at each site varies such that the DNA fragments differ in length between individuals. When sufficient sites are examined, profiles created using minisatellites are therefore individual-specific (Jeffreys *et al.* 1985; Jeffreys *et al.* 1985a), unless the DNA is from clonal organisms in which case the profiles would be identical. The numerous loci of which each profile is comprised are inherited in a Mendelian fashion such that each allele of an offspring being inherited from one of the parents (Parker *et al.* 1998). Therefore, their use in human forensics as a tool to test maternity and paternity in parentage cases was recognised (Jeffreys *et al.* 1985a).

Advantages

In birds, minisatellites were shown to hold high variability between individuals (Burke *et al.* 1987). This provided the tool with which to study the relatedness of individuals within and between populations (Wetton *et al.* 1987) and breeding systems (Birkhead *et al.* 1990; Burke *et al.* 1989) for example to identify parentage in the presence of extra-pair copulations within wild populations (Huyvaert *et al.* 2000; Pilastro *et al.* 2001). This technique provided the opportunity to answer a wide range of biological questions, many of which have been focussed on Passerines due to their ease of study in the wild. In addition to the high variability of minisatellites, standard minisatellite markers can be used

successfully with a wide range of taxa. For example, Jeffrey's multilocus minisatellite probes were shown to produce variable DNA profiles in birds (Burke 1989), mammals (Hoelzel *et al.* 1991), reptiles (Gullberg *et al.* 1999) and fish (Dahle 1994).

Disadvantages

The main disadvantage of minisatellite markers is that they require a large amount of clean, high molecular-weight DNA for analysis (Bruford *et al.* 1998). Large amounts of DNA are a pre-requisite for the development of minisatellite DNA profiles. Jeffreys *et al.* (1985) used 8 ug of DNA to create the first human DNA minisatellite profiles, Longmire *et al.* (1992) used 5 ug of DNA to create whooping crane (*G. americana*) DNA profiles, and similarly, Burke *et al.* (1987) used 5 ug of DNA to create minisatellite profiles in a wide range of avian species. Another pre-requisite is the extraction of high molecular DNA for use in creating minisatellite DNA profiles. The genomic DNA, once cleaved with restriction enzymes, comprise DNA fragments of a high molecular weight (3-12 kb, Parker *et al.* 1998) which are used to create individual-specific DNA profiles. A degraded DNA sample would therefore already be fragmented before being restricted with DNA restriction enzymes, causing erroneous DNA profiles to be development. Therefore minisatellite markers would be unsuitable in cases where only small quantities or degraded DNA are available. Small or degraded samples are often all that is available in many wildlife forensic cases.

A second disadvantage of minisatellites, specifically multi-locus minisatellite probes such as Jeffrey *et al.'s* (Jeffreys *et al.* 1985), is that the probe used to detect the variable regions is repeated at numerous sites within the genome, therefore the comparisons of allelic products between individuals at one locus site is difficult (Parker *et al.* 1998). Furthermore, the minisatellite loci that are detected show levels of variation too great to yield meaningful results when comparing different populations (Bruford *et al.* 1998). For this reason minisatellites are used mostly for individual identification as well as assessing the relationships of individuals within the same population.

1.4.2 Microsatellites

Microsatellites are also known as simple sequence repeats (SSRs), variable number tandem repeats (VNTRs) and short tandem repeats (STRs) (Selkoe *et al.* 2006). They are comprised of short, tandemly-repeated units 1-6 bases in length, and are found in all prokaryotic and eukaryotic genomes analysed to date (Zane *et al.* 2002). Typically, tandem repeats comprise 5-40 units (Selkoe *et al.* 2006), and different sequences of each microsatellite locus (called alleles) are subject to much sequence-length variation (polymorphism). This variation in the number of tandem repeats at a locus is caused by slippage and proof-reading errors during DNA replication, the rate of which is directly proportional to the number of repeats (Ellegren 2004). These mutations in repeat regions do not have a uniform rate and rates differ among loci and among alleles (Ellegren 2004).

Sequences that occur on either side of each microsatellite are termed the flanking region. These regions allow for the design of short (generally in the range of 20 bp) oligonucleotides primers which can anneal on either side of the microsatellite region and amplify the sequence between them using the Polymerase Chain Reaction (PCR). Because alleles at polymorphic loci differ in size, the alleles can be separated by size and visualised electronically using DNA analysers (Ziegle *et al.* 1992). Such techniques are capable of detecting allele-size variation down to a single base pair difference. This process of identifying the allelic composition of each individual at one or more loci is also known as genotyping, and the outcome is a score of the two allele sizes representing the individual's genotype for that locus. When the genotypes from more than one locus are combined, the result is a DNA profile. Like minisatellites, the DNA profiles are individual-specific (unless compared between clonal organisms). Unlike multi-locus minisatellite markers however, the alleles from each locus can be analysed separately. This enables the segregation of alleles between individuals in a family, as well as within and between populations. Ultimately, it allows for a wide range of biological and ecological questions to be addressed. Microsatellite markers have enabled the study of population bottle-necks in amphibian (Jehle *et al.* 2002), avian (Glenn 1997), and mammalian (Ramey *et al.* 2000) populations. In addition, it has allowed for the detection of the level of gene flow among

populations (Pierson *et al.* 2000) and the dynamics of family groups and their helpers in cooperatively breeding birds (Richardson *et al.* 2001).

Advantages of microsatellites over other markers

Microsatellite loci, like minisatellites, are hypervariable and reproducible. Their variability allows for the development of individual-specific profiles, and their reproducibility enable the profiles to be created under the same PCR and other conditions. Microsatellites have discrete loci (Schlötterer 1998) which allow for easy characterisation of the different alleles found within polymorphic loci unlike multi-locus minisatellites where it is not possible to identify loci (Parker *et al.* 1998). In addition, microsatellites are co-dominant (expression of both alleles) (Schlötterer 1998), allowing for the ability to distinguish individuals of both homozygous states and the heterozygous state. This is unlike dominant markers such as RAPDs (Mueller *et al.* 1999). Furthermore, most microsatellite loci are inherited in a Mendelian fashion (Selkoe *et al.* 2006), facilitating their use in relationship studies such as parentage.

Adding to their success in approaching many biology-related questions, microsatellite markers can be used to create DNA profiles from highly degraded DNA samples. This is due to the use of PCR which requires a small amount of template DNA from which target regions are amplified. This is unlike minisatellite markers that require a large amount of high-molecular weight DNA. Microsatellite markers have been shown to be successful in amplifying products such as those extracted from foot pad samples taken from the museum specimens of endangered loggerhead shrikes *Lanius ludovicianus mearnsi* (Mundy *et al.* 1997) as well as Eurasian badger *Meles meles* faecal samples (Frantz *et al.* 2003). One factor contributing to this success is the relatively short length of microsatellite loci (most often 100-300 bp, Selkoe *et al.* 2006) compared to minisatellite loci (2-20 kbp, Burke *et al.* 1987). This allows for a higher chance of unfragmented microsatellite loci, existing within degraded DNA samples that can be amplified.

Disadvantages

Despite the confirmation that microsatellites are the marker of choice in population genetic studies and related areas (Ellegren 2004), their use comes with a number of identified drawbacks. One disadvantage is the variability in the flanking region sequences between and within species at a particular locus. This variability arises because microsatellites are present in non-coding regions of the genome where nucleotide substitution rates are higher than in coding regions (Zane *et al.* 2002). If this variability occurs in the region where the primers were designed, then the ability of the primer to anneal to these sites in other species is greatly reduced. This chance decreases with an increase in genetic distance between the species in which the primers were originally designed, and the species in which the primers are being tested for cross-species amplification (Primmer *et al.* 2005). Consequently, microsatellite markers often need to be developed *de novo,* since the availability of near-universal microsatellite markers is still low (Galbusera *et al.* 2000). Furthermore, any variation in the primer binding site could result in non-amplification of the allele (Hoffman *et al.* 2005). This is known as a null allele.

Similarly, a DNA sample that is highly degraded might provide difficulty during amplification, and could result in the non-amplification of target regions (Sefc *et al.* 2003), a phenomenon known as 'allelic drop-out' (Hoffman *et al.* 2005). Both allelic drop-out and null alleles can result in true heterozygotes appearing as homozygotes, and a true homozygote appearing as a failed PCR run. True heterozygotes may appear as a homozygote due the presence of an amplified allele of one size, and the non-amplification of the other allele. For true homozygotes, a failed PCR run is observed due the non-amplification of both alleles. These outcomes would have negative implications when performing parentage analysis since an offspring might then appear to be incompatible with one or both of its true parents. In order to check for the presence of null alleles and allelic drop-outs, tests can be performed on a genotypic dataset using software such as CERVUS (Marshall *et al.* 1998). They compare observed with expected heterozygosity estimates. Loci identified as having significantly lower than expected heterozygotes are ear-marked as being likely null-allele candidates (Marshall *et al.* 1998). In addition, repeated genotyping,

or using different samples types (tissue versus blood) from the same individual can potentially identify cases of allelic drop-out.

Although variability in the flanking region can cause problems associated with genotyping, variability in the microsatellite repeat composition can cause errors when scoring alleles. Microsatellite repeats can occur in different forms. They can either be 'simple' containing a single repeat type such as 'GGAGGAGGA', 'compound' containing more than one repeat type such as 'GGAGGACTCTCT', or 'interrupted' where the repeat units are separated by a non-variable sequence such as 'GGAGGATGGTCACTCTCT'. A repeat type that differs in repeat composition at a locus can in some cases amplify a fragment of the same allele size between individuals, a phenomenon known as 'homoplasy' (Selkoe *et al.* 2006). Although identical in state, homoplasious alleles are not identical by descent. For example, a compound repeat such as 'GGAGGACTCTCTCTCT' has a repeat unit length of 16 bp. However a different 16 bp combination at the same locus might arise: 'GGAGGAGGAGGACTCT'. Sequencing alleles to detect cases of homoplasy could be used when analysing compound or interrupted repeats, however, undertaking this on a population basis would be time consuming and unpractical. To reduce the risk of homoplasy causing mis-assignment in parentage, compound repeats known to experience homoplasy should be avoided or appropriate precautions, such as sequencing, taken. There is currently little literature on detecting homoplasy in simple repeats, therefore homoplasy remains a problem that is not completely avoidable. But by removing known incomplete or compound repeats that show homoplasy, the negative effect of homoplasious alleles in parentage analyses can be marginally, but not completely, avoided.

Summary

Fortunately, many of the drawbacks presented for microsatellite markers can be reduced by the careful selection of only the most appropriate markers. The hypervariability and versatility of microsatellites has made this marker one of the most widely used in biology, for example just under half (42.5 %) of all papers published in *Molecular Ecology* between 1996-2003 used microsatellite markers (Zhang *et al.* 2003). This trend has been shown to be increasing (Zane *et al.* 2002), and a likely factor contributing to this increase is the range of user-friendly software making the statistical analysis of genotypic data easier than in the

past (Sunnucks 2000). For these reasons, microsatellite markers were selected to be developed in the blue crane to assist the detection of the illegal trade in this species by providing a tool with which parentage analysis can be performed.

1.4.3 Case studies: the detection of illegal trade using microsatellites

In addition to biology-related fields, one field in which microsatellite markers have proven valuable is wildlife forensics cases (Manel *et al.* 2002; Zane *et al.* 2002). The main reason being that ecological studies such as population differentiation and parentage studies using microsatellite markers in wild populations also have an importance in cases dealing in wildlife crime. For example, the study of population differentiation can allow for the origin of an individual to be determined, and in forensics this can be used to determine the origin of illegally traded species and their products (Case study I, below). Parentage testing can provide information regarding the relationship between offspring and parents, and in forensics, this can be used to verify the relationship between offspring and their claimed parents in species in legally protected species where chicks are fraudulently claimed to be the offspring of a legal captive pair (Case study II, below). The application of microsatellite markers in these two areas shall be discussed with the aid of case studies below.

Case study I: Geographic origins of illegally traded species and their products

The African elephant (*Loxodonta africana*) is listed on CITES Appendix I with all commercial trade banned since 1990, but with non-commercial trade occurring only in exceptional circumstances such as for scientific purposes (Hemley 1994). Despite legal measures in place to control the trade in this species, cases of illegal elephant poaching and the sale of tusks on the illegal trade market still exist (Jachmann 2003). The ability to identify the origin of a cache of ivory known to be obtained illegally could help to control the illegal trade, since enforcement efforts could be increased in those areas identified as being frequently targeted by poachers (Wasser *et al.* 2004). For this reason, microsatellite markers were developed in this species with the purpose of differentiating elephant samples from a collection of sites within Africa. Wasser *et al.* (2004) identified geographically-

specific allele frequencies, and was able to place 50 % of the trial samples within 499 km of their actual origin, and 80 % to within 932 km.

The largest illegal cache of ivory confiscated by CITES since 1990 provided an investigation with which the microsatellite markers could be used to determine the geographic location of the ivory (Brown 2006). The cache was discovered in a Singapore airport in 2002 bound for Yokohama, Japan (Brown 2006). Following analysis using 16 microsatellite markers developed for this species (Wasser *et al.* 2004), the ivory was successfully identified as being of Zambian origin and therefore allowed for the identification of the area where high levels of poaching occur (Brown 2006). This information could then be used to increase resources for law enforcement in this area since this has been shown to have a positive impact in reducing the levels of illegal poaching (Jachmann 2003).

A similar application of microsatellites was used for bobcats (*Lynx rufus*), (Millions *et al.* 2006), and tuatara lizards (*Sphenodont spp.*), (Aitken *et al.* 2001). Microsatellite markers were used in distinguishing between individual bobcats occurring on two geographically isolated peninsulas in Michigan. Because the hunting bag limit for each peninsula was different, hunters were presenting carcasses as being from a different peninsula so as to stay below the legal bag limit (Millions *et al.* 2006). Microsatellite markers, however, enabled the origins of the bobcats that were bagged during hunting to be determined, and identify cases where hunters were poaching over the legal bag limit. For tuatara lizards, microsatellites allowed for the geographic location of illegally smuggled individuals to be determined (Aitken *et al.* 2001). The markers allowed for the release of illegally captured lizard on the island from which they were originally caught (Aitken *et al.* 2001).

Case study II: Parentage analysis of peregrine falcons

The need to confirm the relationship between individuals for forensic purposes is required in cases involving captive birds such as peregrine falcons (*Falco peregrinus*), where a member of the captive population is suspected of being obtained through illegal sources, The peregrine falcon is listed on CITES Appendix I (CITES 2006) and international trade

is permitted providing the birds are captive bred and have the necessary permits (Nesje *et al.* 2000). The illegal trade in this species arises when eggs, chicks or full-grown birds are trapped in the wild and sold on the trade market. The 12 microsatellite loci developed in this species were found to have high powers of parentage exclusion, allowing for the verification of relationships with a high degree of certainty (Nesje *et al.* 2000). Interestingly, these markers could also be used to discriminate between full-sibs (chicks from the same parents) and half-sibs (chicks that share only one parent). This could be used to connect stolen nestlings to a particular nest site by comparisons with the other nestlings in cases where parental data is unobtainable (Nesje *et al.* 2000).

1.5 STUDY OBJECTIVES

"The demand for captive birds results in the illegal removal from the wild and trading of birds"

The first of five problems identified in the National Guidelines for Trade and Keeping Cranes in Captivity (SACWG 2003).

"The use of legal permitted cranes as a front for the movement of illegal birds or the pretence that young birds (removed from the wild) are the offspring of a legal captive pair."

The second of five problems identified in the National Guidelines for Trade and Keeping Cranes in Captivity (SACWG 2003).

"To ensure minimal impacts on wild crane populations from crane trade (legal and illegal)"

- one of the six South African Working Group objectives (SACWG 2004).

The development of a set of molecular markers with which to perform parentage tests in the blue crane would assist the detection, control and regulation of the illegal trade in this species by providing the DNA evidence for use in forensic cases. A set of microsatellite markers for parentage testing and other population studies in the blue crane were unavailable at the start of this study. My objective here is the development of a microsatellite markers set that would have sufficient exclusionary power for accurate parentage testing. But before a set of microsatellite markers can be developed for this

purpose, each marker must be assessed for its suitability by undergoing characterisation. This study aims to complete a characterisation procedure for all microsatellite markers tested in this species to ultimately assemble the most suitable set of microsatellite markers for parentage assignment in the blue crane. Upon completion of forensic validation in the future, the microsatellite markers may be used by forensic laboratories when evaluating DNA evidence in a parentage case.

1.5.1 Thesis outline

- Chapter 1: Introduction

Provides a review of the current literature surrounding species within the family *Gruidae*, specifically the blue crane, as well as information pertaining to wildlife laws, molecular markers, and how these two factors contribute a greater understanding as to the requirements for detecting illegal trade in the blue crane.

- Chapter 2: Methods

Contains general methods that were used throughout this study. They are mentioned only once here to avoid repetition in subsequent chapters.

- Chapter 3: Sex determination in three African crane species

The aim of this chapter is two-fold: firstly to identify the most suitable sexing primers in the blue crane as well as the wattled crane and grey-crowned crane; and secondly to identify sex-linked loci among those microsatellite loci tested for polymorphism in these three species. Sex linked markers are unsuitable for parentage analyses, and must therefore be excluded from the set of microsatellite markers developed for this purpose. In addition to these two aims, a brief study investigating the sex-ratio of the South African blue crane population shall be described.

- Chapter 4: A predicted map of *Grus* microsatellite loci based on the chicken genome

This chapter investigates the ability of a predicted *Grus* microsatellite map to identify loci that are in close association in the genome and may potentially be linked. Creating a map based on the sequence similarity between chicken and *Grus* species, when proven accurate, would allow for the identification of an independent set of markers. This is important during the development of a set of microsatellite markers for parentage testing as only randomly associated loci can be used for this purpose.

- Chapter 5: Characterisation of *Grus* microsatellite loci

Using information drawn from chapters 3 and 4, as well as information for a suite analyses described here, this chapter details the selection of a suitable set of microsatellite loci that shall be used for parentage analysis in the blue crane. This chapter also investigates the cross-species utility of *Grus* loci in other avian species, including two other crane species: the wattled crane and grey-crowned crane.

- Chapters subsequent to Chapter 5 include conclusions and future directions of this study. Thereafter, a list of references and appendices are provided.

CHAPTER 2 METHODS

2.1 INTRODUCTION

The methods described in this chapter are split into two sections: 'General experimental protocols' (section 2.2); 'Sourcing and developing microsatellite loci' (section 2.3).

2.2 GENERAL EXPERIMENT PROTOCOLS

Laboratory disposables such as eppendorf tubes and pipette tips were autoclaved before their use in all laboratory procedures. Pipettes were cleaned regularly to reduce the risk of contamination through the pipette shaft. Nitrile gloves were worn during handling of ethidium bromide, SYBR safe gel stain (Invitrogen), Formamide (genotyping and sequencing) and Hoechst dye (calculating DNA concentration).

2.2.1 Collection and storage of DNA samples

Each year, blue crane chicks are caught up for ringing purposes as part of a conservation management strategy, therefore providing an ideal time to obtain blood and feather samples. Blood and tissue sampling kits were provided to SACWG members to use during the ringing season. Those obtained by SACWG prior for previous crane studies had been stored at the Wildlife Breeding Resources Centre (WBRC, Pretoria) and were kindly provided for use in this study. A total of 112 blue cranes, 10 wattled and 10 grey-crowned cranes blood and tissue samples were provided. The time between collection of the sample and DNA extraction ranged between 6-35 months. Most of these samples had originally been collected by SACWG.

Prior to arrival, blood samples had been stored in TES (0.03 M Tris, 0.005 M EDTA, 0.05 M NaCl [pH 8]) buffer and in 1.5 ml eppendorf tubes. After arrival, samples were kept at room temperature in a dark cupboard to avoid DNA degradation (Janse van Rensburg 2005). A database of sample details including ring number, sampler, collection date and location for all the samples was provided by WBRC (Appendix I).

SACWG samples kits

Sampling kits were assembled to be provided to members of SACWG who are involved in ringing crane chicks. This provided the opportunity to take blood samples. Kits were sent out during the 2005 blue crane breeding season (February 2005) and again before the 2006 breeding season (October 2005). Sampling kits were packaged in 250 x 300 mm sealable plastic bags containing:

a) Ten 15 ml Sterlin tubes containing 10 ml Longmire's buffer (Longmire 1997) for blood and tissue sample storage. This was sufficient to preserve 0.5 to 1 ml blood;
b) Ten 1.5 ml eppendorf tubes, each containing one strip of FTA card (Whatman®) approximately 3 mm x 40 mm in size. Strips were cut manually using a sterile blade;
c) Ten sterile blades for tissue sampling when dead birds were found;
d) Five envelopes for storing plucked or moulted feathers;
e) Twenty adhesive labels (Figure 2.1) to label the Sterlin and eppendorf tubes;

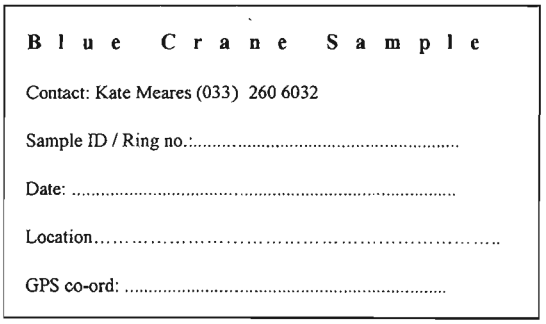

Figure 2.1 Label provided for recording sample information.

f) A permanent marker to record crane details on adhesive labels or envelopes;
g) Two small (150 x 200 mm) sealable plastic bags to store eppendorfs and Sterlin tubes after sample collection;
h) Syringes and needles. Not provided in all kits, many SACWG crane ringers either sourced syringes and needles from veterinary surgeons working in their area or were not qualified in their use;
i) One instruction leaflet (see below) providing details on kit components, as well as guidance on how to avoid contamination when collecting tissue, blood and feather samples.

Upon reaching the lab, samples were stored in a dark cupboard at room temperature.

Instruction leaflet for blood sampling

Collection of samples for DNA analysis

1. Tubes for collection of tissue and/or blood samples

Tubes contain a buffer "Longmire's Buffer" which contains mainly salts and a detergent. Handle with a fair degree of caution. Do not consume.

Tube identification

15 ml clear, plastic Sterlin tubes with blue lids. These contain 10 ml of Longmire's buffer.

Tips for blood and tissue sample collection

Blood:

1. Please add between 0.5 to 1.0 ml of blood to each tube containing the buffer. This amount is roughly one tenth of the volume inside the tube.
2. Please turn the tube up and down a few times to mix the blood in the buffer.
3. If using capillary tubes:
4. Slightly pierce skin with needle so that a small amount of blood leaks. When blood comes out hold a capillary tube at a slight angle to the skin and blood and the blood will be drawn into the tube. If there is sufficient blood and the tube is not more than half full you may use another tube to get more blood.
5. Place capillary tube in the Sterlin tube – you may need to snap the end of the capillary to put the lid back on – place the broken piece of capillary in the tube for safe keeping.
6. Do not use a capillary tube on more than one animal.
7. Do not use the same needle on more than one animal.

Tissue:

1. Do not take tissue samples from live birds. Tissue samples must only be taken from birds that are dead.
2. Please only take tissue samples if unable to take blood.
3. Add a piece (about $1 - 2$ cm^2) of tissue to the buffer.
4. Please try to ensure the tissue remains under the liquid during storage.
5. Please read 'Important considerations' below for further guidance

Envelopes for collection of feather samples for DNA analysis

1. When plucking feathers, please do not handle the tips as this can cause contamination.
2. One envelope for each bird. Please ensure that feathers from different individuals are not mixed.
3. Please read 'Important considerations' below for further guidance

Eppendorfs for collection of blood samples for DNA analysis

Eppendorf identification:

1.5 ml clear, plastic Eppendorfs containing thin strips of Whatmann filter paper.

Tips for blood collection:

1. Slightly pierce skin with needle so that a small amount of blood leaks. When blood comes out, place the strip of filter paper over and allow the filer paper to absorb the blood. Once a small blot of blood appears on the filter paper, remove the filter paper and place it back in the eppendorf.
2. Do not use filter paper on more than one animal.
3. Do not use the same needle on more than one animal.
4. Please read 'Important considerations' below for further guidance

Important considerations:

1. One tube/envelope per sample (i.e.: one blood or tissue sample per tube). Please be careful not to allow blood/tissue from two different birds to mix.
2. Please do not use the same syringe/needle for more than one bird as it will cause contamination.
3. All tubes should be stored at room temperature but please keep them out of sunlight as UV can damage the DNA. They should not be frozen or refrigerated - a dark cupboard is fine.
4. Strips of parafilm can be used to seal the lids of the tubes. Stretch the parafilm over the lids while turning the tube to obtain a tight seal.
5. Please label the tube/envelopes clearly and provide any additional information that may be of use (e.g. ring number, location etc)
6. Please use a permanent marker to write on the tubes/envelopes to avoid the writing being washed off. An alternative option is to stick labels to the tubes.
7. If any further information required, please don't hesitate to call either

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2.2.2 DNA extraction

DNA from blood and tissue samples was extracted using an ammonium acetate method (Bruford *et al.* 1998). This method involves the lysis of the blood sample, an overnight digestion, ethanol precipitation, and finally dilution of the DNA pellet. Protocol is provided in below and is based on Bruford *et al.* (1998). Recipes are provided elsewhere (Appendix II).

DNA extraction with Ammonium Acetate

1. In a 1.5 ml flip-top clean autoclaved eppendorf tube place 250 µl of Digsol buffer and 10 µl of 10 mg/ml Proteinase K (stored at -20 °C). Keep on ice.

2. Remove a sample of blood or tissue (approximately the size of a match-stick head) from the storage container using a sterile toothpick, blot the toothpick with the sample on it onto clean tissue to remove excess buffer.

3. When the blood on the toothpick is blotted dry, place it the eppendorf containing the Digsol buffer and twizzle the toothpick to dislodge the blood. Remove toothpick and discard by placing in bleach.

4. Vortex for 30 seconds, wrap the rack in tissue and cling-film and place in rotating oven at 37 °C overnight.

5. Once digested add 300 µl of 4 M Ammonium Acetate to each sample.

6. Vortex for 30 seconds several times over a period of at least 15 minutes at room temp.

7. Centrifuge for 15 minutes at 13,000 rpm for 10 min to ensure the unwanted precipitate is spun to the bottom of the eppendorf.

8. Transfer supernatant (which contains the DNA) into and autoclaved, labelled eppendorf.

9. Add two volumes of 100 % ethanol to your supernatant. Add more if sample is still dirty.

10. Mix thoroughly by vortexing for 2 seconds and then invert eppendorf gently 10-20 times to precipitate DNA.

11. Centrifuge for 15 minutes at 13,000 rpm

12. Pour and the pipette off all ethanol taking care not to lose DNA pellet.

13. Add about 1 ml of 70 % cold ethanol to rinse the pellet. Pour the ethanol straight off.

14. Pour and the pipette off ethanol.

15. If the pellet dislodges from the bottom of the tube centrifuge for 5 minutes at 15,000 rpm to fix it back to the bottom of eppendorf.

16. Perform a second 70 % ethanol rinse by repeating steps 13 - 15.

17. Dry the pellet at room temp with the eppendorf lid open.

18. Once fully dry add 100-300 µl low TE (here after referred to as $T_{10}E_{0.1}$) to re-suspend the pellet. However, if a very small pellet is observed, only add about 50 µl $T_{10}E_{0.1}$ so that the pellet does not become too diluted for use in PCR.

19. Dissolve the DNA sample in a 37 °C waterbath for about 30 min, occasionally flicking the eppendorf. Ensure the DNA is fully dissolved before quantification.

$T_{10}E_{0.1}$ provides protection against nucleases. DNA can be stored at 4 °C short-term (4 weeks). However keeping samples at -20 °C is advised for long term storage.

2.2.3 DNA visualisation

Method

A 0.8 % agarose gel was used for quantification of extracted whole genome DNA samples. PCR products were run on 2-3 % agarose gels depending on the degree of separation necessary. DNA visualisation took place by staining the agarose gel with either ethidium bromide or SYBR safe gel stain (Invitrogen). The methods used varied between the two laboratories in which the laboratory work was conducted. Gels run at the University of KwaZulu-Natal (UKZN) were stained post-electrophoresis by soaking the agarose gel in an ethidium bromide bath. At the Sheffield Molecular Genetics Facility (SMGF), agarose gels were stained by adding ethidium bromide or SYBR safe to the liquid agarose. A loading buffer (Orange G or Bromophenol blue) was added to each sample before loading. DNA ladders, 100 bp (Generuler) and Phi-X174 DNA/HaeIII Digest (ABgene), were used to determine the size of PCR products. For high molecular weight samples such as extracted DNA, lambda DNA (Helena Biosciences) was used as a size standard. Agarose gels were run between 80 V and 110 V. Visualisation took place in a dark room using a UV light box.

2.2.4 Calculation of DNA concentration

Two methods were used to determine concentrations of extracted DNA:

a) comparisons of band intensities, on agarose gels, between extracted DNA samples and known concentrations of lambda DNA standards;
b) fluorimetry using the FLUOstar OPTIMA (BMG LABTECH)

Lambda DNA standards

Concentrations of extracted DNA samples were estimated by comparing the extracted DNA with lambda DNA of known concentrations. Lambda DNA standards were made at concentrations of 4.0, 8.1, 16.3, 32.5, 75.0, 150.0 and 300.0 ng/µl using serial dilutions. The first seven wells of the 0.8 % agarose gel comprised 2 µl of the respective lambda standard in 10 µl loading buffer Orange G (2 x concentration). Thereafter, samples

consisting of 2 µl extracted DNA and 10 µl loading buffer Orange G (2 x concentration). Agarose gels were stained pre-running using ethidium bromide. Gels were run for 30 – 45 minutes and viewed under UV light. Band intensity of each DNA sample was compared by eye to the seven lambda standards to obtain a concentration estimate.

Fluorimetry

Calf thymus DNA standards were prepared at concentrations 200, 100, 50, 25, 12.5 and 6.25 ng/µl using serial dilutions. Extracted DNA (2 µl) was added to a 96-well BMG black plate (BMG LABTECH); reserving the last seven wells for six calf thymus DNA standards and a negative control (H_2O). Hoechst dye was added to each well before fluorimetry.

The DNA concentrations obtained from fluorimetry and lambda standards were averaged to create a final concentration estimate for each crane sample.

2.2.5 DNA standardisation: preparing 10 ng/µl (PCR-ready) samples

Aliquots from each DNA sample were diluted to 10 ng/µl with low $T_{10}E_{0.1}$ in two 1.5 ml eppendorfs. These dilutions provide a quick source of PCR-ready DNA, as apposed to aliquoting small volumes from the thawed stock DNA. Stock DNA was only thawed when more 10 ng/µl dilutions were needed, thereby reducing the number of freeze-thawing cycles of the stock DNA. This reduced the risk of degradation of the stock DNA which results from frequent freeze-thawing. The stock DNA and the 10 ng/µl dilutions were stored at – 20 °C.

2.2.6 Polymerase Chain Reaction

Described below are the methods followed for all general and temperature gradient PCR reactions. Any deviations from these profiles are described in their appropriate sections.

General PCR profile

Each 10 µl PCR contained approximately 10 ng of genomic DNA, 0.5 µM of each primer, 0.2 mM of each dNTP, 1.0–3.0 mM $MgCl_2$ and 0.05 units of Taq DNA polymerase (BIOTAQ, Bioline Ltd., London, UK) in the manufacturers buffer (Final constituents: 16 mM $(NH4)_2SO_4$, 67 mM Tris-HCI (pH 8.8 at 25 °C), 0.01 % Tween-20). Mineral oil was used to cover each PCR reaction and prevented evaporation. PCR amplification was performed using either a DNA Engine Tetrad 2 thermal cycler (MJ Research, Bio-Rad), DNA Engine Tetrad PTC-225 Peltier thermal cycler (Bio-Rad) or MyCycler Thermal Cycler (Bio-Rad). The PCR program used was 94 °C for 3 min; 35 cycles of 94 °C for 30 s, annealing temperature (T_a) for 30 s, 72 °C for 30 s followed by one cycle of 72 °C for 10 min.

Temperature gradient PCR

Two variables were used for the optimisation of each primer set, $MgCl_2$ concentration and annealing temperatures (T_a). Primer optimisation took place using a 96-well PCR plate (8 rows by 12 columns). This enabled eight primer sets to be examined simultaneously, one in each row. The 12 columns were set for each temperature increment in the T_a gradient. Each row consisted of different primer sets, and for consistency, the same DNA sample (blue crane sample Somerton 1) was used in all optimisation samples.

For optimisation of the $MgCl_2$ concentration, a 2 mM $MgCl_2$ PCR reaction was initially used for all primer sets over a suitable temperature gradient (see next paragraph). If the PCR products revealed high levels of non-specific fragments on a 2 % agarose gel, the concentration of $MgCl_2$ was reduced to 1 mM and the reaction repeated at the same temperature gradient. If the PCR products were faint using 2 mM $MgCl_2$, the concentration was increased to 3 mM.

For optimisation using temperature gradients, the PCR programs used were 94 °C 3 min; 35 cycles of 94 °C for 30 s, T_a gradient (45-60 °C, 50-65 °C, or 55-70 °C), 72 °C for 30 s; ending with one cycle of 72 °C for 10 min. T_a estimates obtained during primer design

For optimisation using temperature gradients, the PCR programs used were 94 °C 3 min; 35 cycles of 94 °C for 30 s, T_a gradient (45-60 °C, 50-65 °C, or 55-70 °C), 72 °C for 30 s; ending with one cycle of 72 °C for 10 min. T_a estimates obtained during primer design using PRIMER v3 (Rozen *et al.* 2000) were used to determine which of the three gradients (see below) would be the most suitable for each primer set. For example, the temperature gradient 45-60 °C was used for those primer sets with an estimated T_a between 50 and 55 °C.

A clean PCR product as visualised on a 2 % agarose gel signalled optimisation of $MgCl_2$ and T_a.

2.3 SOURCING AND DEVELOPING MICROSATELLITE LOCI

Microsatellite loci to be tested for polymorphism in blue crane were sourced by:

a) Acquiring unpublished sequence data obtained from closely related species and using/redesigning primers for cross-species use in blue crane (section 2.3.1)
b) Testing primers in blue crane and/or redesigning primers that were designed from published microsatellite loci developed in a closely related crane species (section 2.3.2)
c) Developing species-specific blue crane microsatellite libraries (section 2.3.3)

Since crane species are separated by a relatively small genetic distance (a delta $T_{50}H$ DNA-DNA hybridisation distance of 0.7, Sibley *et al.* 1990), the likelihood of successful cross-species amplification, from sources a & b above, was regarded as being high since cross-species amplification has shown to be successful even across closely related taxonomic bird groups (Galbusera *et al.* 2000; Primmer *et al.* 1996). This has been shown to be true with Glenn's whooping crane microsatellite primers that have already been tested on a range of other crane species (Glenn *et al.* 1997). The benefits of being able to use loci already developed in other species are that it potentially reduces the costs and time involved to develop a microsatellite library in the focal species.

A microsatellite library (source c) would be developed in blue crane if cross-species testing produced an insufficient number of polymorphic loci for the development of a set of markers to be used in parentage testing and forensic casework.

2.3.1 Unpublished whooping crane sequences

An enriched microsatellite library for the whooping crane was created from 1991 to 1995 (Glenn 1997). Although sequences were not publicly available at the start of this study, 45 microsatellite loci sequences and/or primer sequences were generously provided by Dr. Travis Glenn and Dr. Ken Jones. Of these, eight loci did not have sequence information available, but primer sequences enabled cross-species utility in blue crane to be examined.

2.3.2 Existing *Grus* microsatellite loci

A search for existing microsatellite sequences within the *Gruidae* (crane) family was performed using EMBL database (Kanz *et al.* 2005). The databases 'EMBL', 'EMBL (updates)' and 'EMBL (release)' were selected to search for keywords 'crane' '*Grus*', 'Gruidae' and 'microsatellite'. Seven loci originally isolated in red-crowned crane (Hasegawa *et al.* 2000) had been submitted to the EMBL database were identified.

2.3.3 Blue crane microsatellite libraries

The unenriched and enriched blue crane genomic libraries were prepared at the Sheffield Molecular Genetics Facility (Sheffield University, UK) by Gavin John Horsburgh. For both libraries, a DNA sample from a single blue crane female (Individual J17718; Appendix I) was digested with *Mbo*I (Qbiogene) and the 300-600 bp sized restriction fragments selected and retained for the development stages.

Unenriched

An unenriched library was created in 2005 following the protocol of Dawson (2005b). Fragments were ligated into pUC19 *Bam*HI-BAP (Qbiogene). Transformant colonies were

probed for the motifs (AAAG)n, (GTAA)n, (GATA)n, (CTAA)n and (TAAA)n along with (CA)n after radiolabelling [α32P]-dCTP the denatured PCR-amplified double stranded targets prepared as in Armour *et al.* (1994). The success of isolating positive colonies was low with only one positive clone identified from 480 unenriched colonies. This low success rate was later identified as being caused by an error in the colony-picking stage of the microsatellite library development.

Enriched

As a source of additional blue crane microsatellite loci, an enriched library was prepared in 2005-2006. The enriched library was specifically selected for the tetranucleotide repeat sequences (AAAG)n, (GTAA)n, (GATA)n, (CTAA)n and (TAAA)n using the method of (Armour *et al.* 1994) with modifications suggested by (Gibbs *et al.* 1997). Fifty-six positive clones were isolated and sequenced.

2.3.4 DNA Sequencing

Where possible all loci were sequenced for the blue crane at loci obtained from two microsatellite sources:

1. Existing microsatellite loci previously developed for other crane species (section 2.3.1 and 2.3.2) that successfully amplified cross-species in the blue crane. These loci were sequenced to check for the presence of a microsatellite repeat, thereby confirming amplification of a microsatellite locus required for this study. Sequences also allowed for the redesign of primers, where necessary, based on blue crane sequences.
2. All loci developed from the two blue crane microsatellite libraries (section 2.3.3). These loci were sequenced to allow primer design and tests for polymorphism.

All loci were cloned using a protocol based on 'Cloning of PCR products' by Sambrook *et al.* (2000) followed by a precipitation reaction to obtain clean DNA fragments for sequencing on an ABI 3730. Sequences were initially generated by Gavin Horsburgh at SMGF (University of Sheffield). Those loci for which sequences originally failed to be

generated were re-sequenced by myself. All blue crane sequences generated in this study are provided elsewhere (Appendix III).

An initial PCR was performed and the products run on a 2 % agarose gel to determine fragment size. This identified whether optimisation of the PCR conditions had been achieved. If a weak band was observed, the PCR reaction volume was increased to 20 µl, and the volume of PCR product added during ligation was increased to 6 µl. Ligation using the PCR product can commence when a strong, single PCR product can be seen on an agarose gel, as this indicates the presence of a high number of fragments of the desired length.

When PCR products were non-specific and unsuitable for purification or sequencing due to the presence of multiple fragment sizes, these were re-cloned (by the author) following gel extraction of the correct fragment size. This was achieved by running the fragments on an agarose gel to separate the fragments according to size. The correct fragment size was cut out using a sterile blade for further purification using the 'QIAquick' gel extraction kit protocol (Qiagen). The cleaned fragment was then processed as before from the ligation step of the Sambrook *et al.* (2000) cloning protocol.

PCR purification can be performed using the gel extraction protocol provided with the QIAGEN gel extraction kit. This process will remove dNTPs and primers, a pre-requisite for ligation (step 3, below).

Cloning PCR products for sequencing

a) Ligation: once fragment has been gel extracted a ligation is set up using pGEM-T easy (Promega) as follows:

    5 µl 2× Rapid ligation buffer
    1 µl pGEM-T easy (50 ng/µl)
    3 µl PCR product
    1 µl T4 DNA Ligase (always keep on ice and add last)

Ligation is incubated overnight or weekend at 15 °C in water bath kept in 4 °C room.

b) Transformation:

1. For efficiency of transformation, electroporation was used to enable transformants to be obtained with very weak products.

2. Add ddH_2O to 500 ml then add 7.5 g agar. Autoclave.

3. Place the autoclave agar in microwave with lid loosened and cook until completely melted. Once melted, place in 55 °C water bath and allow to cool to that temp.

4. Add 0.5 ml of Ampicillin (50 mg/ml) and 0.6 ml of Extra Blue™ plus (Qbiogene) (this is an alternative to IPTG/Xgal in Dimethyl Formamide) in 125 ml LB agar (recipe below).

LB agar (1 litre)

5 g	Bacto™ Tryptone (1%)
2.5 g	Bacto™ Yeast extract (0.5%)
5 g	NaCl (1%)

5. Pour plates suing aseptic techniques. A 500 ml yields 20+ plates (~25 ml/plate). Two plates per ligation are needed. Dilute one in case too many transformants are obtained in the other. Once set, dry plates in 55 °C incubator for 30-45 minutes.

c) Transformation by Electroporation:

1. Place the intended number of electroporation cuvettes in -20 °C freezer.

2. LB is needed at this stage (made as above without agar added and autoclaved).

3. In 1.5 ml Eppendorfs place 90 µl of LB for each transformation.

4. Place 3 µl of ligation into a separate Eppendorf with 2 µl ddH_2O, flick and spin down. Do this with all ligations before the start (no need to keep on ice).

5. Add 40 µl of competent cells to ligation/ddH_2O mix, flick to mix and spin down, place on ice (~1 min).

6. Into a chilled electroporation cuvette, place cell mix. Electroporator should be set to 2350 V. After the voltage has passed through the cuvette, remove the cuvette and immediately add 60 µl of LB. Remove mix from cuvette into fresh 1.5 ml Eppendorf. Take 10 µl of mix and add to the Eppendorf containing 90 µl of LB. Mix.

7. Take two agar plates (I mark them N for neat and D for dilute) and add the transformation mix to both.

8. Using aseptic techniques, spread culture as evenly as possible across agar surface. Place Petri dishes agar surface up to allow culture to dry into agar.

9. Repeat for next ligation mix.

10. Place all plates agar surface down in 37 °C incubator. Incubate for ~18 h.

11. In 96 well titre plates add 100 µl LB + 20 % glycerol. Using toothpicks, pick colonies into wells.

12. Incubate plates in 37 °C incubator with shaking at 110 rpm. Cover plates to prevent drying.

d) Sequencing

1. Perform PCR as follows:

1 µl	10× buffer
1 µl	dNTPs
1 µl	M13 forward primer

1 µl	M13 reverse primer
0.2 µl	$MgCl_2$
4.75 µl	ddH_2O
0.05 µl	Taq polymerase
1 µl	bacterial culture
Total 10 µl	

PCR program:

95 °C for 3 min; 30 cycles of 95 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min (can be extended to 2 min if product larger); followed by 2 min extension at 72 °C.

2. Run 4 µl of product on appropriate percentage gel to determine if product present.

3. With rest of sample add 2 µl EXO-sap and incubate on a PCR block for 15 min at 37 °C then for 15 min at 80 °C to inactivate enzymes.

4. Perform Sequence PCR on **forward and reverse** as follows:

1 µl	Big Dye V1.1
1.5 µl	5× sequencing dilution buffer
1.6 µl	Primer (**M13F or M13R**)
3 µl	DNA (exo-sapped)
2.9 µl	ddH_2O
Total 10 µl	

Include a control as follows:

1 µl	Big Dye V1.1
1.5 µl	5× sequencing dilution buffer
2 µl	M13 -21
2 µl	pGEM-3Zf (+)
3.5 µl	ddH_2O
Total 10 µl	

Perform PCR as follows:

96 °C for 1 min; 30 cycles of 94 °C for 10 s, 50 °C for 5 s, then 60 °C for 4 min.

5. Precipitate reactions and sequence as using the protocol below based on Dawson *et al.* (2005a).

Precipitation of BigDye Terminator v1.1 cycle sequencing reactions

a.) Ethanol/EDTA/sodium acetate precipitation

1. Add 2 µl of 125 mM EDTA, 2 µl 3M sodium acetate (pH 5.2), 10 µl autoclaved ddH_20 and 50 µl 100 % ethanol to each tube.

2. Transfer 10 µl of sequencing reactions to the labelled 1.5 ml eppendorf and briefly vortex.

3. Incubate at room temperature for 15 min in a dark drawer.

4. Spin at 13,000 rpm for 15 min. Orientate eppendorfs with the hinges outermost so the location of the DNA pellet is known.

5. Set a P200 pipette to 80 µl and aspirate off the supernatant, taking care not to dislodge the pellet.

6. Add 195 µl 70 % ethanol (pre-cooled at -70 °C).

7. Spin at 13,000 rpm for 5 min

8. Repeat step 6 using a P200 pipette set to 200 µl.

9. Air-dry at room temperature in the dark for 15 to 60 minutes or until dry.

b.) Sequencing on the ABI 3730 Analyser

1. Add 10 µl Formamide to wells and mix (pipette up and down with multi-channel)

2. Denature for 3 min at 95 °C, place on ice until ready to load on the DNA analyser.

3. Sequencing was performed using the ABI 3730 DNA analyser and two sequencing PCR reactions were run for each sample. One reaction used the forward M13 primer, the second used the reverse M13 primer.

The final consensus sequences were created by comparing forward and reverse sequences. Raw sequences were analysed and consensus sequences generated for all loci that amplified a PCR product in blue crane.

2.3.5 Primer design

Primers were designed using the design tool PRIMER v3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi, Rozen *et al.* 2000).

The following criteria were selected:

1. Within the sequence the microsatellite repeat region was identified as the target by manually adding '<' and '>' symbols upstream and downstream of the repeat region, respectively. This ensured that the selected primers flanked the central repeat region.
2. Primer size (bp): min. 18, opt. 20, max. 27. This selected primers of 20 bp in length where possible. However primer length was allowed to vary between 18 and 27 bp.
3. Primer T_m: min. 60, opt. 65, max. 67. The T_m would increase if a pigtail was added to the primer sequence, therefore the optimum T_a and the allele size was always higher than expected for those primers containing a pigtail.
4. Max T_m difference 0.5. Melting and annealing temperatures for both primers must be as similar as possible for PCR amplification to work effectively.
5. 'Max Poly X': 3. 'Max Poly X' describes the maximum number of identical base pairs (e.g.: TTTTTT) acceptable in a primer sequence. A sequence containing more than three base pairs of the same composition was considered unstable because longer mononucleotide SSRs have more opportunity to undergo slipped-strand

mispairing and there will be more mutability in their length than in shorter mononucleotide SSRs (Coenye *et al.* 2005). Long mononucleotide repeat regions should be avoided for primer design since an addition or deletion of one base pair would result in the failure of the primer to anneal and ultimately a failure of the primers to amplify a product. Therefore, a 'Max Poly X' of '3' base pairs was selected.

6. GC clamp: 1. A 'GC clamp' refers to the composition of the two base pairs at the 5' end of the forward primer. The GC bond is stronger than for example an AT clamp. This ensures a greater level of primer stability and increases the chance of successful annealing and ultimately amplification of a PCR product. The number '1' refers the request that a single GC clamp must be present at the 5' end of the forward primer.

If no suitable primers could be selected, the stringency of the settings was lowered:

1. GC clamp was decreased to '0';
2. Minimum and optimum T_m were lowered for both the primer and the Hyb Oligo;
3. 'Max Poly X' was increased to 4, or higher depending on the nature of the sequence.
4. Only if no other suitable position for the primer could be found, 'Max Poly X' was increased further.

Pigtailing

Pigtailing involves the addition of nucleotides with the sequence 'GTTTCTT' to the 5' end of the reverse primer (Brownstein *et al.* 1996). The addition of the 'pigtail' reduces the amount of plus-A (non-template addition of a single 'A' nucleotide to the end of an amplicon by Taq DNA polymerase) and reduces the risk of genotyping errors. Primers were designed with the addition of a pigtail in cases where a high degree of plus-A was observed in PCR products after the initial optimisation.

Fluorescent labels

Forward primers were labelled with a fluorescent label to enable the detection of PCR products by the ABI 3730 DNA Analyser. Each forward primer was labelled with either HEX or FAM fluorescent labels. Loci amplifying products of a similar size were designed with different fluorescent labels to maximise pooling or multiplexing ability.

2.3.6 Individual Genotyping

The genetic composition at each locus can be determined using locus-specific primers that amplify each of the two alleles present at each locus. These alleles can then be scored according to their length in base-pairs using a genotyper (ABI 3730, Applied Biosystems). The genotype is assigned a numerical value (e.g. 112 114 where the respective alleles are 112 and 114 bp in length) representing the alleles present at each locus. Analysing a sufficient number of different loci allows for the development of individual-specific DNA profiles. These profiles can then be used in a variety of areas, one being forensics when the suspect's DNA is compared with DNA found at the crime-scene. In this case, it will ultimately be used for parentage analysis to assist the identification of illegal trade in the blue crane.

ABI 3730 DNA analysis

The ABI 3730 (Applied Biosystems) is a 48 capillary DNA analyser that generates raw genotyping data which can be analysed manually using GENEMAPPER (Applied Biosystems) software. Due to the high sensitivity of the ABI 3730, PCR products were diluted before being loaded on the genotyper to avoid bleed-through. This is caused by spillover from adjacent lanes or spectral bleed-through of a dye to another wavelength (Sobel *et al.* 2002). Capillary-based gels, such as those created using an ABI 3730, are not subject to spillover, but bleed-through can still occur (Sobel *et al.* 2002). Bleed through results in the addition of an allele because of a false band; for example, misinterpreting a bleed-through band as a second allele. To identify which products required diluting, the strength of PCR amplification was checked on a 2 % agarose gel.

Samples exhibiting bright PCR products were diluted to a final concentration of 1:1000 by serial dilution (Figure 2.2). Weaker PCR products were diluted to 1:500 by halving the volume of water added to the second dilution plate. Initially, 20 µl H_2O was added to 5 µl PCR product creating a 1:5 dilution. From this, 5 µl was taken and added to 45 µl H_2O, creating a dilution of 1:50. From this, 0.5 µl was added to 9.5 µl of a Formamide ROX 500 solution, resulting in a final concentration of 1:1000. The Formamide/ROX 500 stock solution was prepared by adding 8 µl ROX 500 to 1 ml Formamide. ROX 500 was used as the internal size marker to generate allele sizes for all samples. Formamide prevents single-stranded DNA, required for the analysis, from reforming into double-stranded DNA. All dilutions were performed in 96-well plates facilitating the use of a multi-channel pipette.

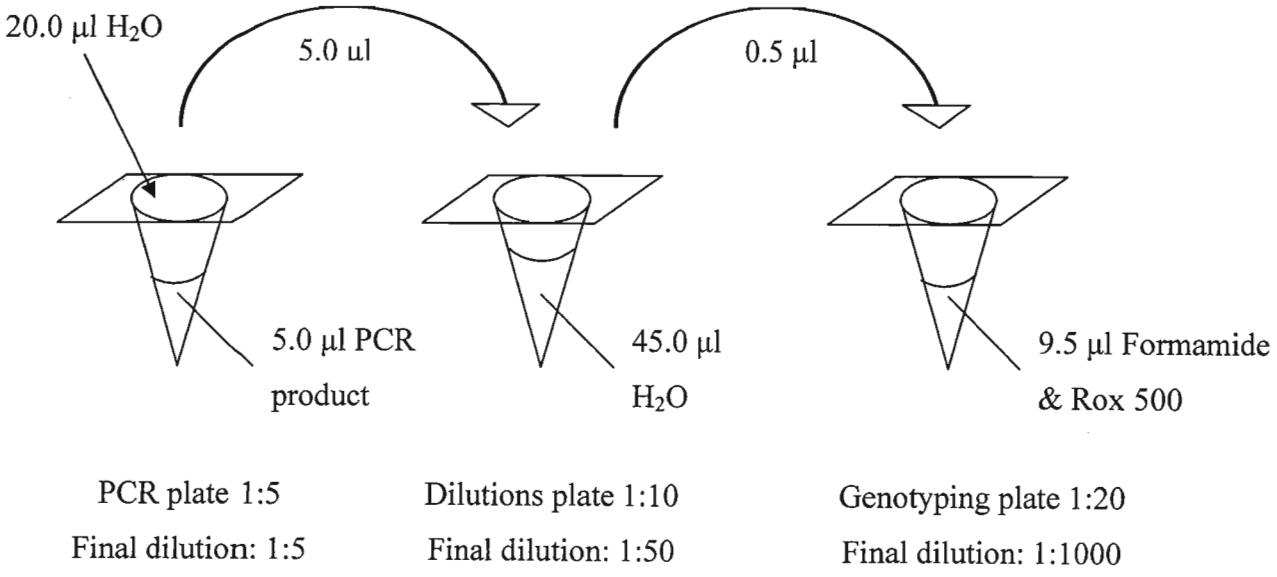


Figure 2.2 Dilution of a PCR product in preparation for genotyping.

To reduce the cost of genotyping, products with different fluorescent labels were pooled within the same reaction. This ensured that products amplified could be separated by fluorescent colour and analysed independently should the allele sizes overlap. Prior to analysing the genotyping plate was covered with a clean rubber genotyping septa and samples were de-natured at 94 °C for 3 min is a repeat. The plate was left on ice for ten minutes then placed in the input stack for analysis.

One negative control (PCR set up with water replacing a DNA sample) and a positive control (PCR reaction using blue crane sample ID: Stoffie) was included in every genotyping plate. The positive control was used as a measure of consistency during allele scoring.

Genotyping success was initially examined for each locus on 10 blue crane samples. This ensured the dilutions were correct and that the PCR reactions were fully optimised. Thereafter, the remaining samples were analysed and the different loci pooled where possible. These were prepared post-PCR by pooling 5 µl of the 1:5 PCR product dilution from each locus into the same well of the second dilution plate (Figure 2.2). The amount of water added was adjusted to ensure a final total well volume of 50 µl.

Raw genotypic data from the ABI 3730 DNA analyser were viewed using GENEMAPPER software (Applied Biosystems). Alleles were scored according to their length in base pairs. The software program created a table listing the two allele sizes present in a sample (e.g.: 112/114). If the individual was a homozygote at a particular locus, two alleles of identical size were given (112/112). A genotype database (Chapter 6) was created in excel which could be exported in the appropriate format for software programs used for analysis of allele frequencies, linkage disequilibrium and Mendelian inheritance (see section 5.2.2), the value '0' (zero) was used where genotypic data were unavailable.

CHAPTER 3 SEX DETERMINATION IN THREE AFRICAN CRANE SPECIES

3.1 INTRODUCTION

The aim of this section is two-fold: firstly to identify the most suitable sexing primers in the blue crane (*Grus paradisea*) as well as the wattled crane (*G. carunculatus*) and grey-crowned crane (*Balearica regulorum*); and secondly to identify any sex-linked loci among those tested for polymorphism in these three species. In addition, a brief study investigating the sex-ratio of the South African blue crane population was undertaken. Results from this study will be useful to those parties undertaking studies requiring sex determination in blue crane, wattled crane and grey-crowned crane in the future.

3.1.1 Molecular sexing

The ability to distinguish males from females in sexually monomorphic species or juvenile young birds that have not developed sexual characteristics provides is a challenge. Observing copulation and courtship displays have been shown to be successful in determining the sex of individuals (Catry *et al.* 1999), however this method is limited to studies during the breading season, and for adult birds only. A method with similar limitations is that of cloacal examinations (Boersma *et al.* 1987) as this technique is only suitable for sexing sexually mature birds.

Recently, PCR-based methods that amplify regions of the avian sex chromosomes have allowed for rapid and accurate sexing of birds of all ages (Fridolfsson *et al.* 1999; Griffiths *et al.* 1998; Huynen *et al.* 2002). With its universal avian species applicability, molecular sexing studies have increased (Hardy 2002), and the technique has shown to provide valuable information to allow for the correct population management strategies for endangered avian species to be implemented (Double *et al.* 1997; Griffiths *et al.* 1995), as well as providing information aiding studies of sex-specific behaviours (Donohue *et al.* 2006), brood sex-ratio manipulation (Bensch *et al.* 1999), and sex-allocation in birds (reviewed in Komdeur *et al.* 2002; Sheldon 1998).

Fundamentals of avian sex differentiation

Molecular sexing is based on the differences of chromosomal composition between males and females. In humans, males are represented as XY since they have one X-chromosome and one Y-chromosome (heterogametic). Females are represented by the symbol XX since two X-chromosomes are present on the female genome (homogametic), with the complete absence of Y-chromosomes. Interestingly, in birds the opposite is true: males are the homogametic sex (ZZ) and females are heterogametic (ZW). Therefore, the use of a marker amplifying a W-specific region would identify females. However, limiting the test to the use of a W-chromosome marker would mean that a male would be indicated by the absence of an amplified product, which might also result from a technical failure. Therefore, the inclusion of an amplifiable Z-chromosome fragment was required to signal PCR success: a characteristic of the universal sex markers P2 and P8 (Griffiths *et al.* 1998) as well as 2550F and 2718FR (Fridolfsson *et al.* 1999).

3.1.2 A review of three sexing primers to be used the three crane species

P2 and P8

The first molecular method to amplify sex-linked products from males and females used a pair of primers that flanked a gene present on both sex chromosomes called the chromobox-helicase-DNA-binding genes (CHD-W and CHD-Z, Griffiths *et al.* 1998). The primers, known commonly as P2 and P8, amplify homologous regions of the CHD-W and the related gene CHD-Z. Because the region amplified by this pair of primers contains a non-conserved intron the PCR products differ in length between the genes. Therefore, the P2 and P8 primers consistently amplify two products in a female (ZW) and one product in males (ZZ) in most species of birds, with the exception of ratites which lack heteromorphic sex chromosomes (Fridolfsson *et al.* 1999). However, in some avian species the difference in size between the fragments of the two genes is small, making it difficult to distinguish males from females on an agarose gel. This problem can be overcome when analysing fragment lengths using more sensitive molecular techniques.

2550F and 2718R

Another set of primers developed to sex non-ratite avian taxa utilised the same conserved CHD gene but amplified the two regions CHD1Z and CHD1W using primers 2550F and 2718R (Fridolfsson *et al.* 1999). Unlike the P2 and P8 primers, they amplified two regions that maintained a consistent size difference. The CHD1W fragments varied between 400 and 450 bp in size and CHD1Z between 600 and 650 bp (Fridolfsson *et al.* 1999). This consistency meant males and females could be easily identified on an agarose gel. However, Dawson *et al.* (2001) noted that this marker has not been tested on as many species as the P2 and P8 markers and therefore the full extent of allele sizes is not yet known. Therefore, the allele sizes could be prone to greater variation and thus an allele size difference smaller than the approximated 200 base pairs. With a decrease in the size difference between sex-specific alleles, the ability to distinguish the different sexes on an agarose gel becomes increasing difficult. Therefore, it was important to test whether this marker is suitable to distinguish between male and female blue crane, as well as wattled crane and grey-crowned cranes using agarose gel.

Gamu007

The locus *Gamu007* developed by Glenn (1997), has been identified as being sex-linked in the whooping crane (Jones *et al.* 1999), a species closely related to the blue crane. Since the genetic distance between whooping crane and blue crane is small (a mean delta $T_{50}H$ DNA-DNA hybridisation distance of 0.7, Sibley *et al.* 1990), the likelihood of this locus being sex-linked in the blue crane was high. This locus was tested along with the two universal primer sets for use as a molecular sexing marker in blue crane.

3.1.3 Sex ratio analysis

A preliminary investigation into the sex ratio of the wild South African blue crane population was briefly performed here. The aim was to determine if there was any skew in sex ratio such that environmental or genetic factors were favouring the production of one sex above the other.

3.2 METHODS

Two universal primers (P2 and P8 as well as 2550F and 2718R) were examined, together with *Gamu007* to determine the most suitable sexing primer for the blue crane, wattled crane and grey-crowned crane. Using the sexing data obtained from primer optimisation above, the sexes of individuals from the three crane species were used to compare male and female genotypes from *Grus* loci to identify sex-linked characteristics. Characteristics of W-linked loci are the failure by males to amplify a PCR product due to the absence of this chromosome in male birds. In contrast, a characteristic of a Z-linked locus is the consistent presence of homozygous genotypes for female birds because they contain only one Z chromosome. A quick check to rule out the possibility of a locus being sex-linked was therefore the presence of heterozygous genotypes in both males and females. Using the sexes of crane individuals identified from primer optimisation, the sex ratio of blue crane individuals in South Africa was calculated. The sex ratio of wattled crane (n = 10) and crowned crane (n = 10) was not calculated due to the small sample size.

3.2.1 Samples

The collection and storage of crane blood, and extraction of DNA have been described elsewhere (section 2.2.1). In total, 103 blue crane samples were sexed. These covered eight of the nine South African provinces: Eastern Cape, Free State, Gauteng, KwaZulu-Natal (KZN), Limpopo, Mpumalanga, Northern Cape and Western Cape. No blue crane samples from the Northern Province were available for sexing. Samples were obtained from birds of all ages. In addition, ten blood samples from both wattled crane (samples from KZN) and grey-crowned crane (samples from Eastern Cape, KZN and Mpumalanga provinces) were provided for sexing. Because the primers P2 and P8 (Griffiths *et al.* 1998) were unavailable for testing in wattled crane and grey-crowned crane, sexing primers 2550F and 2718R (Fridolfsson *et al.* 1999) as well as *Gamu007* (Jones *et al.* 1999) were tested for their utilisation in these species.

3.2.2 Primer testing

All PCR reactions were prepared in 10 µl volumes (see section 2.2.6). A $MgCl_2$ concentration of 2 mM was used for the P2 and P8 primers as well as 2550F and 2718R primers as recommended by the authors (Fridolfsson *et al.* 1999; Griffiths *et al.* 1998). An optimised $MgCl_2$ concentration of 1 mM (Jones *et al.* 1999) was used for *Gamu007* primers. As a blue crane sample of known sex was not available at the time of this study, a blue crane positive control sample could not be used. However, a known female Seychelles warbler (*Acrocephalus sechellensis*) was used as a positive control for the PCR reactions.

P2 and P8 primers (P2: 5'-TCTGCATCGCTAAATCCTTT-3' and P8: 5'-AGATATTCCGGATCTGATAGTGA-3') (Griffiths *et al.* 1998), were amplified using the PCR conditions: denaturation 94 °C for 1 min 30 s followed by 30 cycles of 48 °C for 45 s, 72 °C for 45 s and 94 °C for 30 s; ending with 48 °C for 1 min and 72 °C for 5 min.

For primers 2550F and 2718R (2550F: 5'-GTTACTGATTCGTCTACGAGA-3' and 2718R: 5'-ATTGAAATGATCCAGTGCTTG-3') (Fridolfsson *et al.* 1999), a Touch-down PCR was used. The PCR comprised of an initial denaturing step at 94 °C for 2 min; 9 cycles of 94 °C for 30 s, 60 °C for 30 s, with the T_a decreasing with 1 °C increments per cycle; followed by 35 cycles of 94 °C for 30 s, T_a of 50 °C for 30 s, 72 °C for 40 s, followed by a final extension at 72 °C for 4 min.

Initial allele amplification using locus *Gamu007* was performed using PCR conditions provided by Jones *et al.* (1999). However, poor results were obtained in the first 10 samples analysed, with the presence of multiple amplified fragments when products were run on a 2 % agarose gel. To optimise the marker in the blue crane, a standard temperature gradient profile was used at varying $MgCl_2$ concentrations (section 2.2.6). PCR conditions were optimised at 1 mM $MgCl_2$ concentration with the profile: denaturation at 94 °C for 30 s followed by 30 cycles of 94 °C for 30 s, the optimised T_a of 59 °C for 30 s and 48 °C for 30 s; then lastly 48 °C for 1 min and 72 °C for 5 min.

PCR products were run on 3 % agarose gels stained with ethidium bromide and visualised under UV (section 2.2.3). Products to be genotyped were diluted to 1:1000 and analysed on an ABI 3730 (section 2.3.6) using GENEMAPPER software (Applied Biosystems) at Sheffield University, UK.

Griffiths *et al.* (1998) and Fridolfsson *et al.* (1999) primers were tested for the ability to determine the sex of blue crane (n=103), wattled (n=10) and grey-crowned cranes (n=10) samples, respectively, using both an agarose gel and a DNA analyser. *Gamu007* was tested using genotypes of 103 blue crane samples.

Griffiths *et al.* (1998) primers were not tested in wattled or grey-crowned cranes due the unavailability of blood samples during primer testing. The ability of Fridolfsson *et al.* (1999) primers to sex these species was however tested using an agarose gel and 10 samples from each species. *Gamu007* was tested using genotypes from 10 individuals each of these crane species.

3.2.3 Sex-linked loci

Blue crane individuals were sexed using Griffiths *et al.* (1998) and Fridolfsson *et al.* (1999) primers. Secondly, the genotypes from 35 *Grus* microsatellite loci in blue crane were compared between the sexes to examine for three sex-linked characteristics (below). In addition, genotypes from 27 *Grus* microsatellite loci that amplified in 10 wattled cranes and 10 grey-crowned cranes sample were compared between males and females.

Profiles that were used to examine for the presence the sex-linked characteristics in *Grus* microsatellite loci were:

1. Amplification of a PCR product in females only: W-linked,

2. Homozygous in all females: Z-linked,

3. Heterozygous genotypes in both sexes – not sexed linked.

3.2.4 Sex ratio analysis

Sex ratio analysis using Chi square was undertaken on the South African blue crane population to determine whether significant deviations from an expected 1:1 ratio were present. Sex ratio analysis in the wattled crane and grey-crowned crane was not performed due to a small sample size (n = 10).

3.3 RESULTS

3.3.1 Primer testing

Genotypes for all blue cranes for each of the three sex markers P2 and P8, 2550F and 2718R as well as *Gamu007* are provided in Chapter 6. Genotypes for wattled and grey-crowned cranes at locus *Gamu007* are also provided in Chapter 6.

Primers P2 and P8

Griffiths' primers amplified successfully in blue cranes. However, males and females were indistinguishable on a 3 % agarose gel because amplified products from both sexes were visualised as a single band of approximately 400 bp (Figure 3.1) as determined using a 100 bp ladder (Generuler). To determine sizes of the alleles present in both sexes, PCR products were genotyped (section 2.3.6) and revealed males as having a single product of 391 bp in length, and females as having two products of 385 and 391 bp (Figure 3.1).

Primers 2550F and 2718R

The 2550F and 2718R primers amplified products that could distinguish males from females on a 3 % agarose gel in all three crane species tested (blue crane, Figure 3.2; grey-crowned cranes and wattled cranes, Figure 3.3). Allele sizes present in the 11 blue cranes genotyped were: males one product of 642 bp, females two products: 458 and 642 bp (Figure 3.2b and c). Amplified products from wattled crane and grey-crowned crane samples were not genotyped because blood and DNA samples were not available for genotyping studies in England.

Locus Gamu007

Gamu007 genotypes obtained for males were compared to those obtained for females to determine possible sex-specific alleles amplified from *Gamu007*. Although this marker had determined sexes of whooping cranes (Jones *et al.* 1999), interestingly no sex-specific alleles were observed in the three southern African crane species. Females were consistently homozygous and males displayed both homozygous and heterozygous genotypes. This is in agreement with a Z-linked locus. These factors made this marker inappropriate for use in the three South African crane species.

3.3.2 Sex-linked loci

With the exception of *Gamu007*, a locus previously identified as being sex-linked (Jones *et al.* 1999), no other microsatellite loci were identified as being sex-linked in any of the three species of crane examined here.

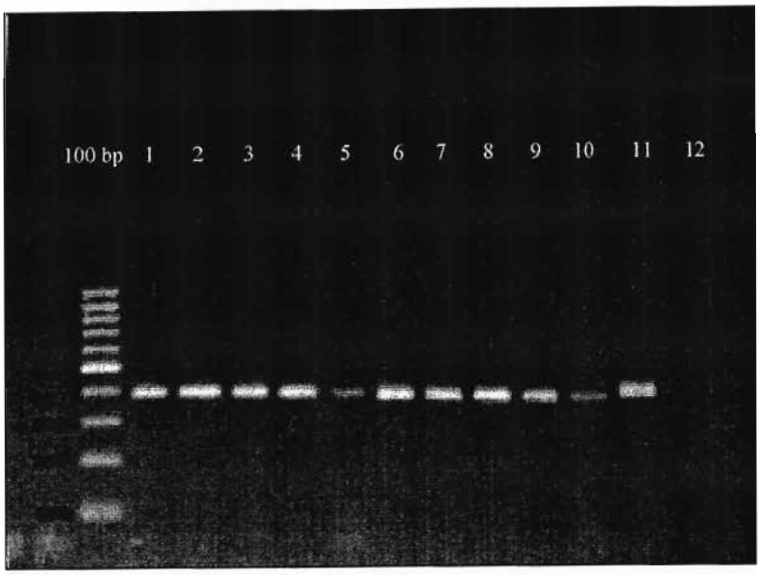
3.3.3 Sex ratio

Sex ratio analysis of 79 unrelated wild blue crane individuals in the South African population showed no significant deviation from an expected 1:1 sex ratio ($P > 0.05$, Table 3.1). Whilst it was not possible to examine sex ratio at a regional or sub-population level, it was interesting to note that a set of samples taken from the Maclear region in the Eastern Cape Province showed a male bias of 11:2 (Table 3.1).

Table 3.1 Sex ratio of the South African blue crane (*Grus paradisea*) population.

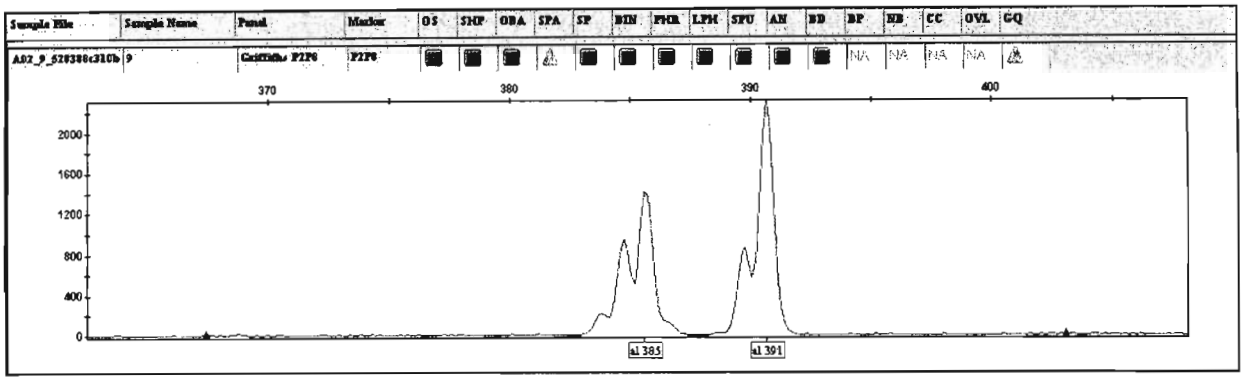
Location	n	Female n =	Male n =	Male:Female sex ratio	Observed % Male	Expected % Male	Chi squared	X^2 p=	significance
Maclear region, Eastern Cape	13	2	11	5.50	84.6	50.0	6.31	0.012	*
Total for South Africa	79	35	44	1.26	55.7	50.0	1.04	0.308	NS

n, sample number
-, could not be calculated due to the absence of female individuals
*significant at the 5 % level
NS, non-significant

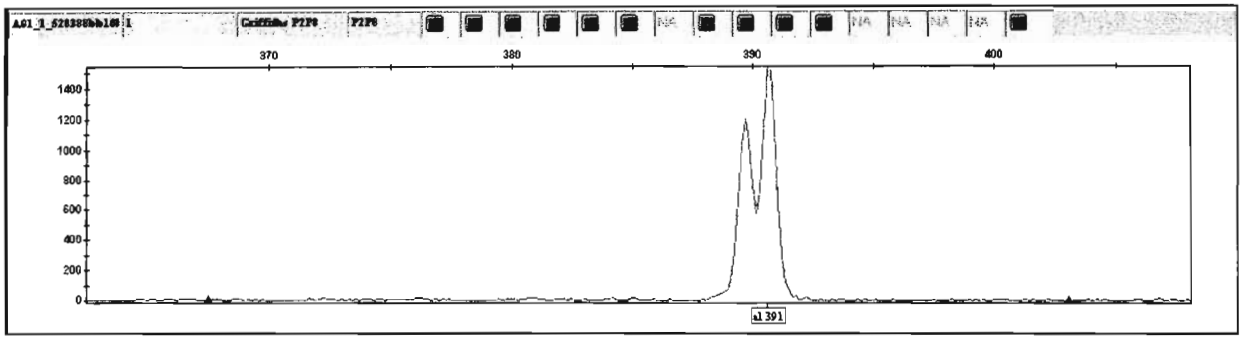


1 - male
2 - male
3 - male
4 - male
5 - male
6 - female
7 - female
8 - male
9 - female
10 - male
11 – positive control (female Seychelles warbler)
12 – negative control (water)

a)

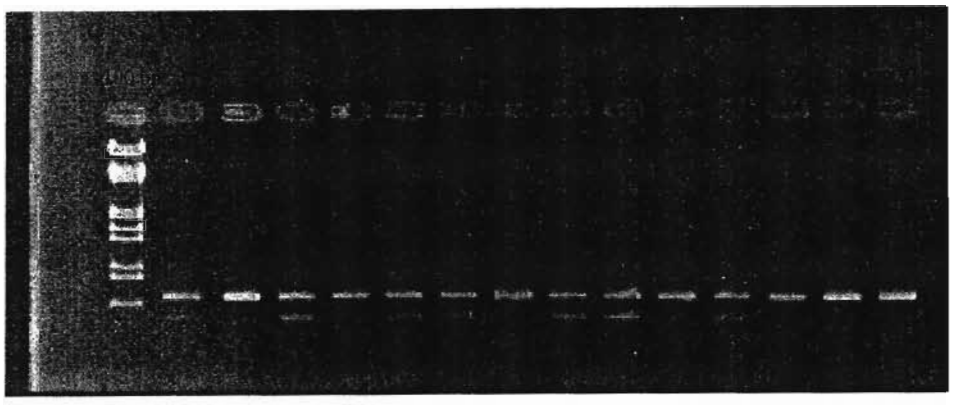


b)



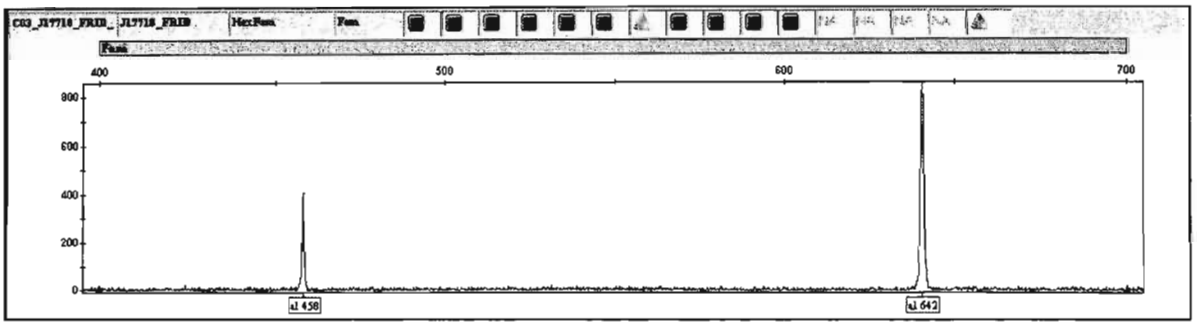
c)

Figure 3.1 Amplification of P2 and P8 primers in the blue crane (*Grus paradisea*); (a) 3 % agarose gel; (b) appearance of a female alleles; (c) appearance of a male allele.

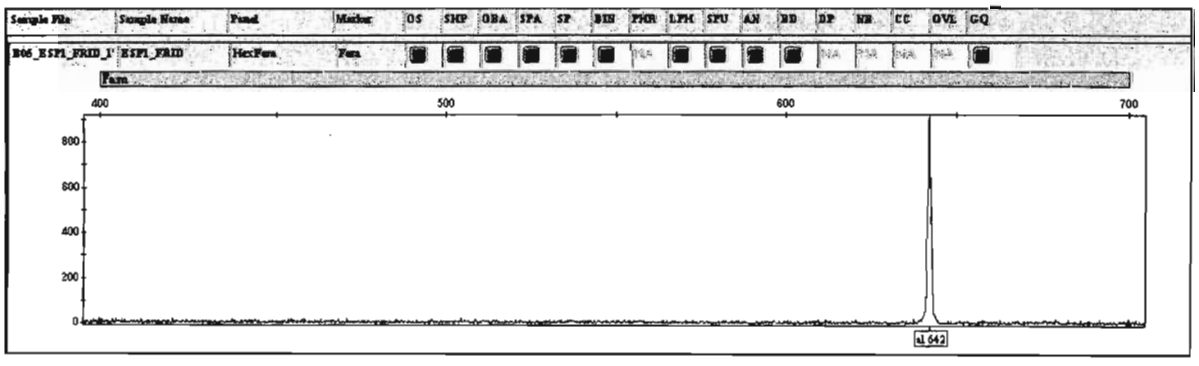


1 - female
2 - male
3 - female
4 - male
5 - female
6 - female
7 – male
8 - female
9 - female
10 - male
11 - female
12 - male
13 - male
14 - male

a)

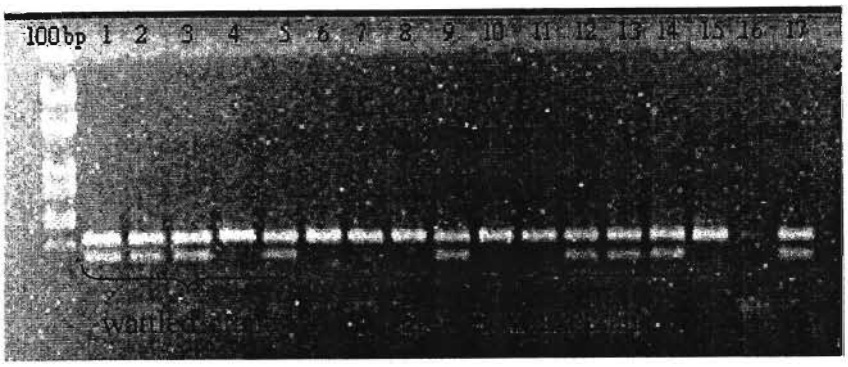


b)



c)

Figure 3.2 Amplification of 2550F and 2718R (Fridolfsson *et al.* 1999) primers in the blue crane (*Grus paradisea*). (a) 3 % agarose gel with a 100 bp ladder; (b) visualisation of the two female alleles; (c) male allele.



1 - female
2 - female
3 - female
4 - male
5 - female
6 - male
7 - male
8 - male
9 - female
10 - male
11 - male
12 - female
13 - female
14 - female
15 -male
16 – negative control (water)
17 – positive control (female Seychelles warbler)

Figure 3.3 Amplification of 2550F and 2718R (Fridolfsson *et al.* 1999) primers in the wattled crane (*Grus carunculatus*) and grey-crowned crane (*Balearica regulorum*). Visualisation took place on a 3% agarose gel and fragment size was estimated using a 100 bp DNA ladder.

3.4 DISCUSSION

Sexing primers

Blue, wattled and grey-crowned crane individuals were successfully sexed using PCR-based methods. The primer pair 2550F and 2718R developed by Fridolfsson *et al.* (1999) was found to provide a reliable, rapid and cost-effective method for sexing the three crane species using an agarose gel. PCR products for blue crane generated from Griffiths P2 and P8 sexing primers (Griffiths *et al.* 1998) could not be separated on an agarose gel and are not recommended for sexing using this technique. Amplified products from both primers could be easily scored on a DNA analyser therefore either of the primers can be used where a DNA analyser is to be used for sex determination. Although locus *Gamu007* has been successfully used for sex determination of whooping cranes (Jones *et al.* 1999), this locus did not show any sex-specific alleles in the blue, whooping or grey-crowned crane. This locus is therefore unsuitable for use toward sexing samples from the three South African crane species.

Sex-linked loci

With the exception of *Gamu007*, no sex-linked loci were identified in 35 loci tested in blue cranes. Therefore, no sex-linked loci were identified in the 28 loci that amplified in 10 wattled crane and 10 grey-crowned crane individuals. *Gamu007* should be excluded from a set of markers for use toward parentage testing and assisting the identification of illegal trade in blue crane.

Sex ratio analysis

Due to a small sample size, the results obtained from the sex-ratio analysis should only be considered a preliminary analysis. The samples within South Africa did not show a significantly skewed sex ratio. Further investigation into the population structure would be useful to determine if, should subpopulations be identified, a sex ratio bias in a subpopulation exists. If this does occur, further investigation should be undertaken to understand whether environmental and/or genetic factors may be driving the sex ratio bias observed.

CHAPTER 4 A PREDICTED MAP OF *GRUS* MICROSATELLITE LOCI BASED ON THE CHICKEN GENOME

4.1 INTRODUCTION

Microsatellite mapping, comprising linkage maps and predicted maps, is the process of assembling genetic markers in the correct order and position on a chromosome. Linkage maps use linkage statistics such as recombination fractions between pairs of loci to assign each locus to a linkage group. Linkage maps have been constructed for a number of species, with efforts focussed mainly on biological models, as well as biomedically and economically important species such as humans, sheep (*Ovis aries*), pigs (*Sus scrofa*) and the red jungle fowl (*Gallus gallus*, hereafter referred to as chicken). Ultimately this tool allowed for the study of gene interactions involved in human diseases, as well as quantitative traits of agricultural interest such as body mass and egg production (Burt *et al.* 1995). Two other species for which linkage maps have been constructed are the great reed warbler (*Acrocephalus arundinaceus*) (Hansson *et al.* 2005) and Japanese quail (*Coturnix japonica*) (Kayang *et al.* 2004), both important model organisms in biological research.

In contrast to linkage maps, predicted maps use available sequences from an unmapped species and assign the location of their orthologous sequences in a species for which a map already exists. To date only one published predicted microsatellite map for an order within the class Aves exists: passerine microsatellite map constructed by Dawson *et al.* (2006). Until recently, the single limiting factor was the absence of an avian species with an entirely sequenced genome. This made it difficult, if not impossible, to predict the location of sequenced loci within a genome by comparing sequences to those having known chromosomal locations. However, in 2004 the International Chicken Genome Sequencing Consortium completed sequencing a large proportion of the chicken genome (International Chicken Genome Sequencing Consortium 2004), and the sequences made publicly available on the Ensembl sequence database (Birney *et al.* 2006).

Although in its infancy, the applications of predictive mapping are the study of quantitative trait loci, understanding karyotype evolution, genome mapping, and the identification of independent set of microsatellite loci (Dawson *et al.* 2006). The latter application is extremely useful when selecting loci to be used in parentage testing, where loci must be independently assorted (Jones *et al.* 2003). This was the motivation behind the construction of a predicted microsatellite map for a non-passerine genus; *Grus* to which the blue crane (*G. paradisea*) belongs. Creating a map based on the sequence similarity between chicken and *Grus* species, when proven accurate, would allow for the identification of an independent set of markers to be selected for parentage testing not only in the blue crane, but potentially other species within this genus.

4.1.1 Can predictive mapping be successful?

Genome evolution represented by chromosomal rearrangements occurs over time causing the likelihood of conserved synteny to decrease as a function of genetic distance (Shetty *et al.* 1999). Consequently, more distantly related species have greater karyotypic differences, as well as differences within the arrangement of genes located on a chromosome. Therefore, genes that are linked in one species may not necessarily be linked in another species which could lead to erroneous conclusions when analysing results obtained from predictive studies. In order for an assessment of the likelihood of predictive microsatellite mapping to be an accurate predictor of locus position within a species, the evolutionary distance between the species/taxonomic group being mapped (cranes for example) and the species on which the map is being based (chicken) must therefore be determined. This will allow for the degree of synteny between the two species to be determined.

One method, fluorescent *in situ* hybridisation (FISH), has been used to study karyotype evolution (genetic distance) in birds (Derjusheva *et al.* 2004; Shetty *et al.* 1999). This technique uses DNA probes from one species, specific for a particular chromosome, and applies this probe to another species to determine the conservation of chromosomal syntenies (the preserved order of genes on a chromosome). Results have shown high homology between two distantly related birds: chicken and emu (Shetty *et al.* 1999). Furthermore, a high conservation of syntenies was revealed between the chicken, pigeon

and passerine birds (Derjusheva *et al.* 2004), as well as between chicken and zebra finch (Itoh *et al.* 2005). Results taken from these analyses support the idea that predictive microsatellite mapping between chicken and other avian species may be a true reflection of microsatellite locations within the genome of the focal species because of the conservation of avian karyotypes over 80 million years of evolution (Shetty *et al.* 1999).

A second method of determining genetic similarity employs linkage map comparisons between distantly related species. Although few avian linkage maps have been constructed, comparisons between a chicken and human linkage map indicated that levels of conserved synteny between these two species appears to be very high (Groenen *et al.* 2000). If this result is extrapolated for genomic evolution in birds, chromosomal synteny between chicken and other avian species is expected to be high because of the smaller genetic distance between avian taxa than chicken and humans (Sibley *et al.* 1990). Ultimately, linkage map comparisons, when available for more avian species, could be examined to identify the level of agreement between linkage and predicted microsatellite maps, thereby determining the accuracy of the predicted map in assigning loci to the correct chromosomal location. Lastly, cytogenetic analysis of a range of passerine families has shown that passerines (2n = 72-84) have a similar karyotype to chicken (2n = 78) (Dawson *et al.* 2006). Therefore, loci identified as being linked based on predictive mapping will likely be a true reflection of their status in passerines.

The support of three methods indicating strong homologies between avian taxa as well as the similarity between passerine and chicken karyotypes, suggested that the likelihood for predictive mapping to assign passerine microsatellite loci to the correct chromosomal locations could be high. This provided the motivation for Dawson *et al.* (2006) to construct the predictive microsatellite map of the passerine genome based on chicken-passerine sequence similarity. Although cranes are non-passerines, the predicted passerine map is discussed here due to its relevance in providing the basis for the construction of the predicted *Grus* microsatellite map.

4.1.2 The predicted passerine map

Passerine species represent a family of birds that are separated by short genetic distances (Sibley *et al.* 1990). Importantly, the likelihood of successful cross-species amplification increases with decreasing genetic distance, supported by high cross-species utility of passerine microsatellite markers in other passerine species (Primmer *et al.* 1996). With more than 500 passerine microsatellite sequences available (Dawson *et al.* 2006), a large selection of loci are available for genetic studies on a passerine species. A requirement in utilising microsatellite loci in population genetic and parentage analyses is the identification and exclusion of linked loci. This can be achieved by selecting loci situated on different chromosomes for use in a set of microsatellite markers.

To identify the location of microsatellite markers within the passerine genome, a predicted microsatellite map was created using the chicken genome as a template. Reasons for using the chicken genome sequence database are highlighted below: (a) no passerine species has had its entire genome sequenced; (b) conserved synteny between chicken and passerine species appears high, with a strong possibility of correct placement of microsatellite loci on passerine chromosomes based on chicken genome sequences.

To test the correct prediction of chromosomal locations of microsatellite loci in passerines, a comparison was made by Dawson *et al.* (2006) between the predicted passerine map and a linkage map developed by Hansson *et al.* (2005) for the great reed warbler, a passerine. Importantly, these two maps were constructed independently of each other using different methodologies. From this comparison, synteny was shown to be conserved between the predicted passerine map and the great reed warbler map (Dawson *et al.* 2006), confirming the ability of the passerine-chicken map to correctly predict chromosomal locations of passerine sequences, as well as to identify linkage of passerine microsatellite loci.

4.1.3 Predicted *Grus* map

The order Gruiformes (non-passerine), to which the blue crane belongs, and Passeriformes (passerines) are separated by a relatively small genetic distance (delta T50H DNA-DNA

hybridisation distance of 20.8, Sibley *et al.* 1990). As predictive mapping of the passerine genome was shown to be accurate based on chicken-passerine sequence similarity, a predictive map constructed for the *Grus* genus within Gruiformes would be expected to show a similar level of accuracy in its ability to correctly assign chromosomal locations to microsatellite markers and ultimately indicate the presence of potentially linked loci. In addition, sequences conserved between blue crane and chicken would suggest an increased likelihood of successful cross-species amplification between this blue crane and other more distantly related avian species.

However, one possible shortfall in the *Grus* map was recognised at the start of this study: crane and chicken karyotypes differ quite substantially: whooping cranes, 2n = 62 (Blederman *et al.* 1982); chicken, 2n = 78 (Burt *et al.* 1995). It may therefore be possible that synteny between these two species may not be as conserved as between chicken and passerines. However, Dawson *et al.* (2006) were able to test the accuracy of the predicted passerine map by comparing it to a linkage map created for a passerine species. The predicted map of the *Grus* genome will therefore only be able to be proven accurate once a linkage map for this genus has been completed. However, analyses of linkage disequilibrium between *Grus* microsatellite loci examined in this study (Chapter 5) revealed neither support nor rejection of the predicted *Grus* map, since chromosomal locations of at least one of the two loci in a linked pair could not be mapped.

4.2 METHODS

Eighty seven unique microsatellite loci were used for mapping. Thirty-eight loci were originally developed in whooping crane (*G. americana*). However, of these 38, the complete sequence was unavailable for 5 loci. Therefore, full locus sequences for these five loci used for microsatellite mapping were those isolated from amplification in blue crane. The five loci are identified by the 'BC' locus prefix (Table 4.2). In addition to whooping crane loci, seven and 42 loci originally developed and sequenced in the red-crowned crane (*G. japonensis*) and the blue crane, respectively were used for predictive mapping.

Loci originally developed in whooping and red-crowned crane sequenced in blue crane were also mapped where possible to determine the level of agreement with loci mapped using sequences from the source species and the blue crane.

Grus sequences were submitted in a single FASTA-formatted text file. Similarity between *Grus* microsatellites (n = 87) and chicken sequences was determined using the BLAST function provided by the Ensembl v40 (Birney *et al.* 2006) sequence database. Search sensitivity was configured to 'Distant homologies', which results in a high sensitivity BLAST search. Configurations of BLAST searches remained at the default setting. A locus was assigned a position (hit) on a chicken chromosome if the sequence similarity E-value was $\leq 1.0e^{-10}$. Furthermore, those sequences with multiple hits at $1.0e^{-10}$ or lower were assigned a position only if the best hit had an E-value at least 10 decimal places lower than the second best hit. Only unique sequences or those identified as being a mappable multiple hit were mapped.

For predictive mapping purposes, information collected from the Ensembl BLASTn chicken genome search included a) chromosome number, and b) start and end position of the locus sequence on the respective chicken chromosomes. These data were placed in an excel document providing the input file for Ensembl KaryoVeiw: a mapping tool available from Ensembl v40 (Birney *et al.* 2006) used to create the microsatellite map of *Grus* loci. Where possible, centromere locations were determined using the position of centromeres provided by the predictive passerine map relative to the position of the loci on the *Grus* map.

4.3 RESULTS

4.3.1 Categories of hits

Chromosomal hits were assigned into one of seven result categories (Table 4.1), with the categorisation based on Dawson *et al.* (2006). The details within this table are magnified in Table 4.2 to reveal the category of hit for each locus, as well as other hit characteristics such as the chromosomal location and the sequence similarity of the *Grus* sequence to the chicken sequence.

Table 4.1 Percentage of microsatellite loci mapped within each of the seven hit categories.

Category of hit	Mapped	Number loci mapped using default settings	Percentage
Unique	Y	24	27.6
Unique hit to unknown chr.	N	3	3.4
Unique hit at 1.0e-10<E<1.0e-5	N	9	10.3
Mappable multiple hit	Y	9	10.3
Mappable multiple hit - unknown chr.	N	2	2.3
Unmappable multiple hit	N	5	5.7
No strong hits	N	35	40.2
Total		**87**	

Unique hits (27 sequences). A hit was regarded as unique if the E-value was $< 1.0e^{-10}$. Of 27 sequences identified as having unique hits, three loci hit a sequence on an unknown chromosome (Table 4.1) and therefore could not be included in the predicted microsatellite map. Future releases of additional chicken genome sequence data should facilitate the mapping of these loci.

Unique hit at $1.0e^{-10} < E < 1.0e^{-5}$ (9 sequences). These sequences were not mapped since hits with E-values $> 1.0e^{-10}$ were considered of insufficient strength for mapping.

Mappable multiple hit (11 sequences). Multiple hits arose when a sequence hit more than one position within the chicken genome. A locus displaying multiple hits was mappable when the E-value of the strongest hit was at least ten decimal places less than the second strongest hit.

No strong hits (35 sequences). Of the 87 sequences BLASTed against the chicken genome, 40.2 % produced no strong hits (Table 4.1).

Table 4.2 Mapping success of *Grus* microsatellite loci within the chicken genome.

Locus	EMBL accession number	Seq. length in source species (bp)	Category of hit	Mapped	Chr.	Start (bp)	E-val
Loci sequenced in whooping crane (Glenn 1997)							
Gamu002	AM084712	132	No strong hits	N			
Gamu003	AM084713	118	Unique	Y	2	3651573	2.40E-11
Gamu004	AM084714	201	No strong hits	N			
Gamu005	AM084716	189	Unique hit at 1.0e-10<E<1.0e-5	N	2		5.40E-05
Gamu006	AM084717	129	Unique	Y	1	92001854	4.00E-12
Gamu007	AM084718	535	No strong hits	N			
Gamu008	AM084719	269	Unique hit at 1.0e-10<E<1.0e-5	N	2		3.10E-09
Gamu009	AM084720	193	No strong hits	N			
Gamu010	AM084721	120	Unique hit at 1.0e-10<E<1.0e-5	N	1		5.50E-08
Gamu012	AM084723	136	No strong hits	N			
Gamu013	AM084724	223	No strong hits	N			
Gamu014	AM084725	191	Unique hit to unkNown chr.	N	Unkn.		2.70E-11
Gamu015	AM084726	166	No strong hits	N			
Gamu016	AM084727	272	Uniique	Y	3	105908138	3.70E-14
Gamu017	AM084728	251	Unique	Y	2	145777060	8.00E-10
Gamu018	AM084729	179	Unique	Y	1	99490376	9.20E-15
Gamu019	AM084730	159	No strong hits	N			
Gamu020	AM084731	132	Unique	Y	4	36801073	3.20E-15
Gamu021	AM084732	150	Unique	Y	7	33351056	1.40E-31
Gamu022	AM084733	189	Unique	Y	8	9755662	7.80E-23
Gamu023	AM084734	188	No strong hits	N			
Gamu024	AM084735	238	Unmappable multiple hit	N	23 and 2		3.2E-6 and 3.5E-6
Gamu025	AM084736	115	No strong hits	N			
Gamu101	AM084738	223	No strong hits	N			
Gamu102	AM084739	139	No strong hits	N			
Gamu103	AM084740	232	No strong hits	N			
SHC-AG-1	AM084741	162	No strong hits	N			
WC-2F-6	AM084742	200	No strong hits	N			
WC-4A-11	AM084743	170	No strong hits	N			
WC-4C-5	AM084744	91	No strong hits	N			
WC-AC-B	AM084745	598	Unique	Y	2	17742915	8.10E-36
WC-AC-R	AM084746	244	Unique hit to unknown chr.	N	Unkn.		4.80E-55
WC-AG-1	AM084711	151	Unique	Y	4	79566340	8.10E-39
Whooping crane loci sequenced in blue crane							
BC AA/GC	AM168499	203	Unique hit at 1.0e-10<E<1.0e-5	N	2		5.30E-06
BC AA-1	AM168498	220	Unmappable multiple hit	N	2		2.10E-21
BC AT/AC-1	AM168500	107	No strong hits	N			
BC Gamu011	AM168506	136	No strong hits	N			
BC SHC-AG-2	AM168522	202	No strong hits	N			
Loci sequenced in red-crowned crane (Hasegawa *et al.* (2001)							
Gj-M08	AB041858	108	Mappable multiple hit	Y	2	40543025	9.30E-26
Gj-M11a	AB041859	187	No strong hits	N			
Gj-M13	AB041860	158	Mappable multiple hit	Y	1	160039700	5.90E-34
Gj-M15	AB041861	114	Mappable multiple hit	Y	4	38245618	1.50E-30
Gj-M34	AB041862	100	No strong hits	N			
Gj-M40	AB041863	487	Mappable multiple hit - unkNown chr.	N	Unkn.		3.30E-35
Gj-M48b	AB041864	220	No strong hits	N			

Table 4.2 cont.

Locus	EMBL accession number	Seq. length in source species (bp)	Category of hit	Mapped	Chr.	Start (bp)	E-val
Loci sequenced in blue crane (Meares *et al.* 2006)							
Gpa01	AM085152	821	Mappable multiple hit	Y	2	36395727	1.60E-65
Gpa02	AM282883	472	Unmappable multiple hit	N	1		5.90E-08
Gpa03	AM282884	512	Unique	Y	3	26926860	2.30E-66
Gpa04	AM282885	336	Unmappable multiple hit	N	1		2.90E-09
Gpa05	AM282886	297	No strong hits	N			
Gpa06	AM282887	293	Unique hit at 1.0e-10<E<1.0e-5	N	4		9.00E-08
Gpa07	AM282888	539	Unique	Y	2	2953557	6.10E-38
Gpa08	AM282889	506	Unique	Y	1	103070385	3.50E-14
Gpa09	AM282890	405	Mappable multiple hit	Y	1	26178250	3.70E-32
Gpa10	AM282891	538	Unique	Y	7	23875726	2.10E-90
Gpa11	AM282892	604	Mappable multiple hit	Y	1	157148833	2.00E-37
Gpa12	AM282893	356	No strong hits	N			
Gpa13	AM282895	187	Unique	Y	4	38305273	4.10E-16
Gpa14	AM282896	450	Mappable multiple hit - unkNown chr.	N	Unkn.		3.20E-31
Gpa15	AM282897	425	Unique	Y	1	34658225	1.50E-34
Gpa16	AM282898	526	Unique	Y	10	6593382	2.50E-52
Gpa17	AM282899	257	No strong hits	N			
Gpa18	AM282900	403	Unique hit at 1.0e-10<E<1.0e-5	N	2		3.40E-08
Gpa19	AM282901	522	Unique	Y	8	23596115	2.70E-33
Gpa20	AM282902	325	Unique hit at 1.0e-10<E<1.0e-5	N	1		1.10E-05
Gpa21	AM282904	312	No strong hits	N			
Gpa22	AM282905	273	Unique hit at 1.0e-10<E<1.0e-5	N	1		1.80E-08
Gpa23	AM282907	280	Unique	Y	1	164736728	3.10E-12
Gpa24	AM282908	528	Unique	Y	9	13450010	5.10E-28
Gpa25	AM282910	232	No strong hits	N			
Gpa26	AM282911	282	No strong hits	N			
Gpa27	AM282913	375	No strong hits	N			
Gpa28	AM282914	324	Unique	Y	1	100493183	1.70E-20
Gpa29	AM282915	454	No strong hits	N			
Gpa30	AM282916	265	Unique	Y	2	104097032	1.30E-12
Gpa31	AM282917	440	Mappable multiple hit	Y	1	93971360	3.30E-26
Gpa32	AM282918	622	Mappable multiple hit	Y	6	21180285	7.20E-42
Gpa33	AM282919	355	No strong hits	N			
Gpa34	AM282920	318	No strong hits	N			
Gpa35	AM282921	636	Unique	Y	1	32456042	1.30E-85
Gpa36	AM282922	388	No strong hits	N			
Gpa37	AM282923	385	Unique hit to unkNown chr.	N	Unkn.		9.40E-16
Gpa38	AM282924	617	No strong hits	N			
Gpa39	AM282925	469	Unique	Y	2	114116902	4.10E-13
Gpa40	AM282926	369	Mappable mulitple hit	Y	2	145769112	4.50E-39
Gpa41	AM282932	342	Unique hit at 1.0e-10<E<1.0e-5	N	3		1.20E-08
Gpa42	AM282933	499	Unmappable multiple hit	N	1		9.90E-10

Chr, chicken chromosome that the sequence hit;

Start, position on the chicken chromosome where the matching *Grus* sequence begins;

E-val, for chicken-*Grus* sequence similarity;

Unkn., hit on an unknown chicken chromosome.

4.3.2 Description of the chromosome map

Of the 87 microsatellite loci examined, 33 (38 %) were successfully mapped to nine of the 30 sequenced and assembled chicken chromosomes, each named 'Gga' (***Gallus gallus*** chromosome) (Figure 4.1). All loci that were assigned chromosomal locations were positioned on the first 10 chicken chromosomes, with the exception of chromosome Gga5 which produced no hits. The first ten chromosomes (excluding Gga5) together constitute ~ 71.19 % of the available chicken genome, making the likelihood of mappable loci being assigned to one of these nine chromosomes high. Gga1 had the highest number of mapped microsatellite loci (n = 11, Figure 4.1).

Centromere locations were determined for five of the nine mapped chromosomes based on the location of passerine loci relative to each chicken centromere. For example: the centromere on Gga1 of the predicted passerine map lies between loci *CmeH2* and *Pdoμ6* (Dawson *et al.* 2006). These loci have start positions of 60031161 and 75545595, respectively (Table 4.3). Therefore, any loci on the *Grus* map with start positions less than 60031161 will lie above the centromere (arm p); those loci with start positions greater than 75545595 will lie on below the centromere (arm q). For the *Grus* map, the centromere for chromosome 1 therefore is situated between loci *Gpa15* and *Gamu006* (Table 4.3).

For chromosome Gga6, Gga9 and Gga10, locations of the centromeres are currently unknown. For Gga8, the centromere on the predicted passerine map was situated between positions 6429857 and 17712523 (Table 4.3). However, locus *Gamu022* on the *Grus* map has a start position of 9755662 which lies between the two passerine values denoting centromere location (Table 4.3) and therefore the position of the centromere relative to this locus on the *Grus* predicted map could not be determined.

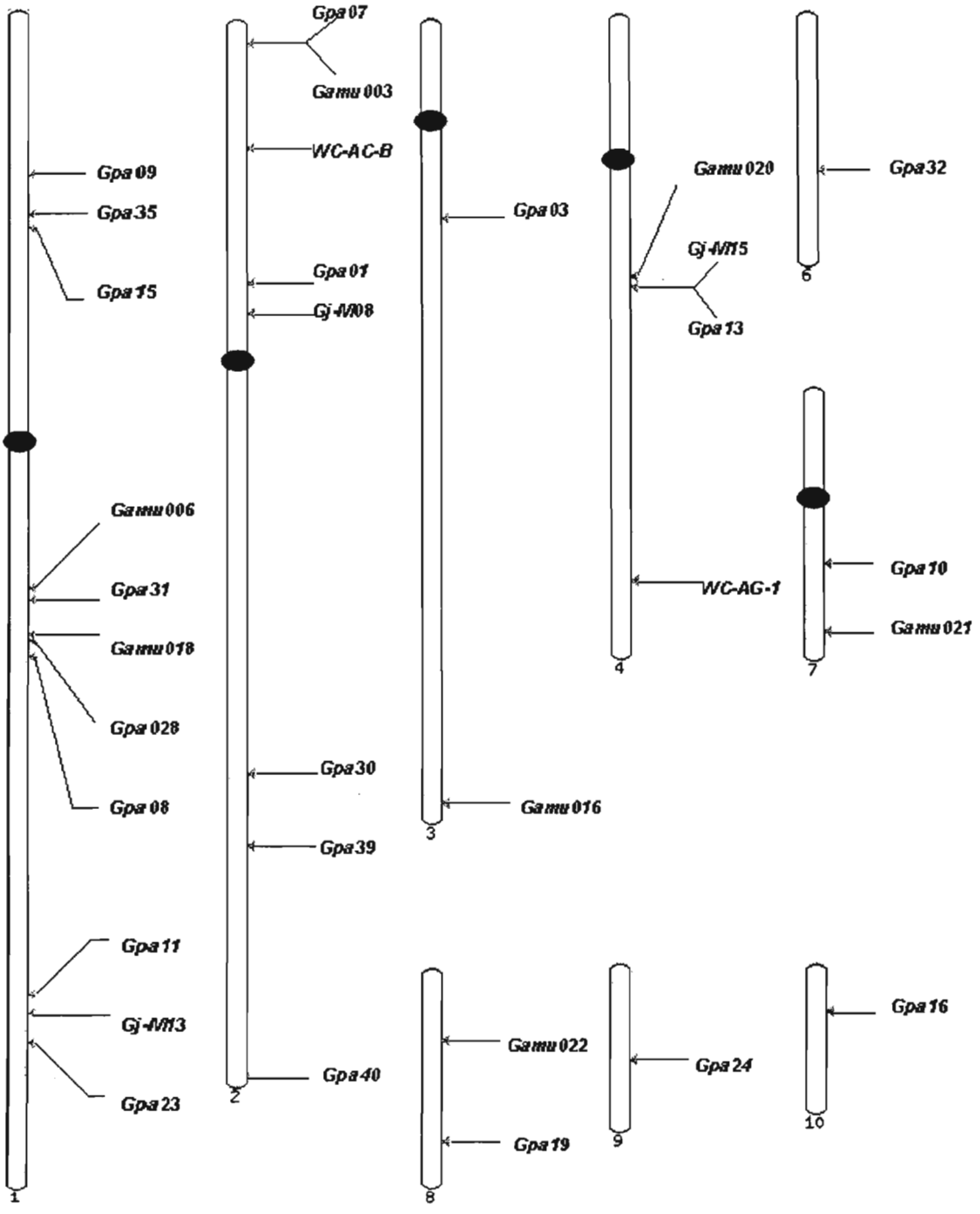


Figure 4.1 Predicted microsatellite map of the *Grus* genome. Numbers beneath each chromosome represent the chicken chromosome number (1 = Gga1)

Table 4.3 Location of centromeres relative to passerine (Table a) and *Grus* (Table b) microsatellite loci.

	Gga1	Gga2	Gga3	Gga4	Gga7	Gga8
Chromosome position relative to centromere	**Passerine locus (start position)**	**Passerine locus (start position)**	**Passerine locus (start position)**	**Passerine locus (start position)**	**Passerine locus (start position)**	**Passerine locus (start position)**
above (arm p)	*CmeH2* (60031161)	*Ck.5A4B* (47550206)	*POCC2* (7436863)	*Ck.1B6G* (20481179)	*POCC1* (6128827)	*ApCo81* (6429857)
centromere						
below (arm q)	*Pdoμ6* (75545595)	*Escμ4* (58831301)	*POCC4* (26179778)	*GCSW10* (34023048)	*PmaTAGAn71* (14215627)	*INDIGO27* (17712523)

(a)

	Gga1	Gga2	Gga3	Gga4	Gga7	Gga8
Chromosome position relative to centromere	***Grus* locus (start position)**	**Grus locus (start position)**	***Grus* locus (start position)**	***Grus* locus (start position)**	***Grus* locus (start position)**	***Grus* locus (start position)**
above (arm p)	*Gpa15* (34658225)	*Gj-M08* (40543025)	no loci above 7436863*	no loci above 20481179*	no loci above 6128827*	*Gamu022* † (9755662)
centromere						
below (arm q)	*Gamu006* (92001854)	*Gpa30* (104097032)	*Gpa03* (26926860)	*Gamu020* (36801073)	*Gpa10* (23875726)	*Gpa19* (23596115)

(b)

*, numbers are from passerine locus positions in table (a)

†, lies within the centromere boundaries provided by mapped passerine loci, therefore centromere location is undetermined

4.3.3 Blue crane sequences versus whooping crane and red-crowned crane sequences

Chromosomal locations of loci mapped using sequences from the blue crane were shown to be in strong agreement with the location of the same loci mapped using sequences from the source species (Table 4.4). Five loci, (*Gamu005, Gamu006, Gamu018, Gamu022 and Gj-M40*, Table 4.4), when mapped using blue crane sequences, were placed on the same chromosome as when mapped using sequences from the source species. Locus *Gamu015* produced no strong hits when mapped using whooping crane loci, but produced a unique hit on Gga4 when mapped using the blue crane sequence even though the sequences used for mapping were of a similar size (Table 4.4), (sequence similarity of 95 %). Similarly, locus *Gamu101* produced no strong hits using the whooping crane sequence but a unique hit at an E-value less than E^{-10} using the blue crane sequence (Table 4.4), a surprising result since the whooping crane sequence was the longer of the two and therefore was expected to be able to produce a stronger hit.

Table 4.4 Level of agreement between loci mapped using sequences from the source species and sequences from the blue crane (*Grus paradsiea*).

	Sequenced in source species					Sequenced in blue crane (*Grus paradisea*)				
Locus	Category of hit	Chr.	Start (bp)	E-val	Sequence length	Category of hit	Chr.	Start (bp)	E-val	Sequence length
Gamu005	Unique	2	3651573	2.4E-11	**189**	Unique at -05<E-value>-10	2	37337132	9.3E-08	188
Gamu006	Unique	1	92001854	4E-12	**129**	Unique at -05<E-value>-10	1	92001968	3.9E-08	125
Gamu015	No strong hits				**166**	Unique	4	87459080	2.6E-12	161
Gamu018	Unique	1	99490376	9.20E-15	179	Unique	1	99490388	6.8E-12	**188**
Gamu022	Unique	8	9755662	7.80E-23	189	Unique at -05<E-value>-10	8	9755733	2.10E-05	**190**
Gamu101	No strong hits				**223**	Unique at -05<E-value>-10	1	133097312	8.00E-05	194
Gj-M40	Mappable multiple hit	Unknown	149256605	3.30E-35	**487**	Mappable multiple hit	Unknown	149256605	3.30E-35	107

Chr, chicken chromosome on which the sequence hit;
Start, start position of sequence on the chicken chromosome;
E-value, measure of sequence similarity between chicken and *Grus* species;
Gamu and *Gj-M*, loci originally developed in whooping crane (*Grus americana*) and red-crowned crane (*G. japonensis*), respectively.

4.4 DISCUSSION

Thirty-three *Grus* loci were successfully mapped to the chicken genome to construct the predictive microsatellite map of the crane genome: the first for a non-passerine genus. This map provided an overview of the relative locations between *Grus* loci. In addition, the map was shown to be reliable in assigning the same position to a locus when using sequences from different species. This suggests that locus positions are conserved among members of the *Grus* genus, and may therefore be used to help create an unlinked set of microsatellite markers for parentage analysis in blue crane as well as other species within this genus.

4.4.1 Important considerations

Karyotypic differences between chicken and cranes must be considered when analysing this predictive map. Although there is evidence to suggest a highly conserved avian karyotype over 80 million years of evolution (Shetty *et al.* 1999) the importance of karyotypic differences between the chicken and the blue crane could only be fully understood upon completion of additional research. This could include a karyotype study of the blue crane to determine the extent of karyotypic difference between these two species. This is because only one species within the *Grus* genus has been the subject of a karyotypic study (whooping crane, Blederman *et al.* 1982); the chromosome number for blue crane is unknown. Secondly, additional research of the importance of karyotypic differences could be determined using comparative analyses between genomes of chicken and blue crane

when available, or even between chicken and other species with a similar karyotype to cranes. Work in the latter direction is underway with large-scale genome analysis proposed for several phylogenetically important avian taxa (as reviewed in Edwards *et al.* 2004).

4.4.2 Future directions

Predictive genome mapping in avian species has been made possible for those species with little sequence data available through the publication of the majority of the chicken genome. The possible applications of this map include identifying sets of unlinked loci for use in genotyping studies, as well as identifying sequences conserved between species. Furthermore, difference in genomes can help the study of genome evolution within avian lineages and between more distantly related groups such as birds, mammals, and reptiles.

Important for this study in the blue crane, the accuracy of the predicted map in assigning loci genetic locations would need to first be tested using comparisons to a linkage map rated for a *Gruiformes* species, as was done for *Passeriformes* in the predicted passerine map (Dawson *et al.* 2006). If proven accurate, the *Grus* map would provide a quick method of mapping microsatellite loci in the blue crane. This could assist in the identification and elimination of linked loci from a set of molecular markers to be used for parentage testing in this species.

The results from the predicted *Grus* microsatellite map shall be compared with linkage disequilibrium results during the characterisation of *Grus* microsatellite loci (Chapter 5). This would examine the level of agreement between these two methods of identifying possible linked loci, and to determine the accuracy of the map constructed here.

CHAPTER 5 CHARACTERISATION OF *GRUS* MICROSATELLITE LOCI

5.1 INTRODUCTION

The ability to extract genetic information to address ecological and population-related questions from a number of molecular markers, such as microsatellites, depends on the characterisation of statistical data (Sunnucks 2000). Statistical data in this study refers to linkage disequilibrium, null allele frequencies and Hardy Weinberg equilibrium studies among others. Markers not exhibiting suitable characteristics have been identified as having important negative effects on the accuracy and outcome of tests employing these markers (Selkoe *et al.* 2006), and must therefore be identified prior to practical applications of a marker set.

This chapter outlines the procedures undertaken to determine which of the *Grus* loci developed in this study, as well as loci developed in other studies and examined here in the blue crane, are suitable for inclusion in a set of markers. This set shall be used to perform parentage analyses in the blue crane (*Grus paradisea*). Initially, the sequences underwent three processes prior to characterisation (section 5.1.1). Thereafter, the analyses described in section 5.1.2 allow for the identification of undesirable characteristics present in loci during the selection of a marker set for forensic casework and parentage testing. In addition, section 5.1.3 aims to identify the extent to which *Grus* loci amplify cross-species with special attention being paid to amplification in humans, where the greatest potential for contamination lies.

The loci to be characterised in the blue crane originated from three sources: loci developed for the whooping crane (*G. americana*) by Glenn (1997), loci developed for the red-crowned crane (*G. japonensis*) by Hasegawa *et al.* (2000), and loci developed for this study of the blue crane. The loci developed for the blue crane were from two microsatellite libraries (an enriched and an unenriched library), both of which were developed by a laboratory technician (Gavin J. Horsburgh) based at the Sheffield Molecular Genetics Facility, UK.

A summary of the characteristics of the loci found to be suitable are compiled in two journal articles (section 5.5) aimed at, and written in the style of, Molecular Ecology Notes. This journal specialises in articles that characterise novel markers or characterise existing markers in a range of different species.

5.1.1 Pre-characterisation procedures

Checking for and deleting additional inserts in crane sequences

During the cloning process, random genomic fragments can be ligated together to form a chimeric sequence which is subsequently ligated into the cloning vector (Dawson *et al.* 2006). If multiple inserts are present, any future work using sequence information (such as chromosome mapping or primer design) may be jeopardised. It is therefore important to detect these chimeric inserts by identifying the enzyme used in fragmenting the DNA, such that only a single individual microsatellite sequence can be used.

Checking for duplicate sequences within and between microsatellite libraries

The aim of identifying duplicate sequences is to distinguish 100 % identical sequences from sequences that represent different alleles of the same locus. Duplicates may arise between independently generated libraries from the different species for which microsatellites were being examined: whooping crane, red-crowned crane and blue crane. Duplicates may also arise in a library when more than one fragment from the same locus is cloned and sequenced. Sequences identified as being 100 % identical were not submitted to European Molecular Biology Laboratories (EMBL).

Since the microsatellite library was developed using DNA from one individual (in this case blue crane sample J17718), it was possible for both alleles at one locus to be unintentionally isolated and sequenced. Importantly, when both alleles of a homozygous locus are isolated, the sequences would be 100 % identical because both the flanking and microsatellite repeat region would match. In contrast, if both alleles of a heterozygous locus are isolated, sequences would be shown to have matching flanking regions but differing

microsatellite repeat regions. These two sequences would still be duplicates of the same locus, but would not have identical sequence composition. Identifying duplicate sequences is therefore necessary to prevent designing and testing primers from the same locus. Although submitting 100 % identical sequences to EMBL is unnecessary, submitting different alleles of the same locus is beneficial as it provides a record of the different alleles that can be present at a particular locus in an individual.

Submitting sequences to EMBL

EMBL is a molecular biology research institution involved in maintaining the EMBL database. This database is a publicly available sequence database which allows for the addition or sourcing of genetic sequence data from different species. Its nucleotide sequence database can be useful when, for example, searching for existing microsatellite loci to be tested for potential in cross-species utilities (section 2.3.2). Furthermore, this database can be used to deposit sequence data obtained from any organism to make it available to other users. On submission a unique sequence-specific identity number, 'EMBL accession number', is generated which is necessary prior to submission of a paper to some journals in which the locus is characterised (e.g. Molecular Ecology Notes).

5.1.2 General locus characteristics

PCR conditions

These conditions refer to the optimised $MgCl_2$ concentrations, annealing temperature (T_a) used to amplify each pair of primers, number of annealing cycles, and the duration time for extension. Optimising these conditions to will allow for easy replication of PCR reactions throughout this study. Optimisation is essential to obtain high quality PCR products for accurate allele scoring during genotyping to minimise the number of genotyping errors known to affect relationship studies such as parentage analysis (Hoffman *et al.* 2005).

Hardy-Weinberg equilibrium

The ability of a locus to conform to Hardy-Weinberg equilibrium (HWE) is tested by determining the level of agreement between observed genotype frequencies and frequencies expected for a population experiencing random mating, no mutation, no drift and no migration (Selkoe *et al.* 2006). Deviations from HWE may be in the form of heterozygosity excess or heterozygosity deficit. A number of factors may cause deviations from HWE such as selection acting on certain alleles, inbreeding, null alleles (see below), biases towards typing particular genotypes and the presence of a sex-linked locus (Marshall *et al.* 1998; Selkoe *et al.* 2006). In addition, large allele drop out is known to cause an observed excess of homozygotes (Selkoe *et al.* 2006) and ultimately deviations from HWE. Large allele drop-out is observed when smaller PCR fragments are preferentially amplified rather than larger fragments, causing the larger fragments to occur in a lower concentration than the smaller fragments. If the concentration is too low, the result may be the failure of the allele to be detected after PCR. Consequently, the true heterozygous individual may appear to be homozygous at that locus.

Failure of loci to meet HWE is not a characteristic on which to exclude loci from a set of markers for genetic studies, but it does provide valuable information suggesting the need for further checks as to the suitability of the locus for parentage and population studies. Loci showing significant deviations from HWE should be checked for linkage disequilibrium, presence of null alleles or whether that locus is sex-linked. Loci exhibiting these factors should be excluded from a set of markers to be used in parentage testing as their ability to cause mis-assignment of parentage have been previously identified (Hoffman *et al.* 2005; Jones *et al.* 2003).

Despite deviations from HWE acting as a red flag for loci to be examined further should they used in parentage studies, loci not in HWE can have a positive role to play in populations studies. However, this is only true if the loci showing significant HWE deviations are not as a result of null-alleles or large-allele drop-outs. For example, recent population bottlenecks can be identified by a significant deficiency of heterozygous individuals (Jehle *et al.* 2002). This is because a heterozygosity deficit can be caused by

inbreeding (Fernandez *et al.* 2004) as a consequence of a small founder population after the bottleneck. Heterozygosity deficits however may also be caused by inadvertently analysing allele frequencies from two or more genetically distinct groups (Selkoe *et al.* 2006), suggesting population structure beyond that which is currently recognised.

Since the level of heterozygosity is also a measure of the population's ability to adapt to different environmental pressures (Amos *et al.* 2001), the ability to identify populations having a significant deficit of heterozygous individuals can help conservation management strategies in severe cases to increase genetic diversity within the population, such as that seen for the genetic management of captive whooping crane populations (Jones *et al.* 2002).

Null alleles

A null allele is any allele that is not detected by the assay used to genotype individuals at a particular locus (Marshall *et al.* 1998). When using microsatellite loci, null alleles arise most commonly when a mutation event has occurred at one or both of the primer binding sites preventing amplification of the allele. As a consequence, true heterozygotes may appear as a homozygote when genotyped, or true homozygotes may fail to produce any PCR product. The result is a lower observed heterozygosity estimate than what is expected. For genetic analyses such as parentage analysis that require low genotyping errors, loci exhibiting a high frequency of null alleles can confound results, and must therefore be identified prior to their use in parentage analyses. The presence of null alleles can be identified either by a significant deficit of heterozygotes, or by the genotypic incompatibility of individuals known to be related. The characterisation process would therefore allow for those loci with a high frequency of null alleles to be identified and assessed for accuracy in parentage analysis using known families.

Mode of inheritance

Determining the mode of inheritance is necessary during the characterisation process. Microsatellite markers used for parentage testing and most population genetic analyses can

only be used if the loci which they amplify are inherited in a Mendelian fashion. This pattern of inheritance defines the manner in which the alleles of a locus are passed on from parent to offspring, such that one allele present in an offspring is maternally inherited and the other paternally. Therefore, when many loci are analysed between the offspring and both parental candidates, an adult and an offspring can be identified as being related or unrelated by the presence or absence of shared alleles.

A survey by Selkoe *et al.* (2006) found an average of one locus out of 15 violated Mendelian inheritance. Therefore, the likelihood of obtaining such loci in this study was high due to the high number of loci tested (n = 28). Loci exhibiting non-Mendelian inheritance may be due to the presence of null alleles, since null alleles have been identified as causing incompatibilities between parent offspring genotypes (Selkoe *et al.* 2006). In addition, genotyping errors could result in allele mis-scoring which would have the same effect on parentage analyses as null alleles.

Linkage disequilibrium

The calculation of linkage disequilibrium between a pair of loci provides a statistical method of identifying linked loci. If two alleles at different loci were in some way associated, e.g. if situated close together on a chromosome such that they were inherited together, the result would be a pair of loci essentially acting as a single genetic unit. Consequently, the outcome of many genetic analyses, including parentage analyses would be compromised by the non-independent relationship between some of the loci involved (Falush *et al.* 2003; Jones *et al.* 2003). Ultimately this would lead to an increase in Type I errors. Therefore, one locus out of a pair displaying linkage disequilibrium is required to be excluded from the set of markers to be used in cases involving for example parentage testing.

Many statistical tests such as those involved in identifying linkage equilibrium involve multiple comparisons. However, analysing the raw data has revealed that the marking of component tests as statistically significant based on their single-test significance values was inappropriate as it yields too many significant results (Rice 1989). Without a correction for

multiple comparisons, a set of P-values would contain an over-estimated number of significant results. In this study, a Bonferroni correction (Rice 1989) was used during the identification of linked pairs of loci. Performing this correction controls for probability of incorrectly rejecting one or more null hypotheses (H_0) while simultaneously maintaining the ability to accurately detect any results rejecting H_0 corrected for multiple comparisons (Rice 1989).

Chromosomal location of genetic markers

Loci lying in close physical proximity on a chromosome may be genetically linked. Genetically linked loci are non-randomly associated and should be avoided during parentage analysis (where markers used are assumed to be randomly associated). For this reason a predicted microsatellite map was constructed to aid the selection of an independent set of markers (Chapter 4), and the results compared to those obtained from linkage disequilibrium analyses to determine the level of agreement between these two methods of determining whether association between pairs of loci exist.

Parentage

Parentage exclusion is the rejection of parental candidates based on parent-offspring incompatibilities found in the genotypic data (Jones *et al.* 2003). Following an example by Jones *et al.* (2003), if a mother and offspring have the diploid genotypes A/A and A/B, respectively, at a single Mendelian-inherited locus, then males with the genotypes A/C can be excluded whereas those with B/C cannot. The use of highly polymorphic loci, such as microsatellites, provides high powers of exclusion due to the low frequency with which the alleles occur in a population. Therefore, the probability of successful exclusion would therefore increase with an increase in the number of polymorphic loci used during parentage analysis.

In this study, the probability of excluding a single randomly-chosen unrelated individual from parentage was calculated using the software program Cervus 2.0 (Marshall *et al.* 1998). This program calculates two exclusion powers: the first parent exclusionary power

calculates the combined power of the set of loci to exclude a randomly-selected unrelated candidate parent from parentage of an arbitrary offspring, given only the genotype of the offspring; second-parent exclusionary power is the combined power of the set of loci to exclude a randomly-selected unrelated candidate parent from parentage of an arbitrary offspring, given the genotype of the offspring and of a known parent of the opposite sex. Values of exclusionary power are given as a proportion of 1.00, where 0.00 indicates no exclusionary power and 1.00 indicates complete exclusion (Marshall *et al.* 1998). The aim of this study was to obtain a set of microsatellite markers that achieve both first and second parent exclusionary powers close to 1.00 so that reliable parentage testing can be performed in the blue crane

5.1.3 Cross-species amplification of *Grus* loci in other species

Loci originally developed in whooping crane (Glenn 1997) and red-crowned crane (Hasegawa *et al.* 2000) were tested for cross-species amplification in the blue crane. In addition, further cross-species amplification of these loci and species-specific loci developed for this study, were described in a selection of nine other species. A selection of eight *Grus* loci had already been analysed in a number of crane species (Glenn *et al.* 1997), including the blue crane. However, only amplification success was published, therefore, amplification was performed again for these loci to determine the level of polymorphism and the suitability of the markers to perform parentage and population studies in the blue crane.

The crane species and other species used in this study were selected to examine the genetic distance over which the loci could cross-species amplify. Determining which loci are polymorphic in wattled crane and grey-crowned crane might assist genetic conservation projects on wattled crane (already underway, Jones *et al.* 2006) and grey-crowned cranes. Locus characteristics were not examined due to the sample size from each species (n = 10) being insufficient to accurately calculate heterozygosity estimates, null allele frequencies and conformity to HWE. The possibility of cross-species amplification in humans would also tested to identify which markers, if any, are at risk of amplifying human products.

Benefits of cross-species studies

The development of microsatellite libraries is costly and time-consuming. Therefore, the possibility of obtaining polymorphic loci through the testing of loci developed in other closely related species provides an attractive alternative method. Moreover, the avian genome is the smallest compared against reptilian, amphibian and mammalian genomes (Hughes *et al.* 1995). As the absolute number of microsatellites correspond positively with genome size (Primmer *et al.* 1997), avian genomes will therefore contain fewer microsatellites than other vertebrate taxonomic classes making the contributions of cross-species amplifying loci more worthwhile.

The number of publications in Molecular Ecology Notes reporting isolation of microsatellites have increased by more than 300 % in the two years between 1999 and 2001 (Zane *et al.* 2002). Therefore, the current chances of finding loci developed in a species closely related to species for which no loci have been developed has been greatly increased. If cross-species utility tests between distantly related species were to increase, the possibility of identifying near-universal markers (those markers that can amplify in a large selection of species from within the same taxonomic class) among those already isolated would also increase. An example of a near-universal locus is *Hru2* developed for the barn swallow (*Hirundo ristuca*) (Primmer *et al.* 1995) identified as being polymorphic in species as genetically distant from the barn swallow as the sage grouse (*Centrocercus urophasianus*) (Primmer *et al.* 1996) (delta $T_{50}H$ DNA-DNA hybridisation distance of 28, Sibley *et al.* 1990).

Due to the numerous benefits, cross-species analysis was performed for the *Grus* loci that had previously been developed in other studies, as well as *Grus* loci developed for this study. However, there are problems associated with cross-species amplification (Primmer *et al.* 2005), such as the presence of null alleles due to variation in the flanking region between species resulting in the failure of the primer to anneal and amplify (Jones *et al.* 2003). The cross-species amplification in this study provided a preliminary investigation of the *Grus* loci across a wide-range of species to determine potential for wide-range cross-species amplification of these loci.

5.2 METHODS

5.2.1 Pre-characterisation procedures

Checking for additional inserts in crane sequences

The restriction enzyme *MboI* (Qbiogene), used to restrict the blue crane DNA during library development, cleaves the DNA such that a cleaved DNA fragment can be identified by having a 'GATC' at the 5' and 3' fragment end. Therefore, this 'GATC' sequence can be used to identify chimeric DNA and the site at which the sequence was ligated into the plasmid vector. To identify these sequences, blue crane sequences (Appendix III) were first arranged in a FASTA-formatted document (the symbol '>' used before the sequence name to signal to the search engine that this line contains non-sequence data). The 5' and 3' 'GATC' sequence was located in each sequence, and the sequences upstream and downstream from the 5' and 3' 'GATC', respectively, were highlighted and recognised as being either chimeric or plasmid. Plasmid DNA was excluded from further analysis ensuring a) primers flank a true microsatellite locus and b) chromosomal locations of microsatellites were identified based on *Grus* microsatellite sequences.

Checking for duplicate sequences within and between microsatellite libraries

To identify all duplicate sequences, a stand-alone BLAST (following the protocol given below by Leviston *et al.* 2004) was performed for all sequences obtained from the blue crane as well as the unpublished sequences provided by Travis Glenn for the whooping crane (Appendix III). This protocol uses MS Dos as the running program to BLAST each sequence against all other sequences in the FASTA-formatted query file. Provided in the output file are aligned sequences and similar matches, if any, identified by the BLAST search. The output of the BLAST assigns an E-value to those sequences identified as having high sequence similarity to the query sequence. Sequences of high similarity (those sequences given an E-value $< e^{-10}$) were imported in to the sequence alignment program MEGA v3.1 (Kumar *et al.* 2004). The flanking regions and microsatellite repeat regions

were compared to determine whether sequences were 100 % identical or two alleles of the same heterozygous locus.

Prefixes used for Grus loci

When submitting sequences to EMBL, sequences generated for the blue crane from the *Grus* loci previously developed, *Gamu* and *Gj* loci, were submitted under the original locus names. The novel blue crane microsatellite sequences, developed for this study, were submitted using the prefix '*Gpa*'(***Grus paradisea***).

5.2.2 Samples used

Blood samples from 103 (102 + 1 duplicate sample) blue cranes were provided by WBRC and SACWG (section 2.2.1, and listed in Appendix I) and DNA extraction followed the ammonium acetate protocol (section 2.2.2). A selection of microsatellite loci originally developed in whooping crane (Glenn 1997), red-crowned crane (Hasegawa *et al.* 2000) and blue crane (developed for this study), respectively, were tested for amplification in the blue crane. A section of unrelated individuals for allele statistic analyses was achieved by excluding individuals that were ringed at the same GPS co-ordinates (the same nest site).

5.2.3 General locus characteristics

PCR conditions and genotyping

PCR reactions were carried out using the optimisation procedure described previously (section 2.2.6). Genotyping was carried out described elsewhere (section 2.3.6).

From the blue crane genomic microsatellite library, 19 microsatellite loci were tested to work with time constraints in finding a suitable set of microsatellite loci for parentage analysis. This number was selected because a large number of whooping crane and red-crowned crane loci had been characterised, and found to be highly informative, in the blue crane prior to the availability of species-specific blue crane microsatellite loci.

Locus statistics

Locus statistics refer to the number of alleles, Hardy-Weinberg equilibrium (HWE) estimates, null allele frequencies and exclusionary powers calculated for each locus. Exclusionary powers were calculated for each polymorphic locus as well as for all the polymorphic loci combined.

Genotypes from all loci that amplified in unrelated blue crane individuals tested were analysed with the software program Cervus 2.0 (Marshall *et al.* 1998) to calculate locus statistics. HWE was calculated by comparing observed heterozygosities with expected heterozygosities, and those that differed significantly were identified as showing significant deviations from HWE. However Cervus 2.0 was unable to calculate HWE estimates for many loci, and the reason given by Cervus 2.0 was due too few individuals present to allow the test to proceed. Therefore, HWE was also calculated using Genepop 3.4 (Raymond *et al.* 1995).

The frequency of null alleles was calculated by comparing expected and observed homozygosities (Marshall *et al.* 1998). Those loci with an excess of homozygotes are given a positive allele frequency, and contrastingly those loci with a deficit of homozygotes are given a negative allele frequency. Only those loci having a positive allele frequency greater that 0.1 were considered as having null alleles.

Mode of inheritance

One blue crane family comprising two parental individuals (mother, 4615; father: 4636) and two offspring (male offspring, 4626; female offspring, 4633) was analysed to confirm that each marker was inherited in a Mendelian fashion. Unfortunately, only a single blue crane family was available for this study. This must be taken into consideration, as the results obtained from the inheritance study using only one family may not be as accurate as a study with a sample size greater than one. The genotypes from each of the family members across all loci tested were entered into a spreadsheet. A locus was confirmed to be inherited in a Mendelian fashion when both alleles from each offspring could be assigned a

parental origin. Importantly, each allele was checked to have a different parental origin i.e. one allele identified as being paternally inherited and the other maternally, in order to fulfil the conditions of Mendelian inheritance.

Linkage disequilibrium

Linkage disequilibrium between all possible pairs of 28 polymorphic *Grus* loci in 56 unrelated blue crane individuals was calculated using Genepop 3.4 (Raymond *et al.* 1995). Two-digit Genepop input files were creating using the file conversion tool available in Cervus 2.0 (Marshall *et al.* 1998). The online version of Genepop was used with the following criteria: 'Test for each pair of loci in each population' with 'Demorization number', 'Number of batches', and 'Number of iterations per batch' set to 1000, 500, and 10000, respectively. Significant ($P < 0.05$) linkage disequilibrium P-values were corrected for multiple comparisons using the sequential Bonferroni method (Rice 1989).

Chromosomal location of genetic markers

The full description of analysis procedures and outcomes of mapping *Grus* microsatellite loci are provided in Chapter 4. Results obtained from the predicted chromosome map are compared here with the results from the linkage disequilibrium to determine the level of agreement between these two independent methods of identifying linked loci.

5.2.4 Further cross-species amplification of *Grus* loci

Twenty-eight polymorphic *Grus* loci (identified as polymorphic in section 5.3.2) were tested for utility in two other African crane species: grey-crowned and wattled crane. In addition, cross-species analysis was examined in non-Gruidae species including houbara bustard (*Chlamydotis undulata*), grey-headed albatross (*Diomedea chrysostma*), Seychelles warbler (*Acrocephalus sechellensis*), Cape parrot (*Poicephalus robustus*) and red jungle fowl (*Gallus gallus*). Two non-avian species tested included the salt-water crocodile (*Crocodylus porosus*) and human (*Homo sapiens*).

PCR reactions for non-*Grus* species took place using the optimised conditions for *Grus* loci (section 5.3.2). Touchdown PCR was performed for wattled and grey-crowned crane (both being *Grus* species) samples. High quality PCR products were required for genotyping to determine levels of polymorphism of *Grus* loci in these two species. Touchdown PCR (Don *et al.* 1991) is a method used to reduce the amount of spurious amplifications by using a range of annealing temperatures to amplify the PCR product, starting with the highest T_a. It begins to exponentially amplify the product which amplified at higher annealing temperatures as the T_a cycles through the lower temperatures. Products amplified at the higher temperatures have less non-specific amplification due to more stringent amplification conditions, resulting in the amplification of clean PCR products that are suitable for genotyping.

The temperature range used in the Touchdown profile for each primer pair was 3 °C above and below the previously optimised T_a. For example, a marker previously optimised at T_a of 52 °C had a Touchdown profile with the highest and lowest T_a of 55 °C and 49 °C, respectively. The PCR profile used was 94 °C for 3 min; 5 cycles of 94 °C for 30 s, highest T_a decreasing with 1 °C increments per cycle, 72 °C for 30 s; followed by 25 cycles of 94 °C for 30 s, lowest T_a for 30 s, 72 °C for 30 s, completing the profile with an extension at 72 °C for 4 min. Genotyping took place as described in section 2.3.6, however samples were analysed using an ABI 3100 DNA analyser, and not an ABI 3730.

Human DNA was initially tested for PCR suitability using unpublished human-derived primers HuMIF1 and HuMIR3 provided by M. Vaez (2006). Each 20 µl PCR reaction contained 2 µl reaction buffer, 0.2 µl 50 mM $MgCl_2$, 0.8 µl 5 mM dNTP, 0.4 µl 10 uM of each primer, 0.4 µl Taq DNA polymerase (BIOTAQ, Bioline Ltd., London, UK) in the manufacturers buffer (Final constituents: 16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl (pH 8.8 at 25 °C), 0.01 % Tween-20), 15.2 µl ddH_20, 0.6 µl 10ng/µl DNA. PCR conditions were 94 °C for 2 min; 40 cycles of 94 °C for 30 s, 58 °C for 27 s, 72 °C for 27 s; followed by a final extension time of 72 °C for 5 min.

5.3 RESULTS

5.3.1 Pre-characterisation procedures

Checking for and deleting additional inserts in crane sequences

The 'GATC' search identified two sequences having vector inserts: *Gamu012*, and *Gamu102* (Figure 5.1). These are indicated by red highlighting. Dark green indicates original primers sequences provided by Glenn (1997). Primers for *Gamu012* were not affected. However, *Gamu102* required a re-designed primer (light green highlights) to exclude the foreign sequence and enable amplification.

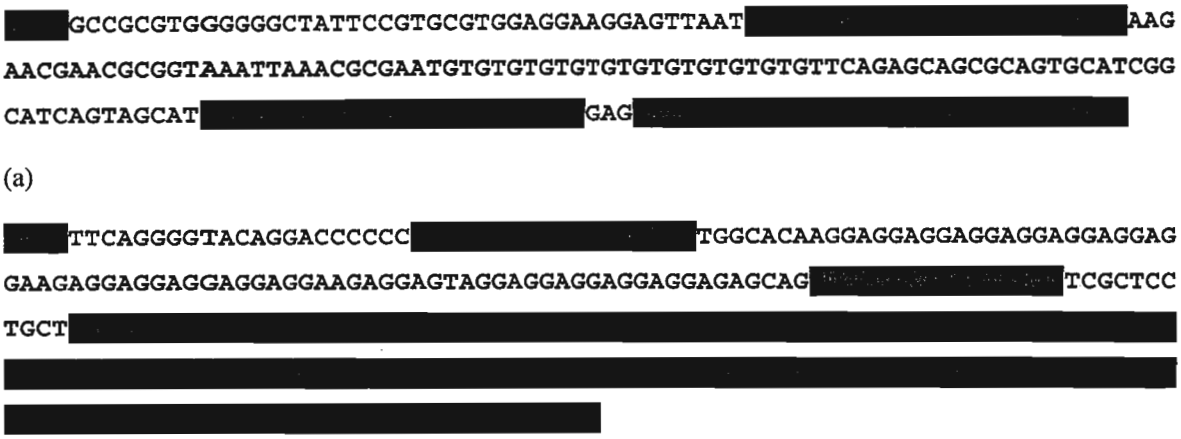


Figure 5.1 Full *Gamu*012 (a) and *Gamu*102 (b) sequences containing a vector insert (red highlighting) after the *MboI* 'GATC' restriction site (light blue highlighting).

Checking for duplicate sequences within and between microsatellite libraries

In this subsection, clones of the same locus having different accession numbers indicate that the sequences contain different alleles (have different allele sizes) of the same locus. In addition, clones of the same locus having the same accession numbers indicate that the two sequences were 100 % identical, and therefore no need to submit both sequences to EMBL.

Whooping crane loci: Thirty-three of the 47 whooping crane sequences (section 2.3.1) were identified as being unique. Two pairs of duplicate sequences were identified as being different alleles of the loci *Gamu004* and *Gamu021* and were submitted individually to EMBL (Table 5.1). A pair of 100 % identical sequences was generated from locus *Gamu011* (Table 5.1). Only one sequence out of this pair was submitted to EMBL.

Table 5.1 Duplicate clones identified in whooping crane (*Grus americana*).

Locus	Clones identifed as being duplicates	EMBL accession no. for whooping crane sequences
Gamu004	WC-AC-O	AM084714
	WC-AC-S	AM084715
Gamu011	ACC-1	AM084722
	WC-AC-II-D-pp	AM084722
Gamu021	WC-3C-3	AM084732
	WC-TG-3-C3	AM084737

Red-crowned crane loci: Seven red-crowned crane sequences were obtained from the NCBI database (section 2.3.2); no duplicates of these loci were identified to loci developed in the other two crane species.

Blue crane loci: Fifty-seven blue crane sequences were isolated from two genomic libraries (section 2.3.3), of which 15 sequences were identified as being duplicates of other sequences (Table 5.2). Therefore, 42 unique species-specific sequences were available for characterising in blue crane. Interestingly, six different alleles were sequenced in the locus *Gpa40* (Table 5.2) and the possible reasons for this are discussed below.

Six alleles of locus *Gpa40*: A single individual was used for the development of the microsatellite library. Therefore, theoretically only two alleles at most for each locus should be present. However, when a locus is duplicated by unequal crossing-over, retroposition, or chromosomal duplication (Zhang 2003), the possibility of finding multiple copies of the locus within the genome and therefore more than two alleles exists. Therefore, it is likely that *Gpa40* underwent a duplication event at least twice in order to observe the six alleles. This hypothesis was tested by BLASTing this locus against the chicken genome to

determine how many chromosomes, or locations within the same chromosome, this sequence hits (methods in section 4.2). This locus was found to hit three chromosomes (Table 5.3), with each hit having a low E-value and therefore strong sequence similarity to the *Gpa40* sequence.

Table 5.2 Duplicate clones identified in blue crane (*Grus paradisea*).

Locus	Clones identifed as being duplicates	EMBL accession no. for blue crane sequences
Gpa12	BC45E11	AM282894
	BC43C11	AM282893
Gpa13	BC41A05	AM282895
	BC43F05	AM282895
Gpa21	BC41C12	AM282904
	BC44H05	AM282903
Gpa22	BC44H03	AM282906
	BC45A01	AM282905
Gpa25	BC41B12	AM282910
	BC44G12	AM282909
	BC45B05	AM282909
Gpa26	BC45E05	AM282912
	BC45B07	AM282911
Gpa34	BC49A09	AM282920
	BC47A04	AM282920
Gpa40†	BC47H11	AM282926
	BC40G12	AM282927
	BC40H03	AM282928
	BC40H08	AM282928
	BC41B11	AM282929
	BC42B06	AM282930
	BC45D05	AM282931
Gpa42	BC41D11	AM282933
	BC49G12	AM282933

†, locus identified as having 6 alleles of different sizes

Table 5.3 Three locations where the microsatellite locus *Gpa40* scored a hit within the chicken genome.

Name	Start position (bp)	End position (bp)	E-val
Chr. 2	145769112	145769401	$4.5E^{-39}$
Chr. 3	102230035	102230221	$2.2E^{-20}$
Z_random	4483297	4483408	$3.6\ E^{-12}$

When amplified using the estimated annealing temperature (calculated from the melting temperature provided after primer design as being 59 °C), genotyping revealed non-specific products. Further optimization under a wide variety of annealing temperatures and $MgCl_2$ concentrations did not improve the possibility for allele scoring. This does not provide evidence to support duplication of locus *Gpa40* within the blue crane genome, since a similar PCR product size-range would still be expected to amplify if duplicate copies of this locus exist. However, this may be a result of the primers not being conserved to this locus, but with multiple sites of a similar sequence being present throughout the genome, thereby enabling the amplification non-specific PCR fragments.

Submitting sequences to EMBL

The 36 whooping crane sequences previously developed for this species (Glenn 1997) were submitted to EMBL by this study on behalf of Dr Travis Glenn (Table 5.4a). These 36 sequences represent 33 unique loci. In addition, 27 sequences generated from the respective whooping crane loci but sequenced in blue crane as part of this study were submitted (Table 5.4a). They were done so using the original locus name but with the species origin given as blue crane to distinguish between whooping and blue crane sequences. Seven unique red-crowned crane loci had previously been submitted to EMBL (Hasegawa et al. 2000). The respective blue crane sequences identified in this study were added to the EMBL database (Table 5.4b). Fifty-two blue crane sequences from the 42 unique species-specific loci developed in the two blue crane microsatellite libraries were submitted to EMBL (Table 5.4c). A summary of the loci currently available on the EMBL sequence database is provided (Table 5.5).

Table 5.4 All Grus microsatellite loci currently on the EMBL sequence database.

Table 5.4a Whooping crane (*Grus americana*) loci and EMBL accession numbers for their respective sequences. Whooping crane loci originally developed by Glenn (1997).

Locus	EMBL accession no. for whooping crane sequences‡	EMBL accession no. for blue crane sequences†	Locus	EMBL accession no. for whooping crane sequences‡	EMBL accession no. for blue crane sequences†
AA/GC	not submitted	AM168499	*Gamu018*	AM084729	AM168511
AA-1	not submitted	AM168498	*Gamu019*	AM084730	AM168512
AT/AC-1	not submitted	AM168500	*Gamu020*	AM084731	not submitted
Gamu002	AM084712	AM168501	*Gamu021*	AM084737	AM168515
Gamu003	AM084713	AM282934	*Gamu021*	AM084732	AM168515
Gamu004	AM084714	AM282935	*Gamu022*	AM084733	AM168513
Gamu004	AM084715	AM282935	*Gamu023*	AM084734	not submitted
Gamu005	AM084716	AM168502	*Gamu024*	AM084735	AM282937
Gamu006	AM084717	AM168503	*Gamu025*	AM084736	AM282938
Gamu007	AM084718	AM168504	*Gamu101*	AM084738	AM168516
Gamu008	AM084719	not submitted	*Gamu102*	AM084739	not submitted
Gamu009	AM084720	not submitted	*Gamu103*	AM084740	AM168517
Gamu010	AM084721	AM168505	*SHC-AG-1*	AM084741	not submitted
Gamu011	AM084722	AM168506	*SHC-AG-2*	not submitted	AM168522
Gamu012	AM084723	AM168507	*WC-2F-6**	AM084742	not submitted
Gamu013	AM084724	AM168508	*WC-4A-11**	AM084743	not submitted
Gamu014	AM084725	AM168509	*WC-4C-5**	AM084744	not submitted
Gamu015	AM084726	AM168510	*WC-AC-B**	AM084745	not submitted
Gamu016	AM084727	not submitted	*WC-AC-R**	AM084746	not submitted
Gamu017	AM084728	AM282936	*WC-AG-1*	AM084711	not submitted

‡, locus sequences not submitted were because only primer sequences were avialable for these loci

†, locus sequence not submitted due to the absence of a sequence caused by the failure of *Grus* primers to cross-species amplify in blue crane

*, loci not tested for polymorphism in blue crane

Table 5.4b Red-crowned crane (*Grus japonensis*) loci and EMBL accession numbers for their respective sequences. Red-crowned crane loci originally developed by Hasegawa *et al.* (2000).

Locus	EMBL accession no. for red-crowned crane sequences	EMBL accession no. for blue crane sequences*
Gj-M08	AB041858	AM168518
Gj-M11a	AB041859	AM282939
Gj-M13	AB041860	not submitted
Gj-M15	AB041861	AM168519
Gj-M34	AB041862	AM168520
Gj-M40	AB041863	AM168521
Gj-M48b	AB041864	AM282940

*locus sequence not submitted due to the absence of a sequence caused by the failure of *Grus* primers to cross-species amplify in blue crane

Table 5.4c Blue crane (*Grus paradisea*) loci and EMBL accession numbers for their respective sequences. Blue crane loci originally developed for this study.

Locus	EMBL accession no. for sequences	Locus	EMBL accession no. for sequences
Gpa01	AM085152	*Gpa24*	AM282908
Gpa02	AM282883	*Gpa25†*	AM282910
Gpa03†	AM282884	*Gpa25†*	AM282909
Gpa04†	AM282885	*Gpa26*	AM282912
Gpa05†	AM282886	*Gpa26*	AM282911
Gpa06†	AM282887	*Gpa27†*	AM282913
Gpa07†	AM282888	*Gpa28†*	AM282914
Gpa08†	AM282889	*Gpa29†*	AM282915
Gpa09†	AM282890	*Gpa30†*	AM282916
Gpa10†	AM282891	*Gpa31†*	AM282917
Gpa11	AM282892	*Gpa32†*	AM282918
Gpa12	AM282894	*Gpa33*	AM282919
Gpa12	AM282893	*Gpa34*	AM282920
Gpa13†	AM282895	*Gpa35*	AM282921
Gpa14	AM282896	*Gpa36*	AM282922
Gpa15†	AM282897	*Gpa37*	AM282923
Gpa16†	AM282898	*Gpa38*	AM282924
Gpa17†	AM282899	*Gpa39*	AM282925
Gpa18†	AM282900	*Gpa40*	AM282927
Gpa19	AM282901	*Gpa40*	AM282928
Gpa20†	AM282902	*Gpa40*	AM282929
Gpa21†	AM282904	*Gpa40*	AM282930
Gpa21†	AM282903	*Gpa40*	AM282931
Gpa22†	AM282906	*Gpa40*	AM282926
Gpa22†	AM282905	*Gpa41*	AM282932
Gpa23†	AM282907	*Gpa42‡*	AM282933

†, loci not tested for polymorphism in blue crane

‡, after EMBL submission this locus was found to be identical to locus *Gpa02*

Table 5.5 Summary of *Grus* microsatellite loci obtained from three species and available on EMBL sequence database

Source species of microsatellite loci	No. sequences	No. unique loci	No. duplicate loci	Total no. sequences available on EMBL	No. blue crane sequences available on EMBL
Whooping crane[1] (*Grus americana)*	37	33	4‡	36	27
Red-crowned crane[2] (*G. japonensis)*	7	7	0	7	6
Blue crane[3] (*G. paradisea)*	57	42	15	52	52
Total	148	82	19	95	85

[1]sequences and primers developed by Glenn (1997)

[2]loci originally developed by Hasegawa *et al.* (2000)

[3]loci developed for this study

‡, one sequence was identified after submission as being a duplicate

5.3.2 General locus characteristics

Of the 36 whooping crane loci tested for successful cross-species amplification in blue crane, 26 (72 %) successfully amplified a PCR product in blue crane (Table 5.6a). Of these, one locus (*AA-1*, Table 5.6a) produced a non-specific PCR product making allele scoring difficult, 13 loci were monomorphic, and 12 were polymorphic. All seven loci originally developed in the red-crowned crane (Hasegawa *et al.* 2000) were able to amplify clean PCR product in blue crane (Table 5.6b). Four loci were found to be monomorphic, and three were polymorphic. Out of 19 loci tested that were developed as part of this study, 15 (80 %) amplified a clean PCR product (Table 5.6c). Of these, 14 were polymorphic and 1 was monomorphic. In total, 28 loci were polymorphic in the blue crane. These loci were examined further to determine a set of suitable markers for parentage analysis based on their performance in various allele statistical analyses such as levels of polymorphism, mode of inheritance and linkage disequilibrium to determine their suitability for inclusion in a set of markers.

Seven *Grus* loci, originally isolated from a whooping crane microsatellite library and tested in this study, have been previously published in a cross-species amplification study by Glenn *et al.* (2007). In their study, *Glenn et al.* (1997) show all seven loci amplifying successfully in the blue crane. However, three of the seven loci (*AAC-1, ATC-1* and *SH(C)-AG-1*) tested in this study did not amplify any PCR product (Table 5.6).

Summary of statistics

Allele statistics were calculated using 17-56 unrelated blue crane individuals. The 28 loci displayed 2-40 alleles (Table 5.7), with an average of 10.68 alleles per locus, in the 17-56 blue crane samples tested.

Table 0.6 Conditions for the detection and amplification of *Grus* microsatellite loci in blue crane (*Grus paradisea*).

Table 0.6a Whooping crane (*Grus americana*) loci tested for polymorphism in the blue crane (*G. paradisea*)

Locus	Accession no. for whooping crane sequence	Accession no. for blue crane sequence	Repeat motif in whooping crane	Repeat motif in blue crane	Fluoro. Label*	Forward and reverse primer sequence (5' - 3')	n	N	$MgCl_2$ (mM)	T_a (°C)	Obs. allele size (bp) in blue crane
AA/GC	p/o	AM168499	-	$(GT)_3$	F-HEX, R-Pig	CCATTTTATCTGTCTCTGTTCA GGGCTTTCACTGATTACTTAA	11	11	1.5	56	211
AA-1 §	p/o	AM168498	-	$(CAAAA)_3$	F-FAM, R-Pig	CCCGTGGGGAATCCAG TCTATTGGTTTTGGCATGGA	87	103	1.5	56	219, 220, 221
AAC-1	p/o	n/s	-	-	F-HEX, R-Pig	GGAATTAACTGGACATGGG GCTATCTGGCACCTAAAAC	11	11	1.5	56	-
AT/AC-1	p/o	AM168500	-	$(TA)_5(CA)_5$	F-HEX, R-Pig	ATACTGGTAGTGCAGCTCC CCCGTCTTTGCTTGTCAT	11	11	1	56	110
ATC-1	p/o	n/a	-	-	F-FAM, R-Pig	GTAAGCCCAGGTATATTTGC AATCCTGATACTACAGTCAGAGAT	11	11	-	-	-
Gamu001	p/o	n/a	$(GT)_{18}(AT)(ATGT)_{24}$	-	F-HEX	TAATTATTATGAAATGGTGAGCA TGGAGCTGAGCGCGTTGA	11	11	-	-	-
Gamu002 †	AM084712	AM168501	$(AC)_{17}$	$(CA)_2$	F-HEX	AACTTTTGCCCATACTTCTGTAGTAAT GTTCCCGCTTCTTTTGCTGTGT	95	103	2	60	172, 174
Gamu003 †	AM084713	AM282934	$(TC)_{11}(AC)_8$	$(GT)_9(GA)_{10}$	F-HEX	CACATTGCCAGACTGTTGTAT ATCCCTGAAGCTAACAATAAACC	99	103	2	56	100, 102, 110, 112, 114, 118
Gamu004	AM084714	AM282935	$(AC)_6G(CA)_{10}$	$(CA)_5$	F-HEX	AAGGGGGGATGCAAGAAGT CCCGAGCCAGCACAAAGGA	11	11	2.5	55	180
Gamu005 †	AM084716	AM168502	$(GT)_{13}$	$(CA)_{10}$ TT $(GT)_5$	FAM	CGGATGTGTACTTGGCTCAGAA TTTCCCTGTGGTTAGTTGTGTG	23	27	2	56	182, 186
Gamu006 †	AM084717	AM168503	$(AC)_{12}$	$(CA)_{10}$	F-HEX	CACCTTTTATTGCGTATGTATTTT GGATTATGTTTTGGTTTTGTTTTT	101	103	2	55	115, 117, 119, 121, 123, 125, 127, 131, 133
Gamu007 ‡ *(PR)*	AM084718	AM168504	$(CA)_{13}$	$(CA)_{12}$ CTTATTCCT GGGCACACTT $(CCT)_3$	F-HEX, R-Pig	TAAAGGAGTGGCTGCTGCTGTG CTGAGGCTCTGCTGTGGGAAAC	95	103	1	59	144, 146, 148, 150, 152
Gamu008	AM084719	n/a	$(CA)_{11}$	-	F-HEX, R-Pig	GGCAGTCACTTTAGGAACCATACA AAAGAGAGATTGCAGAAGATAAGAACT	11	11	-	-	-
Gamu009	AM084720	n/a	$(GT)_{10}$	-	F-HEX, R-Pig	GAGTGGGAGGGGATAGGATGGATT AGCCTGACAGCAAGACCAAAGTAA	11	11	-	-	-
Gamu010	AM084721	AM168505	$(CA)_{11}$	$(CA)_6$	F-HEX, R-Pig	AATCTAATTCTTTTAGATTCTTTTAT GTTTTTTTGAATGAGGAATTAGAT	11	11	3	53	114
Gamu011 †	AM084722	AM168506	$(CCT)_6$	$(CCT)_8$	F-FAM, R-Pig	CAATGGAGCGCCGTCAC CAGTTTCTTGGCCGCTGTT	103	103	1	52	134, 137, 140, 144, 147, 150, 153
Gamu012	AM084723	AM168507	$(GT)_{12}$	$(GAA)_2$ .....$(GT)_4$	F-FAM	GCACGGAATGAATCTTTTCTGTGG GACCAACCATCTGCTCTCCAATTA	11	11	2	56	122
Gamu013 †	AM084724	AM168508	$(GT)_{10}$	$(GT)_{10}$	F-HEX, R-Pig	AATAAGTTTGGAAATGTTTCTCATA TTTTCTGGTCAATACTAAAGC	93	103	2	53	191, 193, 195
Gamu014 † *(VR, PR)*	AM084725	AM168509	$(GT)_{18}(ATGT)_{13}$	$(GT)_4(AT)_8$	F-FAM, R-Pig	CTTGTTCCACCGTTGTCAGC CAGAAGACTGCAATGAACTCCTG	88	103	1	53	118, 120, 122, 124

Table 0.6a cont.

Locus	Accession no. for whooping crane sequence	Accession no. for blue crane sequence	Repeat motif in whooping crane	Repeat motif in blue crane	Fluoro. Label*	Forward and reverse primer sequence (5' - 3')	n	N	$MgCl_2$ (mM)	T_a (°C)	Obs. allele size (bp) in blue crane
Gamu015	AM084726	AM168510	$(CA)_7T(AC)_4$	$(AT)_4$ G $(CA)_6$	F-HEX	CACAAAACTCCCACTAACTTCAAC CAGGAGGATAAGACTAAAACATCAAA	24	27	2	56	159
Gamu016	AM084727	n/a	$(AC)_8$	-	F-HEX, R-Pig	ATTTACATAGAATGTTGCTATTATTG ATTGCCTGCTGATGTTTTATTT	11	11	-	-	-
Gamu017	AM084728	AM282936	$(GT)_9$	$(GT)_6$ $(AT)_4$	F-FAM, R-Pig	ATTGCTGCTTACGAGACTCTG ATACCAGCGTACAAATGTAAGTG	11	11	1	58	259
Gamu018 †	AM084729	AM168511	$(GT)_3TT(GT)_8$	$(GT)_{13}$	F-HEX, R-Pig	TAGCGAGGGTCTGAGGAGAACT ACACCGTTATATCCTTCCCACT	87	103	2	56	182, 184, 188, 190, 192, 194, 196, 204
Gamu019	AM084730	AM168512	$(GT)_9$	$(GT)_3$	F-FAM, R-Pig	TATGAGGAGAGGCTGAGAAAC GTTGGACTCGATGATTCTTATG	22	27	1	53	153
Gamu020	AM084731	n/a	$(GT)_9$	-	F-FAM, R-Pig	TCCGCTGAAGGGCTATTTG CAACTTCCAGCCCACACCAC	11	11	-	-	-
Gamu021 (PR)	AM084732	AM168515	$(GT)_8GC(GT)_4$	$(GT)_6$	F-FAM, R-Pig	CGTGTGAACACTGGCAAAATA CAATGACTGCACCTGGCTAATG	11	11	2	60	145
Gamu022	AM084733	AM168513	$(GT)_9$	$(GT)_8$	F-FAM, R-Pig	TAGGGGAAAGCAAGATGAGAC AAGGACTGCATTTATGGTAGAC	11	11	1	58	197
Gamu023	AM084734	n/a	$(GT)_{10}$	-	F-HEX, R-Pig	CCAATTAGAGATACCACTGCTGTTT ATGCTCTAGGCTCTGCTGCTCTG	11	11	-	-	-
Gamu024 †	AM084735	AM282937	$(GT)_9$	$(CA)_8....(CCCA)_3$	F-FAM, R-Pig	TCTTCTTGCTAATCATCTTTCTAAC GCTGCCAGTACAGACCCTCTT	90	103	1	53	239, 251, 255, 261, 263, 267, 269, 271, 273, 275
Gamu025 †	AM084736	AM282938	$(AC)_{12}$	$(CA)_8$ TA $(CA)_2(TC)_3$	F-FAM, R-Pig	TTAATAAAAATCCACAGTGAAT GTTCTAGACCAGGACTGTTAATA	22	27	1	53	112, 114
Gamu101 † *(PR)*	AM084738	AM168516	$(ATT)_{16}GTT$ $(ATT)_4$	$(ATT)_6$ GTT $(ATT)_4$	F-FAM, R-Pig	CAGTATAAAAACAAACAGGTGAGA TGAAAAAAGTACAGGAGAACATAG	22	27	2	58	200, 249
Gamu102 (PR)	AM084739	n/a	$(AGG)_8AAG$ $(AGG)_5$	-	F-HEX, R-Pig	CCACCAGCACTGGCACA GCTGGCGGGTGAGGAG	11	11	-	-	-
Gamu103	AM084740	AM168517	$(AGG)_7$	$(AGG)_7$	F-HEX, R-Pig	TCAGCCCTTGGATGCGTCAG TGGGAGTCAGGAAAGCAAAGAG	11	11	1	58	249
SHC-AG-1	AM084741	n/a	$(AG)_{10}$	-	F-HEX, R-Pig	GACACACAGACTTCCTTTTGTCTT ATCAATGTTTCTCTCTCTTCCTC	11	11	-	-	-
SHC-AG-2	p/o	AM168522	-	$(CT)_8$	F-HEX, R-Pig	TCCAAGGGTGGTGATATCTTT ATTACAAAAGATAGAGGCTTGGAA	11	11	1	53	210
WC-AG-1	AM084711	n/a	$(AG)_9$	-	F-HEX, R-Pig	TTTCCCTTAGGAGATGCAAAC TTTACTAACACGGCTATTTTTCAA	11	11	-	-	-

*The 5' end of the forward primer in each pair was labelled with a fluorescent phosphoramidite and the "pigtail" (pig) sequence GTTTCTT was added to the some reverse primer, to reduce noise from variable adenylation during the PCR (Brownstein et al. 1996);

n, number of blue crane samples that amplified, N, total number of individuals tested; T_a, annealing temperature; PR, primer redesigned due to initial failure of the locus to amplify in blue crane; VR, vector removed; p/o, only primer sequence available; n/s, no sequence yet; n/a, did not amplify;

§, difficult to score due to the presence of non-specific amplification;

†, loci analysed further for levels of polymorphism;

‡, sex-linked locus.

Table 0.6b Red-crowned crane (*Grus japonensis*) loci tested for polymorphism in the blue crane (*G. paradisea*).

Locus	Accession no. for red-crowned crane sequence	Accession no. for blue crane sequence	Repeat motif in red-crowned crane	Repeat motif in blue crane	Fluoro. Label*	Forward and reverse primer sequence (5' - 3')	n	N	$MgCl_2$ (mM)	T_a (°C)	Obs. allele size (bp) in blue crane
Gj-M08	AB041858	AM168518	$(TC)_{10}$	$(TC)_6$ ATAGTAT $(GT)_2$ AT $(GT)_3$ ATG $(TA)_5$	F-HEX	TCCGTCAAGCTTTTAGTCAT TACAGTTAATGTGGGTGCAA	21	27	2.5	55	100
Gj-M11a §	AB041859	AM282939	$(CA)_{12}$	$(CA)_8$	F-HEX	TGGGGTGCAGTTCAAATAAGCG TTGCATCAAAAAGGACATGC	11	11	5	55	196
Gj-M13	AB041860	n/s	$(AC)_{10}$	-	F-HEX	TCTGCATGCGTCCTGCCTCCAAGA TGCCTTGCACAGGCAGGTGAAATG	11	11	2	55	153
Gj-M15 (PR) †	AB041861	AM168519	$(CA)_{13}$	$(GT)_3$ GCGTGC $(GT)_{11}$	F-FAM, R-Pig	TCTACCAGATATCATCAGAGCTTGC TGCGAATGAACAGATGGCCCCAAGA	103	103	2	59	106, 110, 112, 116, 118, 120
Gj-M34 †	AB041862	AM168520	$(AC)_{19}$	$(CA)_7$....$(GACA)_3$	F-FAM	TGCTCAACATTCATCAGGATTTGGG TCCCTCTGGTGTTGGCTGAAAATAC	102	103	2	59	114, 122, 124, 126, 130
Gj-M40	AB041863	AM168521	$(AC)_9$	$(CA)_9$	F-FAM	TGGGAAATCCTGAAATTCTGCTA TGAGGAATGAGCGATGCTTGTTTCA	11	11	2	55	102
Gj-M48b (PR) †	AB041864	AM282940	$(GT)_{14}$	$(CA)_7$	F-HEX, R-Pig	GATCCTGGGGGTTTTGTTG GGACCCTCCACCGAGAAG	67	103	1	58	204, 206, 208, 210, 212, 214, 216, 218

*The 5' end of the one primer in each pair was labelled with a fluorescent phosphoramidite and the "pigtail" (pig) sequence GTTTCTT was added to the other primer, to reduce noise from variable adenylation during the PCR (Brownstein et al. 1996);

n, number of blue crane samples that amplified; N, total number of individuals tested; T_a, annealing temperature; n/s, no sequence yet; PR, primer redesigned due to initial failure of the locus to amplify in blue crane;

§, difficult to score due to the presence of non-specific amplification;

†, loci analysed further for levels of polymorphism.

Table 0.6c Loci originally developed and tested for polymorphism in 103 blue crane (*Grus paradisea*) individuals.

Locus	Accession no.	Repeat motif	Fluoro Label*	Forward and reverse primer sequence (5' - 3')	n	$MgCl_2$ (mM)	T_a (°C)	Obs. allele size (bp)
Gpa01†	AM085152	$(CA)_8$	F-HEX, R-Pig	TTTGGTTGCCGTCAGAATTG CTGACCTGGGTGTGTTCTGC	102	3	63	291, 293, 297, 299
Gpa02	AM282883	$(CTT)_2$ $(CCTT)_{17}$ CCT CTCT T $(CCTT)_2$	F-HEX	GATCGCTGACAGTTTTTTTC TGTGTGTTAGTGAGGAGTAACG	103	-	-	n-s
Gpa11†	AM282892	$(CT)_5$... $(TTTC)_{34}$... $(TTTC)_2$ C $(CT)_7$ TTTC	F-HEX, R-Pig	CCCTCCTGGAATACATGACAAA AAGTCATTCTCAACAAGAAAGGAAAA	101	3	60	186 - 364
Gpa12†	AM282893	GATG $(GA)_2$ $(GATA)_{11}$	F-FAM, R-Pig	GATCAATGGAAGGATAGGGAGGT TCATCAATCTATTATTTGCCTCAGC	100	2	60	196, 200, 204, 212, 216, 220, 224, 228, 232, 236,
Gpa14†	AM282896	$(TTTC)_{12}$ ... $(TTTTC)_2$ TTTC TTTTC TTTC TTTTC	F-FAM, R-Pig	TTTCGTACTCTGGTCATTGGATTT AATAGGACAGCAGTGCTAAGAAGAAA	95	2	57	278 - 324
Gpa19	AM282901	$(GTTA)_{12}$	F-FAM, R-Pig	GATCTAATAGACTTTAGTTAGTTAGTTAGTTAGTTA TGATTTAGCAGTAGAAGAGAAATAAA	94	-	-	n-s
Gpa24†	AM282908	$(GTTA)_6$	F-HEX, R-Pig	GAGGGAATCTAGCACGCTCCAA TCAAAGCATCGTGTCCATGAAGT	100	2	57	210, 222, 229, 231,239, 243, 247, 251, 255
Gpa26	AM282911	$(GATA)_{11}$	F-FAM, R-Pig	AACTAGGTTAACAGATGCATTAACAGG GGAAGTGGCGGGTATAGGAA	72	2	54	220
Gpa32†	AM282918	$(GT)_{11}$	F-HEX, R-Pig	CCCAGCACACCGTGCATAAG GCAGTCGGTCACATCCTTGG	100	2	57	176, 181, 183, 185, 187, 189, 191, 193
Gpa33†	AM282919	$(TCTTTC)_2$...$(TTTC)_{14}$ $(TCTCTTTC)_{16}$ $(TC)_2$ $(TCTCTTTC)_3$ $(TTTC)_7$	F-FAM, R-Pig	GGCTTAGAAATGGGATACAGTTG CCGTCCAAGCAAGAAGAAA	98	3	60	205 - 416
Gpa34†	AM282920	$(TCTTTC)_2$ TTCTC $(TTTCTC)_{22}$	F-HEX, R-Pig	GCACACAGTGAGGAGACCAGTGA CCTTGATGTGGGAAGACAACTGC	86	2	63	132 - 413
Gpa35†	AM282921	$(CATA)_3$ TAT $(GT)_{13}$ $(TA)_4$	F-FAM, R-Pig	TCATCAGCTTCCAACAGGTCTCC TCAGGCACAATGTATAAGTGTTTGTGG	97	2	57	159, 161, 166, 168, 172, 174, 176, 178, 180, 182,
Gpa36†	AM282922	$(GATA)_{10}$ $(GATG)_2$	F-HEX, R-Pig	TTCATAGACATATGCTTACCTGTTCT ATCCATCCATCTATCTATCTATCTATCTATC	100	2	60	220, 224, 228, 232, 236, 240
Gpa37†	AM282923	$(TC)_2$ CTC $(TTTC)_{43}$ $(TTC)_2$	F-HEX, R-Pig	TGAACGTGTCTGATTTAAGGAA AAAGTAGTCACTAGCCTGGGTTT	98	3	60	120 - 420
Gpa38†	AM282924	$(CTAT)_{13}$	F-HEX, R-Pig	GGGCAGAAGCAAGTCTTTCA GAAGATGTTTGCTGGTTGCAC	102	2	60	180, 184, 188, 192, 196, 200
Gpa39†	AM282925	$(GA)_2$ $(GATA)_{13}$	F-HEX, R-Pig	TGCACAGGTTTGGCCAAGAAG TTCCAAAGTGAAATTAAAGGTGTGTGG	97	2	57	102, 110, 114, 118, 120, 122, 126, 130, 134, 138,
Gpa40	AM282926	$(TTTC)_{15}$ $(T)_{18}$	F-FAM, R-Pig	GGGTGTGGTTTAGGGATGAGGA CAGTCCCTGGCGGACCATAG	103	-	-	n-s
Gpa41†	AM282932	TTCTTTTC $(TTTCTTCC)_6$ $(TC)_5$ $(TTTCTCTC)_3$ TTTC $(TTTCTCTC)_3$...$(TTTC)_2$	F-FAM, R-Pig	AATTCAGTCTAATGTAAATGTCCAAG CCATCATTAATGGAAAGAAAGAG	98	2	54	223, 229, 233, 235, 237, 239, 245, 249, 251, 253,
Gpa42	AM282933	$(GA)_3$ AAAA $(GA)_2$ $(GGAAGAGA)_9$ $(GGAA)_{16}$	F-FAM, R-Pig	TGTGTTAGTGAGGAGTAACGACAATTA GATCCAGGAGTGCTGACAATTT	103	-	-	n-s

*The 5' end of the one primer in each pair was labelled with a fluorescent phosphoramidite and the "pigtail" (pig) sequence GTTTCTT was added to the other primer, to reduce noise from variable adenylation during the PCR (Brownstein et al. 1996);

n, number of blue crane samples that amplified; T_a, annealing temperature; n-s, non-specific amplification

†, loci analysed further for levels of polymorphism

Table 5.7 Summary of characteristics of 28 polymorphic loci amplified in blue crane (*Grus paradisea*).

Locus§	k	n	N	H(O)	H(E)	Excl(1)	Excl(2)	HW (Cervus)	HW (Genepop)	Null freq.
Gamu002	3	52	57	0.212	0.338	0.056	0.162	NA	*	0.2149
Gamu003	6	55	57	0.109	0.381	0.074	0.201	NA	*	0.5809
Gamu005	2	17	17	0.000	0.214	0.022	0.093	NA	*	0.9352
Gamu006	8	55	57	0.709	0.757	0.359	0.536	NS	NS	0.0264
Gamu011	7	56	57	0.643	0.721	0.320	0.501	NS	NS	0.0566
Gamu013	3	52	57	0.462	0.441	0.096	0.212	NA	NS	-0.0449
Gamu014	4	49	57	0.408	0.688	0.247	0.405	**	NS	0.2420
Gamu018	8	49	57	0.653	0.691	0.285	0.466	NS	*	0.0134
Gamu024	8	52	57	0.596	0.755	0.364	0.547	NS	**	0.1038
Gamu025	2	16	17	0.000	0.226	0.024	0.097	NA	*	0.9442
Gamu101	2	16	17	0.063	0.175	0.014	0.078	NA	*	0.4491
Gj-M15	6	56	57	0.732	0.800	0.415	0.594	NA	NS	0.0430
Gj-M34	4	56	57	0.821	0.680	0.241	0.398	**	NS	-0.1115
Gj-M48b	8	40	57	0.675	0.720	0.315	0.494	NS	*	0.0261
Gpa01	4	55	57	0.527	0.571	0.174	0.339	NS	NS	0.0372
Gpa11	40	56	57	0.786	0.972	0.864	0.927	NA	**	0.1019
Gpa12	10	56	57	0.821	0.860	0.539	0.703	NA	NS	0.0134
Gpa14	14	51	57	0.843	0.873	0.576	0.732	NA	NS	0.0161
Gpa24	7	55	57	0.527	0.623	0.207	0.353	NS	*	0.0815
Gpa32	8	55	57	0.800	0.806	0.437	0.614	NS	*	-0.0094
Gpa33	39	53	57	0.887	0.965	0.839	0.912	NA	**	0.0375
Gpa34	26	49	57	0.857	0.941	0.758	0.862	NA	*	0.0425
Gpa35	11	52	57	0.769	0.808	0.444	0.620	NS	NS	0.0191
Gpa36	6	55	57	0.509	0.616	0.208	0.374	NS	**	0.0946
Gpa37	41	53	57	0.868	0.966	0.841	0.913	NA	**	0.0488
Gpa38	6	55	57	0.709	0.785	0.386	0.566	NS	NS	0.0483
Gpa39	10	53	57	0.830	0.812	0.446	0.622	NA	NS	-0.0173
Gpa41	6	54	57	0.574	0.598	0.194	0.357	NS	NS	0.0265
Summary:										
Mean number of alleles per locus:						10.68				
Mean proportion of individuals typed:						0.88				
Mean expected heterozygosity:						0.67				
Total exclusionary power (first parent):						**1.00**				
Total exclusionary power (second parent):						**1.00**				

k, number of alleles; n, number of unrelated blue crane individuals that amplified a PCR product; N, number of individuals tested; H(O), observed heterozygosity; H(E), expected heterozygosity; Excl (1), primary exclusionary power; Excl (1), secondary exclusionary power; HW, deviation from Hardy Weinburg equilibrium calculated using Cervus 2.0 (Marshall *et al.* 1998) and Genepop (Raymond et al. 1995); Null freq, null allele frequency.

§, *Gamu* loci originally developed in whooping crane *Grus americana* (Glenn 1997); *Gj-M* loci originally developed in red-crowned crane *G. japonensis* (Hasegawa et al 2000); *Gpa* loci originally developed as part of this study.

NA, not able to be calculated due to insufficient information; NS, non-significant

*significant at the 5 % level; **significant at the 1 % level

Hardy-Weinberg equilibrium

In total, 17 loci were found to show significant deviations from HWE. Cervus 2.0 (Marshall *et al.* 1998) identified two loci (*Gamu014* and *Gj-M48b*) as having significant ($P < 0.01$) deviations from HWE (Table 5.7). In contrast, Genepop 3.4 (Raymond *et al.* 1995) identified 15 loci as having significant deviations from HWE below the 5 % level (Table 5.7). Interestingly, the two loci identified by Cervus as having significant deviations from HWE were identified as being non-significant when Genepop 3.4 was used. H(O) ranged between 0.000 (*Gamu005* and *Gamu025*) and 0.887 (*Gpa33*); H(E) ranged between 0.175 (*Gamu101*) and 0.972 (*Gpa11*) (Table 5.7).

The 15 loci identified by Genepop as having significant deviations from HWE were shown to have a deficit of heterozygotes; of these, seven loci were also predicted to have high null allele frequencies (Table 5.7). Similarly, *Gamu014* identified by Cervus as showing significant deviations from HWE had a deficit of heterozygotes and a high null allele frequency (Table 5.7). In contrast, locus *Gj-M34* identified by Cervus as showing significant deviations from HWE had an excess of heterozygotes and consequently a low null allele frequency (Table 5.7). Of the two loci that had an excess of heterozygous individuals (*Gamu013* and *Gpa39;* Table 5.7), only locus *Gj-M34* had a significant excess (Table 5.7).

Null allele frequency

Eight loci showed a high frequency of null alleles (Null freq > 0.1, Table 5.7). These loci were also identified as having significant ($P < 0.05$) deviations from HWE. This would be expected since the presence of null alleles is often indicated by an excess of homozygotes. Therefore it would be expected that those loci having a high null allele frequency would also have significant deviations from HWE. The highest null allele frequency was 0.9442 (*Gamu025*), a locus for which no heterozygotes were observed and had the smallest number of alleles ($k = 2$) (Table 5.7). In this locus, 16 out of 17 individuals amplified a product. All loci with two alleles showed high null allele frequencies and consequently significant deviations from HWE (Table 5.7). *Gj-M34* showed as having the lowest null

allele frequency (-0.1115) as a consequence of a significant ($P < 0.01$) excess of heterozygotes.

Exclusion

Locus *Gpa11* showed the greatest first parent exclusionary power (0.864), with locus *Gamu101* showing the lowest (0.014) (Table 5.7). Second parent exclusion power ranged between 0.093 (*Gamu005*) and 0.927 (*Gpa11*). The total exclusionary power for the 28 microsatellite loci was calculated to be 1.000 for both first and second parent exclusionary powers (Table 5.7).

Inheritance patterns

Seventeen of the 28 polymorphic loci were consistent with Mendelian patterns of inheritance (Table 5.8). Of the remaining 11 loci, eight had insufficient genotyping data after two repeated genotyping runs for the detection of the inheritance pattern. An additional three loci (*Gpa39, Gamu003* and *Gamu018*) were identified as showing characteristics of a non-Mendelian locus (Table 5.8). Although the genotype of the female parent from *Gamu003* and *Gamu018* are absent, there was an absence of shared alleles between the father and one offspring (*Gpa39*, son; *Gamu003,* daughter) (Table 5.8). Similarly, an absence of shared alleles between the son and both the parents was found for locus *Gpa39* (Table 5.8).

Table 0.8 Genotypes of four related blue crane (*Grus paradisea*) individuals across 28 loci (*Gpa01 – Gj-M48b*) to reveal patterns of inheritance. Black, loci displaying non-Mendelian inheritance, and the alleles causing parent-offspring incompatibilities; grey, loci for which the inheritance pattern could not be determined due to an absence of genotypic data.

Sample	Relationship	Sex*	*Gpa01*	*Gpa11* †	*Gpa12*	*Gpa14*	*Gpa24*	*Gpa32*	*Gpa33*
4636	father	male	297 297	230 284	0 0	298 306	247 251	185 191	235 267
4615	mother	female	291 293	198 220	232 236	286 324	247 247	187 189	209 239
4626	offspring	male	291 297	198 230	224 232	286 306	247 247	185 187	209 235
4633	offspring	female	293 297	220 230	232 236	286 298	247 251	185 189	209 235

Sample	Relationship	Sex*	*Gpa34*	*Gpa35*	*Gpa36*	*Gpa37*	*Gpa38*	*Gpa39*	*Gpa41*
4636	father	male	237 255	172 172	220 228	222 246	192 200	118 130	229 245
4615	mother	female	267 375	172 176	228 228	226 226	188 188	114 130	245 245
4626	offspring	male	255 267	172 176	228 228	226 246	188 192	130 134	229 245
4633	offspring	female	237 267	172 176	228 228	226 246	188 200	114 118	229 245

Sample	Relationship	Sex*	*Gamu002* †	*Gamu003* †	*Gamu005* †	*Gamu006* †	*Gamu011*	*Gamu013*	*Gamu014* †
4636	father	male	173 173	114 114	186 186	115 123	140 146	193 193	0 0
4615	mother	female	0 0	0 0	0 0	115 125	140 149	191 193	0 0
4626	offspring	male	173 173	114 114	0 0	115 115	140 146	191 193	122 122
4633	offspring	female	173 173	100 100	0 0	115 115	140 149	193 193	122 122

Sample	Relationship	Sex*	*Gamu018*	*Gamu024* †	*Gamu025* †	*Gamu101* †	*GjM15*	*GjM34*	*GjM48b*
4636	father	male	192 194	0 0	0 0	0 0	106 116	124 130	206 206
4615	mother	female	0 0	0 0	0 0	0 0	106 106	124 124	0 0
4626	offspring	male	188 196	251 261	0 0	0 0	106 106	124 130	0 0
4633	offspring	female	192 196	251 273	113 113	200 200	106 116	124 130	206 218

Gpa, loci developed from blue crane genomic library (section 2.3.3)
Gamu, loci developed from whooping crane genomic library (section 2.3.1)
Gj-M, loci developed from a red-crowned crane genomic library (section 2.3.2)
*Sexes were confirmed using P2/P8 and 2550F/2718R primers (section 3.2)
†, loci previously identified as having a high null allele frequency (section 5.3.2)

Linkage disequilibrium

Four sets of loci were identified as showing significant deviations from linkage equilibrium after corrections for multiple comparisons: *Gpa12* and *Gpa36* (P = 0.00233); *Gamu006* and *Gamu013* (P = 0.00073); *Gamu024* and *Gj-M15* (P = 0.00152); *Gj-M15* and *Gj-M48b* (P = 0.00164). When each of these loci were checked for its location on the predicted microsatellite map, only *Gamu006* and *Gj-M15* could be assigned a unique hit on a chromosome (Gga1 and Gga4, respectively, Figure 4.1, page 71). Therefore, the level of agreement between the predicted microsatellite map and linkage disequilibrium results could not be examined.

5.3.3 Further cross-species amplification of *Grus* loci

Amplification of human DNA

The two human DNA control samples were confirmed to be PCR quality as a clear product was observed on a 2 % agarose gel when the samples were amplified using unpublished human primers HuM1F1 and HuM1R3 provided by M. Vaez (Figure 5.2). The expected product size in human ranges between 200-300 bp (Vaez 2006, pers. comm.). The products amplified in the two human samples were approximately 271-281 bp determined by the Phi-X174 DNA/HaeIII Digest (ABgene).

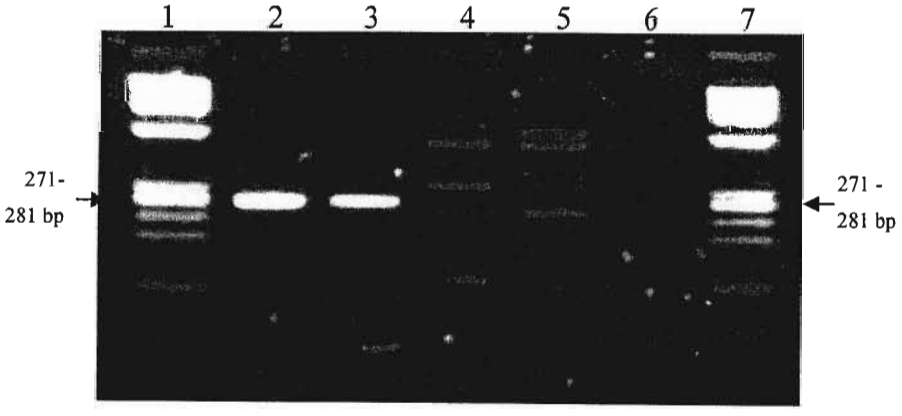


Figure 5.2 Amplification of human-derived primers Hum1F1 and Hum1R3 in humans (*Homo sapiens*) and the blue crane (*Grus paradisea*). 1, phiX174HaeIII ladder; 2, human (male); 3, human (female); 4, blue crane; 5, blue crane; 6, water; 7, phiX174HaeIII ladder

Amplification of Grus *loci*

Locus *Gamu011* amplified in six avian species (Table 5.9). In addition, this locus was observed to amplify across the greatest evolutionary distance (a delta $T_{50}H$ DNA-DNA hybridisation distance of 28, Sibley *et al.* 1990). Similarly, *Gamu003* (Table 5.9) and *Gpa11* (Table 5.10) amplified in five species each. No cross-species amplification was observed for locus *Gamu013* (Table 5.9). None of the 28 *Grus* loci tested were able to amplify a product in humans (Table 5.9 and Table 5.10).

With the exception of *Gamu013*, all loci (n = 27) identified as polymorphic in blue crane amplified a product in wattled and grey-crowned cranes (Table 5.9). Furthermore, a large number of *Grus* loci were shown to successfully cross-species amplify in grey-headed albatross (29 % of *Grus* loci tested, Table 5.9 and Table 5.10) and Cape parrot (32 % of *Grus* loci tested, Table 5.9 and Table 5.10).

Level of polymorphism of Grus loci in wattled cranes and grey-crowned cranes

Of twenty-eight loci that were genotyped in 10 wattled cranes and 10 grey-crowned cranes, 27 were polymorphic (Table 5.11). *Gamu013* amplified non-specific PCR products and could therefore not be assigned allele sizes. Similar allele sizes in 26 loci were observed between the blue, wattled and grey-crowned cranes. Interestingly, *Gj-M48* allele sizes in blue crane were approximately 100 bp higher than those observed for the other two crane species (Table 5.11).

Table 5.9 Cross-species amplification of 10 whooping crane (*Grus americana*) and three red-crowned crane (*G. japonensis*) loci in nine species spanning seven taxonomic orders.

Species	Order	n	Genetic distance to *blue crane* ‡	*Gamu 002*	*Gamu 003*	*Gamu 005*	*Gamu 006*	*Gamu 011*	*Gamu 013*	*Gamu 014*	*Gamu 018*	*Gamu 024*	*Gamu 025*	*Gamu 101*	*Gj-M15*	*Gj-M34*	*Gj-M48b*
Grey-crowned crane* *Balearica regulorum*	*Gruiformes*	*10*	0.7	+	+	+	+	+	-	+	+	+	+	+	+	+	+
Wattled crane* *Grus carunculatus*	*Gruiformes*	*10*	0.7	+	+	+	+	+	-	+	+	+	+	+	+	+	+
Houbara bustard† *Chlamydotis undulata*	*Gruiformes*	*1*	16.9	-	+	-	-	-	-	-	-	-	-	-	-	-	-
Grey-headed albatross† *Diomedea chrysostma*	*Procellariiformes*	*1*	20.1	-	+	+	-	+	-	-	-	-	-	-	-	-	-
Seychelles warbler† *Acrocephalus sechellensis*	*Passeriformes*	*1*	21.6	-	-	-	-	+	-	-	-	-	-	-	-	-	-
Cape parrot† *Poicephalus robustus*	*Psittaciformes*	*2*	23.1	-	+	+	-	+	-	-	+	-	-	-	-	+	-
Red jungle fowl† *Gallus gallus*	*Galliformes*	*1*	28	-	-	-	-	+	-	-	-	-	-	-	-	-	-
Salt water crocodile† *Crocodylus porosus*	*Crocodilia*	*1*	>28	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Human† *Homo sapiens*	*Primates*	*2*	>28	-	-	-	-	-	-	-	-	-	-	-	-	-	-

*amplified using touchdown PCR and genotyped using an ABI 3730 DNA Analyser;

†, amplified using PCR conditions optimised in blue crane and visualised on a 2 % agarose gel;

n, number of individuals tested;

‡, delta $T_{50}H$ DNA-DNA hybridisation distances based on (Sibley & Ahlquist 1990);

+, amplification of a specific product similar in size to that observed in blue crane;

-, no amplification, non-specific amplification or amplification of a product over 200 bp bigger or smaller than the size observed in blue crane (*G. paradisea*);

Gamu and Gj-M loci originally developed in whooping crane (*G. americana* , Glenn 1997) and red-crowned crane (*G. japonensis* , Hasegawa *et al.* 2000), respectively.

Table 5.10 Cross-species amplification of 14 blue crane (*Grus paradisea*) loci in nine species spanning seven taxonomic orders.

Species	Order	n	Genetic distance to blue crane‡	*Gpa01*	*Gpa11*	*Gpa12*	*Gpa14*	*Gpa24*	*Gpa32*	*Gpa33*	*Gpa34*	*Gpa35*	*Gpa37*	*Gpa38*	*Gpa39*	*Gpa41*
Grey-crowned crane* *Balearica regulorum*	*Gruiformes*	10	0.7	+	+	+	+	+	+	+	+	+	+	+	+	+
Wattled crane* *Grus carunculatus*	*Gruiformes*	10	0.7	+	+	+	+	+	+	+	+	+	+	+	+	+
Houbara bustard† *Chlamydotis undulata*	*Gruiformes*	1	16.9	+	+	-	-	-	-	-	-	-	-	-	-	-
Grey-headed albatross† *Diomedea chrysostma*	*Procellariiformes*	1	20.1	-	+	-	-	-	-	-	-	+	+	+	+	-
Seychelles warbler† *Acrocephalus sechellensis*	*Passeriformes*	1	21.6	-	-	-	-	-	-	-	-	-	-	-	-	-
Cape parrot† *Poicephalus robustus*	*Psittaciformes*	2	23.1	+	+	-	-	+	+	-	-	-	-	-	-	-
Red jungle fowl† *Gallus gallus*	*Galliformes*	1	28	-	-	-	-	-	-	-	-	-	-	-	-	-
Salt water crocodile† *Crocodylus porosus*	*Crocodilia*	1	>28	-	-	-	-	-	-	-	-	-	-	-	-	-
Human† *Homo sapiens*	*Primates*	2	>28	-	-	-	-	-	-	-	-	-	-	-	-	-

*amplified using touchdown PCR and genotyped using an ABI 3730 DNA Analyser;

†, amplified using PCR conditions optimised in blue crane and visualised on a 2 % agarose gel;

n, number of individuals tested;

‡, delta $T_{50}H$ DNA-DNA hybridisation distances based on (Sibley & Ahlquist 1990);

+, amplification of a specific product similar in size to that observed in blue crane;

-, no amplification, non-specific amplification or amplification of a product over 200bp bigger or smaller than the size observed in blue crane

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Table 5.11 Cross-species amplification of 28 *Grus* loci in wattle crane (*Grus carunculatus*) and grey-crowned crane (*Balearica regulorum*)

Locus	Obs. allele size (bp) in 103 blue crane (*Grus paradisea*) samples	Obs. allele size (bp) in 10 grey-crowned crane (*Balearica regulorum*) samples	Obs. allele size (bp) in 10 wattled crane (*G. carunculatus*) samples
Gamu002	172, 174	171, 173, 175	167, 171, 173, 175
Gamu003	111, 113	109, 111, 113, 119	111, 113
Gamu005	182, 186	177, 188	177, 179, 181, 183, 188
Gamu006	115, 117, 119, 121, 123, 125, 127, 131 & 133	115, 119, 123, 125, 131	119, 121, 123, 125
Gamu011	134, 137, 140, 144, 147, 150, 153	137, 140, 143	131, 137, 143
Gamu013	191, 193, 195	n-s	n-s
Gamu014	118, 120, 122, 124	119, 121, 123	119, 123, 125, 131, 143
Gamu018	182, 184, 188, 190, 192, 194, 196, 204	182, 192, 194	182, 190, 192
Gamu024	239, 251, 255, 261, 263, 267, 269, 271, 273, 275	239, 251, 255, 267, 269	239, 251, 255, 269
Gamu025	112, 114	109, 115, 182	109, 115, 117, 120, 190
Gamu101	200, 249	186, 198, 200	198, 200, 204, 206
Gj-M15	106, 110, 112, 116, 118, 120	113, 117, 121	111, 113, 119
Gj-M34	114, 122, 124, 126, 130	115, 125, 127, 131	115, 123, 125, 127
Gj-M48b	204, 206, 208, 210, 212, 214, 216, 218	120, 128, 130	120, 130, 134, 136, 138
Gpa01	291, 293, 297, 299	291, 293, 297, 299	291, 293, 297
Gpa11	186 - 364	200, 234, 248, 258, 262, 266, 270, 274, 310	224, 230, 234, 245, 248, 254, 258, 266, 270, 278, 282, 294, 348
Gpa12	196, 200, 204, 212, 216, 220, 224, 228, 232, 236, 240	196, 200, 204, 228, 232, 236, 240	192, 196, 200, 212, 232
Gpa14	278 - 324	246, 250, 254, 262, 266, 270, 290, 294, 304, 208	262, 270, 294, 298, 308, 316, 358
Gpa24	210, 222, 229, 231,239, 243, 247, 251, 255	243, 247, 251	243, 247, 251
Gpa32	176, 181, 183, 185, 187, 189, 191, 193	177, 181, 183, 187, 191	179, 181, 183, 189
Gpa33	205 - 416	206, 214, 218, 266, 268, 310, 391	206, 214, 218, 254, 314, 318, 326, 334, 336, 344
Gpa34	132 - 413	132, 138, 144, 152, 164, 176, 220, 272, 280, 286, 292	164, 248, 268, 280, 286, 292, 298, 304, 320, 322
Gpa35	159, 161, 166, 168, 172, 174, 176, 178, 180, 182, 184, 186	159, 161, 173, 177, 179, 183	161, 169, 173, 185
Gpa36	220, 224, 228, 232, 236, 240	228, 232, 236	220, 224, 228, 232, 236
Gpa37	120 - 420	149, 197, 199, 205, 260, 327	121, 123, 129, 131, 133, 137, 141, 147, 223
Gpa38	180, 184, 188, 192, 196, 200	185, 189, 193	181, 185, 193, 197
Gpa39	102, 110, 114, 118, 120, 122, 126, 130, 134, 138, 142	98, 118, 122, 126, 130, 176, 198, 202, 216, 222, 224, 234, 250	102, 106, 118, 126, 198
Gpa41	223, 229, 233, 235, 237, 239, 245, 249, 251, 253, 261	187, 191, 207, 211, 215, 219, 227, 237, 245	203, 211, 233, 245, 249, 253, 257, 265

n-s, non-specific amplification

5.4 DISCUSSION

5.4.1 Pre-characterisation procedures

Checking for and deleting additional inserts in crane sequences

Identifying vector and chimeric sequences is an essential step following the development of a genomic microsatellite library. Any further steps in the characterisation process could be affected if these sequences ligated to a crane sequence were to remain undetected. For example, primers designed in a vector or across chimeric fragments would fail to amplify in the source species, resulting in the loss of a potentially highly informative locus. Inserts identified in *Gamu012* and *Gamu102* were excluded from primer design, allowing for the successful amplification of this locus before characterisation.

Checking for duplicate sequences within and between microsatellite libraries

The identification of duplicate sequences ensured that primers are designed from only one of the duplicate sequences. Ultimately, this allowed only unique loci to be tested for polymorphism, avoiding unnecessary use of expensive laboratory consumables when characterising duplicate loci. Furthermore, it was necessary to identify duplicate sequences prior to their submission to the EMBL sequence database. This ensured only unique sequences were submitted.

Submitting sequences to EMBL

Full sequence data for the three respective source species from 82 unique microsatellite loci are now available on the EMBL sequence database. More than half of these (n = 42) were loci generated in this study. Similarly, 52 of the 85 blue crane sequences were generated for this study and made available on EMBL. This study has provided a large selection of loci to test for amplification in other *Grus* species and could aid genetic studies in these species by potentially eliminating the need to develop a microsatellite library.

5.4.2 General characterisation

Seven *Grus* loci, originally isolated in the whooping crane, were shown to successfully cross-species amplify in a previous study by Glenn *et al.* (1997). However, when tested again in this study, three of these did not amplify a PCR product (*AAG-1, ATC-1* and *SHC-AG-1,* Table 5.6). One possible reason could be due to erroneous primer sequences used in this study and subsequent failure of the primers to amplify products at these loci. Alternatively, only a single blue crane sample was used to test the amplification of eight loci in Glenn *et al.*'s (1997) study, making the possibility for undetectable errors in their techniques high due to their sample size of one blue crane individual.

PCR conditions

Due to a range of $MgCl_2$ and T_a conditions used when optimising each primer pair, those pairs failing to amplify a product were considered unlikely to be as a result of an insufficient range of PCR conditions tried. One reason for a primer pair failing to amplify a product could be due to the primer sequence. For example, primer sequences for whooping crane loci were designed using whooping crane sequences. Interestingly, a high number of whooping crane primers (31 %, Table 5.6a) failed to produce a PCR product in blue crane. This could be because the whooping crane and blue crane locus sequences were not sufficiently conserved between species. This would lead to the primer being unable to anneal to the respective blue crane site to allow for amplification of the intended microsatellite region. Alternatively, primer sequences may anneal to non-specific sites, producing PCR products with a high degree of length variability, making allele scoring difficult or impossible.

A second factor leading to the failure of a PCR primer to amplify could relate to the region in which the primer sequence was designed (section 2.3.5). If a primer sequence was to be designed over a few units of the microsatellite repeat, the chance of the primer failing to amplify is increased due to the variable nature of microsatellite repeat regions. For example, the reverse primer for locus *Gpa19* was designed over the microsatellite repeat region because the length of the sequence downstream from the repeat region was too short

for alternative primer locations to be chosen. Consequently, non-specific amplification was observed for this locus in the blue crane (section 5.3.2, Table 5.6).

Thirdly, a mononucleotide repeat sequence is susceptible to length mutations (Coenye *et al.* 2005). A primer designed over this region is therefore more likely to result in non-specific amplification due to the high likelihood of this sequence will be different in other individuals. For example, the forward primer for locus *Gpa02* contained a mononucleotide repeat comprising a string of seven 'T's. The non-specific amplification observed in the 103 samples suggests this mononucleotide repeat differs among those blue crane individuals tested, resulting in non-specific amplification due to the mis-annealing of primer.

Hardy-Weinberg equilibrium

Of the 17 loci identified as having significant deviations from HWE using both Cervus and Genepop, eight showed a deficit of heterozygotes most likely attributed to a high null allele frequency. However, the remaining nine loci that were not in HWE did not have nigh null allele frequencies, suggesting that another factor(s) may be causing the deviations from expected heterozygosity estimates. Of these nine loci, eight showed significant deviations from HWE in the form of a heterozygosity deficit, and one locus a heterozygosity excess. The reasons for heterozygote excesses are provided below, followed by reasons for heterozygote deficits.

Factors identified by Selkoe *et al.* (2006) that are known to cause deviations form HWE, such that there is heterozygote deficit, are: strong inbreeding, selection acting for or against certain alleles, or large allele drop-out. Similarly, deviations from HWE in the form of an excess of homozygotes may be due to inadvertently sampling genetically distinct groups (Selkoe *et al.* 2006). However, with the exception of selection acting against certain alleles and large allele drop-out, the other factors would be expected to cause heterozygosity deficits across all loci (Selkoe *et al.* 2006). This however was not observed in the 28 polymorphic *Grus* loci tested. Therefore, it could be that selection is acting on certain alleles in these six loci, or that smaller PCR fragments are being selectively amplified as

apposed to the larger fragments. The latter could be identified by repeating PCR and genotyping runs or increasing the DNA concentration especially for loci that are homozygous for smaller allele sizes.

Heterozygosity excess was observed at loci *Gamu013*, *Gj-M34* and *Gpa39* (Table 5.7). Whilst it is likely that this observed result is due to sampling, heterozygosity excesses can be caused by recent population bottlenecks (Luikart *et al.* 1999). This is unexpected since population bottlenecks are known to cause an increase in inbreeding and consequently a reduction in the number or heterozygous individuals (Frankham *et al.* 2002). However, when a bottleneck occurs, the reduction in allele number is faster than the reduction in heterozygosity (Primmer 2005). Therefore, in a population that has recently undergone a bottleneck the observed heterozygosity should be higher than expected; providing the expected heterozygosity was calculated from the observed number of alleles and under the assumption of a constant population size (Luikart *et al.* 1999). Other factors known to cause heterozygosity excess are inbreeding depression (Brown 1979; cited in Charlesworth *et al.* 1999) and incest avoidance (Tarr *et al.* 2000). However, one would expect these factors to influence many loci and not just a single locus as seen in the blue crane. Therefore, the cause of the heterozygosity excess in this locus remains unknown, but could be a factor relating to sampling.

Loci that exhibit significant deviations from HWE are not required to be excluded from a set of markers for parentage analysis (Selkoe *et al.* 2006). Therefore, deviations from HWE were not used in this study to exclude markers from the marker set. Similarly, two loci showing significant heterozygote deficits were included in the set of markers for conservation genetic analyses in the corncrake (*Crex crex*) (Gautschi *et al.* 2002). Loci identified as being in non-HWE, however, may have a high null allele frequency, and it is the extent to which null alleles may cause mis-assignment of parentage that must be determined to assess whether the locus will contribute accurate genotypic data toward parentage analyses. This is examined by studying inheritance patterns in known families to check whether null alleles may cause incompatibilities between parent-offspring relationships (below).

Inheritance patterns and null alleles

Analysis of the single blue crane family identified three out of the 28 loci as showing non-Mendelian inheritance patterns: *Gpa39, Gamu003* and *Gamu018.* This is higher than the estimated 1 in 15 loci having non-Mendelian inheritance (Selkoe *et al.* 2006). Furthermore, it was in close agreement with other studies such as the identification of one microsatellite locus that showed non-Mendelian inheritance out of 15 loci characterised for parentage testing in the Seychelles warbler (Richardson *et al.* 2001). A further eight loci could not be tested for inheritance patterns due the absence of genotypic data from one or more family member. Although *Gpa39*, *Gamu003* and *Gamu018* loci appear to be inherited in a non-Mendelian fashion, it is known that the presence of null alleles or mutations may cause a Mendelian-inherited locus to appear as non-Mendelian. However, the null allele frequencies at loci *Gpa39, Gamu003* were not high. Therefore, the presence of null alleles seems an unlikely explanation for the observed inheritance incompatibilities at these two loci.

The presence of a null allele is identified by a true heterozygote showing as a homozygote, or a true homozygote showing as failure to amplify (Selkoe et al. 2006). This cannot be the case for *Gpa39* as all four family members were heterozygous. In *Gamu003* and *Gamu018*, the failure of the mother to amplify suggests a possibility of the presence of a null allele or a mutation. However, in *Gamu018,* there were no shared alleles between the heterozygous father and son at this locus. These results suggest that null alleles did not cause incompatibilities between these two individuals, but are perhaps the result of a mutation event. This is supported by a low null allele frequency calculated using Cervus 2.0 (Marshall et al. 1998).

In *Gamu003* however, there were no shared alleles between the homozygous father and the homozygous daughter. However, the homozygosities observed in these samples could be due the non-amplification of an allele, and this observation would tie in with the high null allele frequencies observed for this locus. Moreover, in *Gamu003, Gamu005* and *Gamu025*, the estimated null allele frequencies were the highest among all loci analysed at 0.5809, 0.9352 and 0.9442, respectively (Table 5.7). This could provide one possible

explanation for the high homozygosities observed in the single blue crane family at locus *Gamu003*, as well as for the low amplification success at loci *Gamu005* and *Gamu025* within the family tested. However, null allele frequencies at ~0.9000 as seen in the latter two loci suggest that little or no amplification should have been observed for all blue crane individuals sampled. Despite this, almost all unrelated individuals tested for amplification at these two loci produced a PCR product (Table 5.7). Therefore, null allele frequencies calculated in this study using Cervus may be an over-estimation since a high proportion of individuals, although identified as being mostly homozygous, could still be genotyped at these three loci.

A second possible reason for the incompatibilities between parent-offspring genotypes found at loci *Gpa39, Gamu003* and *Gamu018* could be due to genotyping errors, since microsatellite genotyping is error prone (Hoffman *et al.* 2005). Errors have been shown to perturb estimates of parentage (Marshall *et al.* 1998). As a way of identifying errors in genotypic data, samples runs should be repeated to check for genotyping consistencies. However, genotypes for these three loci were consistent after two repeated genotyping runs, suggesting that genotyping errors are not the cause of the parent-offspring genotype incompatibilities here.

Thirdly, observed incompatibilities could have been due to a germline mutation event which was inherited in its offspring. This can result in an allele being present in the offspring that is not found in either parent. On average, microsatellites have a mutation rate of 10^{-3} (Balloux *et al.* 2002), but this rate changes depending on allele size, sex of the individual and mutational direction, among others. Mutation rates generally increase with an increase in allele size (Brohede *et al.* 2002), and mutations are more frequently paternally derived than maternally (Ellegren *et al.* 1997).

In the nine loci where an absence of genotypic data was observed, inheritance patterns could be identified if more families are tested. However, seven out of the nine loci having missing genotypic data were previously identified as having high null allele frequencies. Therefore, there is a strong possibility that true homozygotes will show non-amplification and true heterozygotes will show homozygous genotypes, or non-amplification, in the new

families tested. There is evidence that suggests this is occurring in the single family tested since the seven loci show a high number of homozygotes and repeated amplification failures. Therefore, if these seven loci are susceptible to high null allele frequencies, additional inheritance studies in more families could yield erroneous results since any incompatibilities or compatibilities in parent-offspring genotypes may be attributed to null alleles. Therefore, the seven loci which show both a high null allele frequency and an absence of an identified inheritance pattern should not be used for parentage testing, as their reliability for this use is questionable. In addition, loci *Gpa39*, *Gamu003* and *Gamu018* identified as being inherited in a non-Mendelian fashion in the single blue crane family should be excluded for parentage testing, as has been done in other studies, three examples of which are: the exclusion of the single non-Mendelian locus from the set of markers for Seychelles warbler parentage analyses (Richardson *et al.* 2001); the use of only Mendelian loci in parentage studies of the yellow warbler (*Dendroica petechia*) (Dawson *et al.* 1997) and the peregrine falcon (*Falco peregrinus*) (Nesje *et al.* 2000).

Linkage disequilibrium

Cervus 2.0 (Marshall *et al.* 1998) identified four sets of loci showing significant levels of linkage disequilibrium (section 5.3.2). When linkage disequilibrium results were compared with the results of the predicted *Grus* microsatellite map, one locus or both loci from each pair could not be assigned a position on the map. Therefore, pairs showing linkage disequilibrium could not be confirmed as being in close proximity on the same chromosome when analysing the predicted microsatellite map. Therefore the elimination of one locus from each pair was achieved based on other locus characteristics discussed for each pair below:

1. *Gpa12 & Gpa36*

Gpa12 showed higher levels of first and second parent exclusion, and can add greater powers of exclusion to the final set of markers. In addition, *Gpa12* was not shown to deviate from HWE, whereas Genepop 3.4 (Raymond *et al.* 1995) identified *Gpa36* as

having significant deviations from HWE ($P < 0.05$). For these two reasons, *Gpa12* was chosen over *Gpa36* for inclusion in the marker set.

2. *Gamu006* & *Gamu013*

Similar to the previous pair of loci, *Gamu006* showed higher levels of first and second parent exclusion than *Gamu013*. However, *Gamu006* showed a higher frequency of null alleles, which may lead to erroneous parentage assignments. Therefore *Gamu013* was chosen over *Gamu006* for inclusion in asset of microsatellite markers for parentage analysis.

3. *Gamu024* & *Gj-M15* and *Gj-M15* & *Gj-M48b*.

Gj-M15 was identified as being linked to *Gamu024* and *Gj-M48b*. Loci *Gamu024* and *Gj-M48b* showed significant deviations from HWE whereas *Gj-M15* did not. *Gamu024* had a high frequency of null alleles suggesting this locus be avoided for parentage analyses. Therefore, since *Gj-M15* did not show any undesirable characteristics its inclusion in the marker set is favoured above *Gamu024* and *Gj-M48b*.

Markers suitable for parentage analysis

After characterisation, the 16 *Grus* microsatellite loci that fulfilled the requirements for parentage analysis were *Gpa01, Gpa11, Gpa12, Gpa14, Gpa24, Gpa32, Gpa33, Gpa34, Gpa35, Gpa37, Gpa38, Gpa41, Gamu011, Gamu013, Gj-M15* and *Gj-M34*. This set maintains a strong first and second parent exclusion power of 0.9999 and 1.0000, respectively, providing a valuable tool for parentage testing in blue crane. These exclusionary powers were higher than those used in many other parentage studies. McInnes *et al.* (2005) reported a set of markers with a 0.64 and 0.87 first and second parent exclusionary powers for use in conservation research of Carnaby's cockatoo *Calyptorhynchus latirostris*; Primmer *et al* (1995) reported a mean exclusionary power of 0.9996 for parentage testing in the European swallow *Hirundo rustica*; Nesje *et al.*(2000) developed a set of markers with a 0.94 and 0.99 first and second parent exclusionary

powers which would be used to identify the illegal trade of wild-bred peregrine falcons *Falco peregrinus.* Therefore, exclusionary powers obtained for the set of 16 loci in blue crane would be suitable for use toward parentage analysis in this species.

Chromosomal location of the selected 16 loci

The chromosomal locations of the 16 loci were previously identified using the predicted *Grus* microsatellite map created using the chicken genome (Chapter 4, Figure 4.1). The aim was to identify loci in possible close association that had not been detected using linkage disequilibrium analyses. Six of the 16 loci were able to be mapped, and were mapped on five different chromosomes. *Gpa11* and *Gpa35* were both mapped to chromosome one, however, they were situated on different arms of the chromosome therefore a likelihood of non-random association between these loci was low. Loci *Gpa01, Gpa24, Gpa32, Gj-M15* mapped to chromosomes 2, 9, 6 and 5, respectively, suggesting that a non-random association between these loci is unlikely, which is a key requirement when undertaking parentage analysis. This showed that the linkage disequilibrium results were in agreement with the predictive mapping results in identifying these loci as being unlinked

When mapping passerine microsatellite loci to the chicken genome, Dawson *et al.* (2006) identified six Seychelles warbler microsatellite loci mapping to chromosome 3, and a further four to chromosome 1. Fortunately, not all of the loci from each linkage group were used in the parentage study as many loci had low variability (Richardson *et al.* 2001). Although this example shows the passerine map (Dawson *et al.* 2006) being used to identify possible linked passerine microsatellite loci that have already been used in parentage and population studies, the analysis of chromosomal locations of the 16 *Grus* microsatellite loci represents one of the first studies to assign microsatellite loci to predicted chromosomal locations before their use in empirical parentage and population studies.

5.4.3 Further cross-species amplification of *Grus* loci

Amplification of human DNA

With the successful demonstration that human DNA is unable to be amplified using 28 polymorphic *Grus* microsatellite markers, these markers can therefore be used without the caution required when avoiding contamination of crane DNA samples with human DNA. It also excludes the possibility that any PCR amplification is a result of human DNA contamination when testing crane samples: a factor which must be taken into consideration where genotypic errors care known to have undesirable consequences in studies such as parentage testing.

Amplification of Grus *loci in other cranes and non-Gruidae species*

Three loci (*Gamu003, Gamu011* and *Gpa11*) which amplify in a wide range of the avian species tested may have potential use in other avian species genetically distant from the order *Gruiformes*, helping to provide a source of microsatellite loci in those species for which microsatellite loci are currently unavailable. In addition, this analysis also provided the extent to which each locus is able to cross-species amplify. This can help to determine the likelihood of a locus amplifying in a species not tested for amplification in this study by assessing whether the genetic distance of this species lies within the genetic-distance boundaries of successful cross-species amplification for each locus.

Although *Gamu011* was shown to amplify in six species up to a DNA-DNA hybridisation distance of 28 (delta $T_{50}H$ units based on Sibley *et al.* 1990), this locus failed to amplify in the houbara bustard (*Chlamydotis undulata*) at a DNA-DNA hybridisation distance of 16.9 (delta $T_{50}H$ units based on Sibley *et al.* 1990). But cross-species amplification is more likely to occur between closely-related species (Primmer *et al.* 1996). Therefore, *Gamu011* was expected to amplify in the houbara bustard since amplification in species at a greater evolutionary distance than bustards to cranes were observed. However, nine other loci showed no amplification in this species, yet amplified a product in more genetically distant species. This might suggest that the quality of the Houbara bustard DNA sample was below

PCR grade. However, the quality of the DNA was confirmed to be of a high molecular weight on an agarose gel prior to use in PCR, and PCR products were obtained from this sample for three other loci. The nine other loci showing no amplification in Houbara bustard but in more distantly related species showed consistent amplification in one or both of two species: Cape parrot and grey-headed albatross.

Despite the non-amplification of *Gamu013*, 27 loci have been identified as polymorphic in the wattled crane and grey-crowned crane. One locus, *Gj-M48b*, was shown to have markedly different allele sizes between blue crane and the other two crane species. The larger allele sizes in the blue crane could be due to an insertion of a roughly 100 bp fragment near the microsatellite repeat region such that the primers amplify a product larger than what is observed in blue crane. Alternatively, the microsatellite repeat region in the blue cranes could be substantially larger than that observed in wattled cranes and grey-crowned cranes, resulting in a larger PCR product being amplified. Any further study might amplify the sequence information at locus *Gj-M48b* for wattled cranes and grey-crowned cranes, to determine the exact causes for this size difference.

Seven additional loci to those shown in tables 5.9 and 5.10 are available for use in wattled and grey-crowned cranes, due to the successful cross-species amplification of *G. americana* loci *AA-1, AC-1, AT/AC-1, AAC-1, ATC-1, AAGC-1* and *SH-AG-1* in these two species (Glenn *et al.* 1997). An additional locus in Glenn *et al.*'s (1997) study, *ACC-1,* was in fact found to be a duplicate of *Gamu011*, and was shown to successfully cross-species amplify in these two species both in this study and Glenn *et al.*'s (1997) study. Unfortunately, Glenn *et al.*'s (1997) study did not include a description of the level of polymorphism observed at the eight loci in the 15 Gruidae species tested, therefore further work would be required to determine the suitability of these loci for genetic studies in these two species.

With the suite of polymorphic loci available for use in wattled crane, the rarest of the six African crane species (Meine *et al.* 1996b), these loci can be put toward gathering genetic information required for the conservation of this species (genetic conservation using whooping crane loci already underway, Jones *et al.* 2006). No genetic studies have currently been undertaken for the grey-crowned crane. These 27 loci could be a valuable tool in providing genetic information when undertaking these studies as well as for future

possible conservation actions to conserve the wattled and grey-crowned crane, and may eliminate the need to develop an expensive and time-consuming genomic microsatellite library in these species.

5.5 SUMMARY

The summary of the above nine characterisation procedures are provided in the subsequent two papers written in the style of and to be submitted to the journal Molecular Ecology Notes. These papers have been edited by a team of researches including myself. The two blue crane microsatellite libraries were developed chiefly by Gavin J. Horsburgh. However, I was involved with sequencing some of the clones, and all post-library-development and characterisation procedures.

The characteristics described in each paper form the foundation for development validation of *Grus* microsatellite loci to allow for their ultimate use toward assisting the identification of illegal trade in blue crane.

The two papers presented below are in draft format and are currently under revision. Any citations of these papers should refer to the published articles themselves, rather than the draft papers presented in this thesis. The authorships have not been decided and may change. They are provided here to show other researches who contributed in some way in this study.

PRIMER NOTE

Characterization of 14 blue crane *Grus paradisea* (Gruidae, AVES) microsatellite loci for use in detecting illegal trade

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Abstract

Fourteen polymorphic microsatellite loci were isolated and characterized in the blue crane *Grus paradisea*. Loci were isolated from an unenriched and a tetranucleotide enriched genomic library. When characterized in 56 unrelated wild blue crane individuals from South Africa, loci displayed 4-41 alleles with observed heterozygosities ranging between 0.51-0.89. These loci are aimed at assisting the identification of illegal trade in the blue crane but have wider population and conservation applications in this species.

Keywords: AVES, blue crane, Gruidae, *Grus*, microsatellite

The blue crane (*Grus paradisea*) is endemic to southern Africa and has the smallest geographical range of the 15 crane species (Ellis *et al.* 1996). Although this species is still found throughout most of its historic range, it has experienced a significant and rapid decline in numbers over the last 20 years (McCann *et al.* 2002). We describe the identification of a set of microsatellite loci developed to assist identification of illegal trade. Specifically the removal of chicks and eggs from wild birds, subsequently falsely claimed as captive bred (Meares 2006).

Blood was collected from 112 blue crane individuals from nine South African provinces. DNA was extracted using an ammonium acetate salt extraction method (adapted from Bruford *et al.* 1998). For both libraries, DNA from a single female blue crane (individual J17718) was restricted with *Mbo*I (Qbiogene) and 300-600 bp fragments selected. Two libraries were prepared: an unenriched and an enriched library. The latter was enriched for tetranucleotides $(AAAG)_n$, $(GTAA)_n$, $(GATA)_n$, $(CTAA)_n$ and $(TAAA)_n$ using the modified (Gibbs *et al.* 1997) protocol of Armour *et al.* (1994). Fragments were ligated into pUC19*Bam*HI-BAP (Qbiogene). Transformant colonies from both libraries were probed for the above motifs plus $(CA)_n$ using radiolabeled $[\alpha^{32}P]$-dCTP probes (Armour *et al.* 1994). Only one autorad positive was identified from 480 unenriched colonies (locus *Gpa01*, Table 1) and 209 (31%) positives were identified from 672 tetranucleotide-enriched colonies. Fifty-six clones were sequenced in total (Accession Nos. AM282883-AM282933, Meares 2006).

Primers for 19 loci were tested for polymorphism in 56 unrelated blue cranes. Each 10 µl PCR contained approximately 10 ng of genomic DNA, 0.5 µM of each primer, 0.2 mM of each dNTP, 1.0–3.0 mM $MgCl_2$ (Table 1) and 0.05 units of *Taq* DNA polymerase (BIOTAQ, Bioline Ltd.) in the manufacturers buffer (Final constituents: 16mM $(NH_4)_2SO_4$, 67mM Tris-HCI (pH 8.8 at 25°C), 0.01% Tween-20). PCR amplification was performed using either a DNA Engine Tetrad 2 thermal cycler (MJ Research) or a DNA Engine Tetrad PTC-225 Peltier thermal cycler (Bio-Rad). PCR conditions were 94 °C for 3 min; 35 cycles of 94 °C for 30 s, primer-specific annealing temperature (Table 1) for 30 s, 72 °C for 30 s and one cycle of 72 °C for 10 min. Genotypes were assigned on an ABI 3730 DNA Analyser using GENEMAPPER software (Applied Biosystems).

Table 1 Characterization of microsatellite loci in the blue crane (*Grus paradisea*)

Locus	EMBL Accession number	Primer sequence (5' – 3') §	T_a (°C)	$MgCl_2$ (mM)	Repeat motif	*n*	A	Observed allele size ranges (bp) ‡	H_O	H_E	HW	Null
Gpa01	AM085152	F: HEX - TTTGGTTGCCGTCAGAATTG R: CTGACCTGGGTGTGTTCTGC	63	1.5	$(CA)_8$	55	4	291 - 299	0.53	0.57	NS	0.04
Gpa11†	AM282892	F: HEX - CCCTCCTGGAATACATGACAAA R: AAGTCATTCTCAACAAGAAAGGAAAA	60	3	$(TTTC)_{34}$	56	40	186 - 364	0.79	0.97	NA	0.1
Gpa12	AM282893	F: FAM - GATCAATGGAAGGATAGGGAGGT R: TCATCAATCTATTATTTGCCTCAGC	60	3	$(GATA)_{11}$	56	10	196 - 240	0.82	0.86	NA	0.01
Gpa14	AM282896	F: FAM - TTTCGTACTCTGGTCATTGGATTT R: AATAGGACAGCAGTGCTAAGAAGAAA	57	2	$(TTTC)_{12}$	51	14	278 - 324	0.84	0.87	NA	0.02
Gpa24	AM282908	F: HEX -GAGGGAATCTAGCACGCTCCAA R: TCAAAGCATCGTGTCCATGAAGT	57	2	$(GTTA)_6$	55	7	221 - 255	0.53	0.62	NS	0.08
Gpa32	AM282918	F: HEX -CCCAGCACACCGTGCATAAG R: GCAGTCGGTCACATCCTTGG	57	2	$(GT)_{11}$	55	8	177 - 193	0.80	0.81	NS	-0.01
Gpa33	AM282919	F: FAM - GGCTTAGAAATGGGATACAGTTG R: CCGTCCAAGCAAGAAGAAA	60	2	$(TTTC)_{14}(TCTCTTTC)_{16}$	53	39	205 - 417	0.89	0.97	NA	0.04
Gpa34	AM282920	F: HEX -GCACACAGTGAGGAGACCAGTGA R: CCTTGATGTGGGAAGACAACTGC	60	3	$(TTTCTC)_{22}$	49	26	139 - 413	0.86	0.94	NA	0.04
Gpa35	AM282921	F: FAM - TCATCAGCTTCCAACAGGTCTCC R: TCAGGCACAATGTATAAGTGTTTGTGG	57	2	$(GT)_{13}(TA)_4$	52	11	160 - 186	0.77	0.81	NS	0.02
Gpa36	AM282922	F: HEX -TTCATAGACATATGCTTACCTGTTCT R: ATCCATCCATCTATCTATCTATCTATCTATC	60	2	$(GATA)_{10}(GATG)_2$	55	6	220 - 240	0.51	0.62	NS	0.09
Gpa37	AM282923	F: HEX -TGAACGTGTCTGATTTAAGGAA R: AAAGTAGTCACTAGCCTGGGTTT	60	2	$(TC)_2CTC(TTTC)_{43}$ $(TTC)_2$	53	41	152 - 396	0.87	0.97	NA	0.05
Gpa38	AM282924	F: HEX -GGGCAGAAGCAAGTCTTTCA R: GAAGATGTTTGCTGGTTGCAC	60	3	$(CTAT)_{13}$	55	6	180 - 200	0.71	0.79	NS	0.05
Gpa39	AM282925	F: HEX -TGCACAGGTTTGGCCAAGAAG R: TTCCAAAGTGAAATTAAAGGTGTGTGG	57	2	$(GA)_2(GATA)_{13}$	53	10	110 - 142	0.83	0.81	NA	-0.02
Gpa41	AM282932	F: FAM - AATTCAGTCTAATGTAAATGTCCAAG R: CCATCATTAATGGAAAGAAAGAG	54	2	$(TTTCTTCC)_6(TC)_5$ $(TTTCTCTC)_3(TTTCTCTC)_3$ $(TTTC)_2$	54	6	229 - 261	0.57	0.60	NS	0.03

T_a, annealing temperature; *n*, number of unrelated individuals; *A*, number of alleles; H_O, observed heterozygosity, H_E, expected heterozygosity; HW, deviations from Hardy-Weinberg; Null, null allele frequency;

†, locus has high null allele frequency of equal or above 0.1; §, the 5' end of each reverse primer had a "pigtail" sequence GTTTCTT added, to reduce noise from variable adenylation during the PCR (Brownstein *et al.* 1996); ‡, the seven bases of the pigtail are included in the observed allele size;

NS, non-significant; NA, not available.

Of 19 microsatellite loci assessed for polymorphism, 14 loci were polymorphic displaying between 4 and 41 alleles (Table 1). Based on genotyping individuals of known sex (32 males and 24 females), no loci were found to be sex-linked. Pairwise tests for linkage disequilibrium (GENEPOP v3.4, Raymond *et al.* 1995), corrected for multiple comparisons (Rice, 1989) identified one pair of loci (*Gpa*12 and *Gpa*36) which displayed linkage disequilibrium. Although no loci showed departures from Hardy-Weinberg equilibrium (HWE) when estimated using CERVUS v2 (Marshall *et al.*, 1998) (Table 1) HWE could not be calculated for seven of the 14 loci due to the large number of alleles displayed and/or the small sample numbers genotyped. One locus *Gpa11* (Table 1), displayed high predicted null allele frequencies and a significant deficit of heterozygotes. A single blue crane family comprising both parents and two chicks was examined for Mendelian inheritance. This was observed at all loci except *Gpa39* where one chick possessed an allele not present in either parent, possibly due to a mutation event.

Fourteen loci polymorphic in blue crane were tested for cross-species utility in nine species covering seven taxonomic orders (Table 2). PCR reactions were performed as above and PCR products visualized on a 2 % agarose gel. Wattled and grey-crowned crane samples were genotyped to determine levels of polymorphism since no species-derived microsatellite markers are currently available for these two species. One locus, *Gpa11*, produced a specific PCR product of the expected size in five species (Table 2) and may have potential use in a wide range of avian species.

Fourteen polymorphic microsatellite markers have been successfully characterized for the blue crane. These loci are aimed at detecting illegal trade and have a number of wider population and conservation genetic applications.

Table 2. Cross-species utility of 14 blue crane (*Grus paradisea*) microsatellite loci in nine species and covering seven taxonomic orders.

Species	Order	n	Genetic distance to blue crane‡	*Gpa 01*	*Gpa 11*	*Gpa 12*	*Gpa 14*	*Gpa 24*	*Gpa 32*	*Gpa 33*	*Gpa 34*	*Gpa 35*	*Gpa 36*	*Gpa 37*	*Gpa 38*	*Gpa 39*	*Gpa 41*
Touchdown PCR temperature range (°C)				66-60	63-57	63-57	60-54	60-54	60-54	63-57	63-57	60-54	63-57	63-57	63-57	60-54	57-51
Grey-crowned crane*	*Gruiformes*	*10*	0.7	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Balearica regulorum				(4)	(9)	(7)	(10)	(3)	(5)	(7)	(11)	(6)	(3)	(6)	(3)	(14)	(9)
Wattled crane*	*Gruiformes*	*10*	0.7	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Grus carunculatus				(3)	(13)	(5)	(7)	(3)	(4)	(10)	(10)	(4)	(5)	(9)	(4)	(5)	(8)
Houbara bustard†	*Gruiformes*	*1*	16.9	+	+	-	-	-	-	-	-	-	-	-	-	-	-
Chlamydotis undulata																	
Grey-headed albatross†	*Procellariiformes*	*1*	20.1	-	+	-	-	-	-	-	-	+	-	+	+	+	-
Diomedea chrysostma																	
Seychelles warbler†	*Passeriformes*	*1*	21.6	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Acrocephalus sechellensis																	
Cape parrot†	*Psittaciformes*	*2*	23.1	+	+	-	-	+	+	-	-	-	-	-	-	-	-
Poicephalus robustus																	
Red jungle fowl†	*Galliformes*	*1*	28.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gallus gallus																	
Salt water crocodile†	*Crocodilia*	*1*	na	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Crocodylus porosus																	
Human†	*Primates*	*2*	na	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Homo sapiens																	

+, amplification, () no. of alleles; -, no amplification, non-specific amplification or amplification of a product over 200 bp larger or smaller than the size observed in blue crane; n, number of individuals examined; *amplified using touchdown PCR (temperature range provided under each locus) and genotyped using an ABI 3730 DNA Analyser; †, amplified using PCR conditions optimised in blue crane and visualised on a 2 % agarose gel; ‡, delta $T_{50}H$ DNA-DNA hybridisation distances based on (Sibley *et al.*1990);
na, not available.

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References

Armour J, Neumann R, Gobert S, Jeffreys A (1994) Isolation of human simple repeat loci by hybridization selection. *Human Molecular Genetics* **3**, 599 - 605.

Brownstein M, Carpten J, Smith J (1996) Modulaton of non-templated nucleotide addition by *Taq* DNA polymerase: Primer modifications that facilitate genotyping. *BioTechniques*, **20**, 1004-1010.

Bruford MW, Hanotte O, Brookfield JFY, Burke T (1998) Multilocus and single-locus DNA fingerprinting. In: *Molecular Genetic Analysis of Populations: A Practical Approach* (ed. Hoelzel AR). IRL Press, Oxford.

Ellis DH, Gee GF, Mirande CM (eds) (1996) *Cranes: Their Biology, Husbandry and Conservation.* U.S. Department of the Interior, National Biological Service, Washington, DC and International Crane Foundation, Baraboo, Wisconsin.

Gibbs M, Dawson D, McCamley C, Wardle A, Armour J, Burke T (1997) Chicken microsatellite markers isolated from libraries enriched for simple tandem repeats. *Animal Genetics*, **28**, 401-417.

Marshall TC, Slate J, Kruuk LEB, Pemberton JM (1998) Statistical confidence for likelihood-based paternity inference in natural populations. *Molecular Ecology* **7**, 639 - 655.

McCann K, Morrison K, Byers A, Miller P, Friedman Y (eds) (2002) *Blue crane (Anthropoides paradiseus). A population and habitat viability assessment workshop. Final workshop report.* Conservation Breeding Specialist Group (SSC/IUCN), Villiersdorp.

Meares KM (2006) *Characterising Grus microsatellite loci in the blue crane Grus paradisea* MSc thesis, University of KwaZulu-Natal, South Africa.

Raymond M, Rousset F (1995) GENEPOP (version 1.2) population genetics software for exact tests and ecumenism. *Journal of Heredity*, **86**, 248-249.

Rice WR (1989) Analyzing Tables of Statistical Tests. *Evolution* **43**, 223-225.

Sibley C, Ahlquist J (1990) *Phylogeny and Classification of Birds. A Study in Molecular Evolution.* Yale University Press, New Haven.

PRIMER NOTE

Cross-species utility of microsatellite loci in blue crane *Grus paradisea* (Gruidae, AVES).

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Abstract

Fifteen polymorphic microsatellite loci originally isolated in *Grus americana* and *G. japonensis* were characterized in the blue crane *G. paradisea*. Loci were characterized in 56 unrelated blue crane individuals from the South African population. Each locus displayed 2-8 alleles, with the observed heterozygosity ranging between 0.06-0.82. These loci can be used for parentage analyses and population analysis in the blue crane.

Keywords: AVES, blue crane, cross-species utility, Gruidae, *Grus*, microsatellite

This investigation was part of a larger study developing microsatellite loci for the blue crane aimed at the use of parentage analysis to investigate illegal trade (Meares 2006, Meares *et al.* in prep). The blue crane (*G. paradisea*) belongs to the family Gruidae where microsatellite loci from two other species had previously been developed. The species are separated by relatively small genetic distances (mean delta $T_{50}H$ DNA-DNA hybridisation distance of 0.7; Sibley *et al.* 1990). Cross-species utility of microsatellite loci is generally

high between birds separated by small genetics distances (Primmer *et al.* 1996). Therefore, 43 *Grus* microsatellite loci were examined for utility in the blue crane.

The loci investigated were originally isolated from whooping crane (*G. americana*; Glenn 1997, Glenn *et al.* 1997) and red-crowned crane (*G. japonensis*; Hasegawa *et al.* 2000). Blue crane blood samples were extracted using ammonium acetate (adapted from Bruford *et al.* 1998). Sequences were generated for all loci that amplified a product, enabling the design of blue crane specific primers. Amplified products were ligated into pGEM (pGEM T-easy kit; Promega) and sequenced. Primers were re-designed for five loci using PRIMER v3 (Rozen *et al.* 2000) (Table 1).

Each 10 µl PCR contained approximately 10 ng genomic DNA, 0.5 µM each primer, 0.2 mM each dNTP, 1.0-3.0 mM $MgCl_2$ (Table 1) and 0.05 units *Taq* DNA polymerase (BIOTAQ, Bioline Ltd.) in the manufacturers buffer (Final concentrations: 16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCI (pH 8.8 at 25 °C), 0.01 % Tween-20). PCR amplification was performed using either a DNA Engine Tetrad 2 thermal cycler (MJ Research) or a DNA Engine Tetrad PTC-225 Peltier thermal cycler (Bio-Rad). PCR amplification conditions were 94 °C 3 min; 35 cycles of 94 °C for 30 s, locus-specific annealing temperature (Table 1) for 30 s, 72 °C for 30 s and one cycle of 72 °C for 10 min. Genotypes were assigned on an ABI 3730 DNA Analyser using GENEMAPPER software (Applied Biosystems).

Twelve of the 43 loci failed to amplify or amplified a non-specific product and 16 loci were monomorphic in 11 individuals tested. Samples from 56 unrelated cranes were examined at 15 polymorphic loci and amplified a product in between 16 and all individuals (Table 1). The number of alleles ranged from 2–8 (Table 1). Locus *Gamu007* was heterozygous in males only (n=16/36), all females were homozygous (n=28), suggesting

Table 1 Characterization of polymorphic microsatellite loci in the blue crane (*Grus paradisea*)

Table 1a

Locus	*Grus* species EMBL accession number	*G. paradisea* EMBL accession number	Primer sequence (5' – 3') with 'pigtail' underlined[1]	T_a (°C)	$MgCl_2$ (mM)
Gamu loci originally developed in whooping crane, *G. americana*, (Glenn 1997)					
Gamu002	AM084712	AM168501	F: HEX – AACTTTTGCCCATACTTCTGTAGTAAT	60	2.0
			R: GTTCCCGCTTCTTTTGCTGTGT		
Gamu003	AM084713	AM282934	F: HEX – CACATTGCCAGACTGTTGTAT	56	2.0
			R: ATCCCTGAAGCTAACAATAAACC		
Gamu005	AM084716	AM168502	F: FAM – CGGATGTGTACTTGGCTCAGAA	56	2.0
			R: TTTCCCTGTGGTTAGTTGTGTG		
Gamu006	AM084717	AM168503	F: HEX – CACCTTTTATTGCGTATGTATTTT	55	2.0
			R: GGATTATGTTTTGGTTTTGTTTTT		
Gamu007§	AM084718	AM168504	F: HEX – TAAAGGAGTGGCTGCTGCTGTG	59	1.0
			R: GTTTCTT - CTGAGGCTCTGCTGTGGGAAAC		
Gamu011	AM084722	AM168506	F: FAM-CAATGGAGCGCCGTCAC	52	2.0
			R: GTTTCTT - CAGTTTCTTGGCCGCTGTT		
Gamu013	AM084724	AM168508	F: HEX –AATAAGTTTGGAAATGTTTCTCATA	53	2.0
			R: GTTTCTT - TTTTCTGGTCAATACTAAAGC		
Gamu014§	AM084725	AM168509	F: FAM – CACAAAACTCCCACTAACTTCAAC	56	1.0
			R: GTTTCTT - CAGAAGACTGCAATGAACTCCTG		
Gamu018	AM084729	AM168511	F: HEX – TAGCGAGGGTCTGAGGAGAACT	56	1.0
			R: GTTTCTT - ACACCGTTATATCCTTCCCACT		
Gamu024	AM084735	AM282937	F: FAM – TCTTCTTGCTAATCATCTTTCTAAC	53	1.0
			R: GTTTCTT - GCTGCCAGTACAGACCCTCTT		
Gamu025	AM084736	AM282938	F: FAM – TTAATAAAAATCCACAGTGAAT	53	1.0
			R: GTTTCTT - GTTCTAGACCAGGACTGTTAATA		
Gamu101b§	AM084738	AM168516	F: FAM – CAGTATAAAAACAAACAGGTGAGA	58	2.0
			R: GTTTCTT - TGAAAAAAGTACAGGAGAACATAG		
Gj-M loci originally developed in red-crowned crane, *G. japonensis*, (Hasegawa *et al.* 2000)					
Gj-M15§	AB041861	AM168519	F: FAM –TCTACCAGATATCATCAGAGCTTGC	59	2.0
			R: GTTTCTT - TGCGAATGAACAGATGGCCCCAAGA		
Gj-M34	AB041862	AM168520	F: FAM – TGCTCAACATTCATCAGGATTTGGG	59	2.0
			R: TCCCTCTGGTGTTGGCTGAAAATAC		
Gj-M48b§	AB041864	AM282940	F: HEX –GATCCTGGGGGTTTTGTTG	58	1.0
			R: GTTTCTT – GGACCCTCCACCGAGAAG		

Table 1b

Locus	Repeat motif observed in *G. paradisea*	*n*	A	Expected allele size (bp)	Observed allele sizes (bp) [2]	H_O	H_E	H-W	Null allele frequency	Mendelian Inheritance
Gamu002†	$(CA)_2$	52	3	174	171, 173, 175	0.21	0.34	NA	0.21	NA
Gamu003†	$(GT)_9(GA)_{10}$	55	6	114	100, 102, 110, 112, 114, 118	0.11	0.38	NA	0.58	NA
Gamu005†	$(CA)_{10}$ TT $(GT)_5$	17	2	188	176, 186	0.00	0.21	NA	0.94	NA
Gamu006	$(CA)_{10}$	55	8	125	115, 117, 121, 123, 125, 127, 131, 133	0.71	0.76	NS	0.03	Yes
Gamu007	$(CA)_{13}$	33	5	143	144, 146, 148, 150, 152	0.52	0.65	NS	0.08	NA
Gamu011	$(CCT)_8$	56	7	136	134, 137, 140, 143, 146, 149, 152	0.64	0.72	NS	0.06	Yes
Gamu013	$(GT)_{10}$	52	3	182	191, 193, 195	0.46	0.44	NA	-0.04	Yes
Gamu014†	$(GT)_{18}$	49	4	117	118, 120, 122, 124	0.41	0.69	**	0.24	NA
Gamu018	(GT)13	49	8	188	182, 184, 188, 190, 192, 194, 196, 204	0.65	0.70	NS	0.01	NA
Gamu024†	$(CA)_8$...$(CCCA)_3$	52	8	243	239, 251, 261, 263, 267, 269, 273, 275	0.60	0.76	NS	0.10	NA
Gamu025†	$(CA)_8TA(CA)_2(TC)_3$	16	2	111	109, 113	0.00	0.22	NA	0.94	NA
Gamu101b†	$(ATT)_6GTT$ $(ATT)_4$	16	2	194	186, 201	0.06	0.18	NA	0.45	NA
Gj-M15	$(GT)_{11}$	56	6	111	106, 110, 112, 116, 118, 120	0.73	0.80	NA	0.04	Yes
Gj-M34	$(CA)_7$	56	5	127	114, 122, 124, 126, 130	0.82	0.68	**	-0.11	Yes.
Gj-M48b	$(CA)_7$	40	8	198	204, 206, 208, 210, 212, 214, 216, 218	0.68	0.72	NS	0.03	Yes‡

T_a, annealing temperature; *n*, number of individuals where a product was amplified in 56 unrelated individuals tested; *A*, number of alleles; H_O, observed heterozygosity, H_E, expected heterozygosity; H-W, deviations from Hardy-Weinberg equilibrium; NS, non-significant; NA, could not be calculated with Cervus 2.0 Marshall *et al.* (1998); significant at the 0.05** probability level;
§, alternative primer set used to that published: re-designed to allow for successful PCR amplification; †, locus has high null allele frequency of equal or above 0.1; [1]The 5' end of one primer in each pair was labelled with a fluorescent phosphoramidite and the "pigtail" sequence GTTTCTT was added to the other primer, to reduce noise from variable adenylation during the PCR (Brownstein *et al.* 1996);
[2]The seven bases of the pigtail are included in the observed allele size; [3]Observed in a single family (mother, father and two chicks genotyped); ‡, genotype of mother unavailable.

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this locus was Z-linked (in agreement with Jones *et al.* 1999). Allele frequency analyses were performed using CERVUS v2.0 (Marshall *et al.* 1998), males only at *Gamu007.* Hardy-Weinberg Equilibrium (HWE) probabilities could not be calculated for eight of the 16 loci (using CERVUS v2.0, Table 1) due to the large number of alleles displayed and/or the small sample numbers genotyped. Of the remaining eight loci, *Gj-M34* deviated significantly from HWE. Seven loci displayed high null allele frequencies and a deficit of heterozygotes (Table 1). Pairwise tests for linkage disequilibrium (GENEPOP v3.4, Raymond *et al.* 1995), corrected for multiple comparisons (Rice, 1989) identified three loci with significant deviations (*Gamu006* & *Gamu013*; *Gamu024* & *Gj-M15*; *Gj-M15* & *Gj-M48*).

The 15 loci were tested for cross species utility in a further nine species (Table 2). Touchdown PCR (using temperatures provided in Table 2) was performed to amplify wattled crane and grey crowned crane samples. This would allow for high quality PCR products for analysis of polymorphism since no species-derived microsatellite markers are currently available for these species. PCRs were performed as above for the other seven species with products visualized on a 2 % agarose gel. For locus *Gamu013*, cross-species amplification was only observed in blue crane. Locus *Gamu011* amplified in six avian species (Table 2) and could have potential use in a wide range of avian species.

Fifteen polymorphic *Grus* microsatellite markers have been successfully characterized in the blue crane. These loci are made available for use in a range of population and conservation applications.

Table 2. Cross-species utility of *Grus* microsatellite loci in nine species covering seven taxonomic orders.

Species	Order	n	Genetic distance to blue crane‡	*Gamu 002*	*Gamu 003*	*Gamu 005*	*Gamu 006*	*Gamu 007*	*Gamu 011*	*Gamu 013*	*Gamu 014*	*Gamu 018*	*Gamu 024*	*Gamu 025*	*Gamu 101b*	*Gj-M15*	*Gj-M34*	*Gj-M48b*
Touchdown PCR temperature range (°C)				63-57	59-53	59-53	57-52	62-56	49-55	56-50	59-53	56-50	59-53	56-50	61-55	62-56	62-56	61-55
Grey-crowned crane*	*Gruiformes*	*10*	0.7	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+
Balearica regulorum				(4)	(4)	(2)	(5)	(3)	(3)		(3)	(3)	(5)	(3)	(3)	(3)	(4)	(3)
Wattled crane*	*Gruiformes*	*10*	0.7	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+
Grus carunculatus				(4)	(2)	(5)	(4)	(4)	(3)		(5)	(3)	(4)	(4)	(4)	(3)	(4)	(5)
Houbara bustard†	*Gruiformes*	*1*	16.9	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
Chlamydotis undulata																		
Grey-headed albatross†	*Procellariiformes*	*1*	20.1	-	+	+	-	-	+	-	-	-	-	-	-	-	-	-
Diomedea chrysostma																		
Seychelles warbler†	*Passeriformes*	*1*	21.6	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
Acrocephalus sechellensis																		
Cape parrot†	*Psittaciformes*	*2*	23.1	-	+	+	-	-	+	-	-	+	-	-	-	-	+	-
Poicephalus robustus																		
Red jungle fowl†	*Galliformes*	*1*	28	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
Gallus gallus																		
Salt water crocodile†	*Crocodilia*	*1*	na	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Crocodylus porosus																		
Human†	*Primates*	*2*	na	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Homo sapiens																		

+, amplification; ( ) number of alleles provided where examined for polymorphism; -, no amplification, non-specific amplification or amplification of a product over 200 bp larger or smaller than the size observed in blue crane; n, number of individuals tested;

*amplified using touchdown PCR (temperature range provided under each locus) and genotyped using an ABI 3730 DNA Analyser; †, amplified using PCR conditions optimised in blue crane and visualised on a 2 % agarose gel; ‡, delta $T_{50}H$ DNA-DNA hybridisation distances based on (Sibley & Ahlquist 1990); na, not available;

Gamu and Gj-M loci originally developed in whooping crane (*G. americana*, Glenn 1997) and red-crowned crane (*G. japonensis*, Hasegawa *et al.* 2000), respectively.

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References

Brownstein M, Carpten J, Smith J (1996) Modulaton of non-templated nucleotide addition by *Taq* DNA polymerase: Primer modifications that facilitate genotyping. *BioTechniques*, **20**, 1004-1010.

Bruford MW, Hanotte O, Brookfield JFY, Burke T (1998) Multilocus and single-locus DNA fingerprinting. In: *Molecular Genetic Analysis of Populations: A Practical Approach* (ed. Hoelzel AR). IRL Press, Oxford.

Glenn TC (1997) Genetic bottlenecks in long-lived vertebrates: Mitochondrial and microsatellite DNA variation in american alligators and whooping cranes. PhD thesis, University of Maryland, USA.

Glenn TC, Ojerio RS, Stephan W, Braun MJ (1997) Microsatellite DNA loci for genetic studies of cranes. *Proceedings of the North American Crane Workshop*, **7**, 36-45.

Hasegawa O, Ishibashi Y, Abe S (2000) Isolation and characterisation of microsatellite loci in the Red-crowned crane *Grus japonensis*. *Molecular Ecology*, **9**, 1677-1678.

Jones K, Glenn T (1999) Screening the Gamu7 microsatellite locus to determine the sex of captive whooping cranes. *Promega Notes Online: http://www.promega.com/enotes/applications/ap0006_tabs.htm.*

Marshall TC, Slate J, Kruuk LEB, Pemberton JM (1998) Statistical confidence for likelihood-based paternity inference in natural populations. *Molecular Ecology*, **7**, 639-655.

Meares KM (2006) *Characterising Grus microsatellite loci in the blue crane Grus paradisea* MSc thesis, University of KwaZulu-Natal, South Africa.

Meares KM, Horsburgh GJ, Dawson DA, Perrin M, Burke T, Taylor T (in prep) Characterisation of 14 blue crane *Grus paradisea* (Gruidae, AVES) microsatellite loci for use in detecting illegal trade.

Primmer CR, Møller AP, Ellegren H (1996) A wide-range survey of cross-species microsatellite amplification in birds. *Molecular Ecology*, **5**, 365-378.

Raymond M, Rousset F (1995) GENEPOP (version 1.2) population genetics software for exact tests and ecumenism. *Journal of Heredity*, **86**, 248-249.

Rice WR (1989) Analyzing Tables of Statistical Tests. *Evolution* **43**, 223-225.

Rozen S, Skaletsky HJ (2000) In: *Bioinformatics Methods and Protocols: Methods in Molecular Biology*, (eds Krawetz S, Misener S) pp. 365-386. Humana Press, Totowa, NJ.

Sibley C, Ahlquist J (1990) Phylogeny and Classification of Birds. A Study in Molecular Evolution. Yale University Press, New Haven.

CHAPTER 6 CONCLUSION & FUTURE DIRECTIONS

6.1 GRUS MICROSATELLITE MARKERS

The cranes (AVES: *Gruidae*) are among the world's most threatened birds (Meine *et al.* 1996a). The blue crane (*Grus paradisea*) is listed on CITES appendix II and the IUCN Red List of Threatened Species (IUCN 2006). The illegal removal of chicks from the wild has been recognised as a factor causing population declines in this threatened species (McCann *et al.* 2002). Chicks are removed from the wild and fraudulently incorporated into captive populations under the pretence that they are the offspring of a legal captive pair. This occurs since permits to keep cranes require that the cranes be captive bred (except in rare occasional cases such as injury of a wild bird). There was a need for the development of a genetic-based tool that would enable the identification of such illegal trade in cranes. Specifically, the genetic tools that could be used for parentage analysis to check the validity of the relationship between the chick and its claimed parents.

Molecular markers can assist with the enforcement of wildlife legislation through methods that include, but are not limited to, species identification, individual identification, determining the population of origin, and parentage analysis (Millions *et al.* 2006). The latter application was the focus of this study. Microsatellite markers were selected above other molecular markers to be used in parentage analysis in the blue crane and aimed at a future forensic role. Microsatellite markers have a number of advantages over other markers. First, microsatellite markers are co-dominant allowing for the expression of both alleles (Schlötterer 1998). Second, many microsatellite loci are inherited in a Mendelian fashion which allows for the relationship between individuals to be studied, which is an important characteristic and pre-requisite for markers to be used in parentage analysis. Third, their hyper-variability allows these markers to make valuable contributions toward individual identification when a set of variable markers are used simultaneously. In addition, microsatellite loci are amplified using PCR, therefore only a small amount of DNA is required at the start of the analysis procedure. This becomes important when only a small sample is available; such is the case in many wildlife forensic cases.

Microsatellite markers used in this study were derived from three sources: (i) a microsatellite library previously developed for the whooping crane (*G. americana*), (Glenn 1997); (ii) a microsatellite library previously developed for the red-crowned crane (*G. japonensis*), (Hasegawa *et al.* 2000); and (iii) two genomic microsatellite libraries developed for this study. Thirty seven whooping crane loci, seven red-crowned crane loci and 57 blue crane loci were available for characterisation in the blue crane. Of these, 62 loci were examined for polymorphism. Genotyping identified 28 polymorphic loci, 20 monomorphic loci, and 14 non-amplifying loci in the blue crane. Genotypic data obtained from the blue crane at the 28 polymorphic loci were taken forward to perform characterisation studies to select a set of loci for parentage analysis.

A number of technical and biological hurdles when using microsatellite markers for parentage analysis have been recognised (Jones *et al.* 2003). Fortunately, many of these hurdles can be overcome by careful statistical analysis and selection of loci during the characterisation process. A number of characterisation procedures were carried out to determine which of the other loci tested for polymorphism in the blue crane needed to be excluded due to characteristics that are unfavourable for parentage analysis.

6.1.1 Characterisation

The first characterisation procedure undertaken in this study was the identification of sex-linked loci. Sex-linked loci should be excluded from parentage analyses since only autosomal loci that are inherited in a Mendelian fashion can be used to infer relationships between individuals. Prior to the identification of sex-linked loci, the various sexing markers available for avian species were tested for the suitability of sexing blue cranes. The primers described by Fridolfsson *et al.* (1999) were tested in blue, wattled (*G. carunculatus*) and grey-crowned crane (*Balearica regulorum*), and were found to be suitable in distinguishing males from females on an agarose gel. Primers described by Griffiths *et al.* (1998) were tested in the blue crane only. They could not distinguish males from females using an agarose gel due the small allele size differences between the two alleles amplified. However, the amplified product using these primers could successfully differentiate the sexes using a DNA analyser, as was found for the PCR product amplified

using Fridolfsson *et al.* (1999) primers. A whooping crane locus, *Gamu007*, previously identified as being sex-linked (Jones *et al.* 1999) was also Z-linked in the blue crane. However, it did not show any sex-specific alleles in blue, wattled and grey-crowned cranes and therefore could not be used for sex determination in these species. Using the sexes of the three crane species determined using Griffiths *et al.* (1998) primers and analysing the genotypes on a DNA analyser, the 48 microsatellite loci that amplified a product were checked for sex-linkage. With the exception of *Gamu007* identified previously as being sex-linked, no loci were identified as being sex-linked. Therefore, *Gamu007* was excluded from further analysis for suitability for parentage analysis.

A second characterisation procedure was the identification of linked loci. Linked loci may be associated such that they are inherited as a single genetic unit (Dawson *et al.* 2006). Using loci that are in linkage disequilibrium decreases the expected probability of exclusion and the accuracy of parentage assignments (Chakraborty *et al.* 1983). Therefore, the inclusion of both loci in a set of markers for parentage analysis should be avoided. The identification of linked loci was achieved using linkage disequilibrium analysis, which identified four pairs of linked loci (*Gpa12* & *Gpa36*; *Gamu006* & *Gamu013*; *Gamu024* & *Gj-M15*; *Gj-M15* & *Gj-M48b*). The location of these linked loci on the predicted *Grus* microsatellite map based on the chicken genome was checked to identify possible linkage i.e. loci in close proximity on the predicted map suggests potential linkage (Dawson *et al.* 2006) and therefore only one loci out of a pair or group of loci in close proximity on the same chromosome should be taken further for characterisation. In the four pairs of linked loci identified by statistical analysis, both loci of each linked pair unfortunately could not be mapped on the predicted microsatellite map. Therefore, the level of agreement between the linkage disequilibrium results and the mapped loci could not be examined. In order to select the most appropriate locus to exclude from of each of these pairs, the 28 polymorphic loci were characterised using (i) patterns of inheritance and (ii) frequency of null alleles. This is because it may be possible that both loci in a linked pair may be unsuitable for parentage analysis, based on these two factors. Furthermore, loci exhibiting non-Mendelian inheritance should be excluded from parentage analyses as they can have severe effects on results, such as only one parental allele being passed on to all offspring (Selkoe *et al.* 2006). Similarly, loci exhibiting a high null allele frequency can cause erroneous parentage

assignments and should be excluded from marker sets for parentage analysis (Jones *et al.* 2003; Selkoe *et al.* 2006). Therefore, loci identified as having these characteristics have been excluded from many biological and forensic-related studies (Lillandt *et al.* 2001; Nesje *et al.* 2000).

The 28 polymorphic loci in the blue crane were tested for inheritance patterns in a single blue crane family. Three loci were identified as having incompatibilities between parent and offspring genotypes, which suggested that these loci are inherited in a non-Mendelian fashion. Until further research can be undertaken to investigate what is occurring at these loci, these three loci were excluded from the set for parentage analysis. The inheritance patterns could not be identified at eight loci due to the absence of genotypic data in one or more parental individual. These eight loci were also excluded from the parentage analysis set. Of the 17 loci that conformed to Mendelian inheritance, two were identified as having a high null allele frequency, of which one (*Gamu006*) showed significant linkage disequilibrium and possible linkage to a locus with suitable characteristics. Locus *Gamu006* was therefore excluded. Because the other locus (*Gpa11*) was identified as having Mendelian inheritance patterns, with members of the family all being heterozygous, the effect of null alleles in confounding parentage analysis at this locus was considered negligible based on results from the single family tested. This locus was therefore not excluded from the set of markers.

Following the exclusion of microsatellite loci considered unsuitable, the remaining 16 microsatellite loci were: *Gpa01, Gpa11, Gpa12, Gpa14, Gpa24, Gpa32, Gpa33, Gpa34, Gpa35, Gpa37, Gpa38, Gpa41, Gamu011, Gamu013, Gj-M15* and *Gj-M34*. These loci were identified in this study as being randomly associated; showing Mendelian inheritance, and a low frequency of null alleles (with the exception of *Gpa11*). This set provides strong first and second parent exclusion powers of 0.9999 and 1.0000, respectively, providing a valuable tool for parentage testing in the blue crane. These exclusionary powers are higher than many sets used in other biological studies (Primmer *et al.* 1995; Richardson *et al.* 2001) and wildlife forensic cases (Nesje *et al.* 2000). Six of the 16 loci were able to be mapped on five different chromosomes on the predicted *Grus* microsatellite map. *Gpa11* and *Gpa35* were both mapped to chromosome one, however, they were situated on

different arms of the chromosome therefore a likelihood of non-random association between these loci was low. Loci *Gpa01, Gpa24, Gpa32, Gj-M15* mapped to chromosomes 2, 9, 6 and 5, respectively, suggesting that a non-random association between these loci is unlikely. Non-random association is a key requirement when undertaking parentage analysis. These results showed that the results from linkage disequilibrium that identified these loci as being unlinked were in agreement with the predictive mapping results. In addition, the analysis of chromosomal locations of the 16 *Grus* microsatellite loci represented one of the first studies to assign microsatellite loci to predicted chromosomal locations before their use in empirical parentage and population studies.

6.1.2 Cross-species analysis

The 28 loci identified as being polymorphic in the blue crane were also tested for cross-species amplification in wattled crane, grey-crowned crane, five other avian species covering five taxonomic orders, the salt-water crocodile (*Crocodylus porosus*) and human (*Homo sapiens*). Three loci, *Gamu003*, *Gamu011*, *Gpa11*, were shown to cross-species amplify in a wide range of avian taxa with the cut-off evolutionary distance for amplification being 23.1 Δ $T_{50}H$ (*Gamu003 & Gpa11*) and 28 Δ $T_{50}H$ (*Gamu011*), (distances based on delta $T_{50}H$ DNA-DNA hybridisation distance, Sibley *et al.* 1990). Primmer *et al.* (1996) found a much shorter average cut-off of 10-15 Δ $T_{50}H$ in a wide-range survey of cross-species amplification of microsatellite markers in birds. However, the maximum evolutionary distance across which *Grus* loci could amplify was in agreement with Primmer *et al.*'s (1996) findings of 28 Δ $T_{50}H$. These loci could be a valuable source of polymorphic loci in closely-related avian species for which no species-specific microsatellite libraries currently exist. Studies on avian species that require a suite of microsatellite loci could use the information from this study on amplification ability in cross-species analysis to determine whether loci might be suitable to be examined in their species.

Levels of polymorphism at the 28 loci were determined in the wattled and grey-crowned crane. Wattled and grey-crowned cranes both occur in southern Africa (Maclean 1985), and the wattled crane is the rarest of the six crane species that occur in Africa (Jones *et al.*

2006). Of the 28 loci tested, 27 were polymorphic in both species and one locus did not amplify. The 27 polymorphic loci can provide a selection of loci with which to compliment an existing suite of microsatellite markers in the wattled crane, a species for which a few whooping crane loci have previously been used for a population study (Jones *et al.* 2006). In addition, these 27 loci can provide a source of loci with which to undertake genetic studies in the grey-crowned crane, a species for which no genetic studies currently exist.

This study has characterised 27 markers available to the blue, wattled and grey-crowned crane. However, there is an additional source of loci that can be used in these species from the blue-crane species-specific microsatellite library. Due to time limitations, these were not tested for polymorphism in this study. Only 19 of the 57 blue crane species-specific loci developed for this study were tested for amplification and polymorphism in the blue crane. Of these, only 14 were tested for polymorphism in the wattled and grey-crowned crane. Cross-species amplification of blue cane loci in these species was shown to have a high level of success, suggesting that more polymorphic loci for wattled and grey-crowned crane can be sourced by testing the remaining blue crane loci not tested in this study.

6.2 FUTURE DIRECTIONS

6.2.1 Validation of markers for forensic use

The ultimate use of the microsatellite loci developed and analysed in this study is to build forensic capacity to assist the detection of illegal trade in the blue crane (*Grus paradisea*) using parentage analysis. The markers will be available to be used for forensic cases should a case arise where the relationship between offspring and their claimed legal captive parents comes under investigation. Forensic cases require robust and validated techniques to provide indisputable evidence in a court of law. In order for markers characterised in this study to be of use in a forensic setting, their ability to perform in a precise, accurate and reproducible manner must be verified. This can be achieved by the validation of the selected set of microsatellite loci.

Validation procedures arose when shortfalls in scientific techniques were recognised during the initial cases involving DNA evidence (Giannelli 2006). Consequently, extensive standardisation procedures have been developed by organisations such as the International Society for Forensic Genetics (ISFG) and the Scientific Working Group on DNA analysis (SWGDAM) for molecular techniques used in forensic cases. In South Africa, the organisation that sets the standards required for validation is the South African National Accreditation System (SANAS). Understandably, most validation protocols were designed for human forensic casework such as the validation of STR markers for human identification, with the subsequent publication of detailed validation reports (see Collins *et al.* 2004; Moretti *et al.* 2001).

Two types of validation, developmental and internal, are required before the genetic markers can be implemented for forensic use (SWGDAM 2004). Developmental validation is the demonstration of the accuracy, precision, and reproducibility of a procedure by the manufacturer, technical organization, academic institution, government lab, or other party (SWGDAM 2004). Recommended procedures to be carried out during developmental validation of microsatellite markers include: the determination of general locus characteristics (Chapter 5), species specificity, population studies, examining PCR-based procedures, stability, mixture studies, sensitivity of amplification, precision and accuracy studies, reproducibility and the analysis of case-type samples. The latter five procedures are also carried out for internal validation. This type of validation is conducted by each forensic DNA testing laboratory and provides an in house demonstration of the reliability and limitations of the procedure (SWGDAM 2004). Additional procedures to be carried out for internal validation include: contamination assessments and qualifying tests. Once the markers have been validated using the above procedures, they will be able to be used by forensic-accredited laboratories to provide robust DNA evidence for cases involving the illegal trade in the blue crane.

6.2.2 Research on population structure of the blue crane

Population studies of endangered species often aim to identify genetically distinct populations that may require management as separate entities (Frankham *et al.* 2002). The

main reason for this is the potential that each population has to evolve adaptations to local environmental pressures (Frankham *et al.* 2002). For example, a population supplementation program that translocated individuals unknowingly across geographically defined breeding barriers could result in outbreeding depression (Storfer 1999), resulting in the 'contamination' of the gene pool with genes adapted to different environmental conditions. Population analyses performed for the wattled crane (*Grus carunculatus*) using 12 microsatellite loci identified the South African population as being genetically distinct from the south-central African population (Jones *et al.* 2006). This has direct management implication for this species. Should translocation or re-introductions be required, plans should consider the geographic origin and the genetic diversity of translocated individuals and their impact on the target population. With a set of molecular markers, such as the ones developed in this study, the population structure of blue cranes could be analysed. This could determine whether there are different populations that should be managed as separate entities, thereby providing the genetic information should population supplementation or reintroduction be required in the future.

6.2.3 Studbook management

Very little data on the status of the illegal removal of chicks from the wild exists (Morrison 2002). However, this could be improved by requiring owners of captive blue cranes to have their birds 'fingerprinted', for incorporation into studbook records (McCann *et al.* 2002), since these data could be called upon when the relationship between individuals needs to be determined to verify or refute claimed parentage of the bird in question. Microsatellite markers used for maintaining studbooks have been shown to be valuable for the conservation of the endangered whooping crane (*G. americana*) (Jones *et al.* 2002). The motivation for incorporating DNA data into the studbook was not trade-related, but to provide a tool for maintaining genetic diversity within captive populations. Comprehensive pedigrees were constructed for captive populations of whooping cranes using genotypic data obtained from 11 microsatellite markers originally developed in this species (Jones *et al.* 2002). The maintenance of genetic diversity in captive populations is difficult due to potential for inbreeding and genetic drift. An understanding of the relationship between individuals can allow for selective mating of genetically different individuals to produce

offspring with levels of heterozygosity greater than those of the founder population. This will boost the populations gene diversity (Jones *et al.* 2002). Increased gene diversity is especially advantageous as it allows for the selection of genes to assist adaptation to novel environmental conditions (such as the presence of new diseases) in future generations (Frankham *et al.* 2002). Ultimately, it can assist in reducing the captive population's risk of extinction. Blue crane captive populations could benefit in a similar manner by including genetic data into studbooks to assess the most advantageous breeding strategies.

6.3 CONCLUDING REMARKS

This study successfully identified a highly informative set of 16 microsatellite markers for use in accurate parentage analysis in the blue crane. Upon completion of the validation procedures required for forensic casework, these markers could assist the detection of illegal trade in this species. Ultimately, providing the genetic forensic capacity to detect the illegal trade in the blue crane could provide a sufficient deterrent, assisting to reduce the illegal removal of crane chicks from the wild. This will help to reduce one of the factors identified as causing a decline in the population size in South Africa's endangered national bird.

In addition to their potential in forensic casework, these markers will prove useful to future researchers wishing to examine population structure, reproductive ecology and behaviour. In addition, the markers can be applied to genetic studies aimed at the genetic conservation of both wild and captive blue crane populations. Furthermore, a suite of loci were shown to be polymorphic in two other southern African crane species, which could serve to assist genetic conservation studies in these species.

REFERENCES

Aitken N, Hay JM, Sarre SD, Lambert DM (2001) Microsatellite markers for tuatara (*Sphenodon spp.*). *Conservation Genetics* **2**, 183-185.

Allan D (1995) Habitat selection by blue cranes in the Western Cape Province and the Karoo. *South African Journal of Wildlife Research* **25(3)**, 90-97.

Allan D (2001) The blue crane, imperilled national icon. *Africa Birds and Birding*, 30-39.

Amos W, Balmford A (2001) When does conservation genetics matter? *Heredity* **87**, 257-265.

Armour J, Neumann R, Gobert S, Jeffreys A (1994) Isolation of human simple repeat loci by hybridization selection. *Human Molecular Genetics* **3**, 599 - 605.

Balloux F, Lugon-Moulin N (2002) The estimation of population differentiation with microsatellite markers. *Molecular Ecology* **11(2)**, 155-165.

Bensch S, Westerdahl H, Hansson B, Hasselquist D (1999) Do females adjust the sex ratio of their offspring in relation to the breeding sex ratio? *Journal of Evolutionary Biology* **12**, 1104-1109.

Birkhead TR, Burke T, Zann R, Hunter FM, Krupa AP (1990) Extra-pair paternity and intraspecific brood parasitism in wild zebra finches (*Taeniopygia guttata*), revealed by DNA fingerprinting. *Behavioural Ecology and Sociobiology* **27**, 315-324.

Birney E, Andrews D, Caccamo M, Chen Y, Clarke L, Coates G, Cox T, Cunningham F, Curwen V, Cutts T, Down T, Durbin R, Fernandez-Suarez XM, Flicek P, Graf S, Hammond M, Herrero J, Howe K, Iyer V, Jekosch K, Kahari A, Kasprzyk A, Keefe D, Kokocinski F, Kulesha E, London D, Longden I, Melsopp C, Meidl P, Overduin B, Parker A, Proctor G, Prlic A, Rae M, Rios D, Redmond S, Schuster M, Sealy I, Searle S, Severin J, Slater G, Smedley D, Smith J, Stabenau A, Stalker J, Trevanion S, Ureta-Vidal A, Vogel J, White S, Woodwark C, Hubbard TJP (2006) Ensembl 2006. *Nucleic Acids Research* **34**, 556-561.

Blederman BM, Lin CC, Kuyt E, Drewien RC (1982) Genome of the whooping crane. *The Journal of Heredity* **73**, 145-146.

Boersma PD, Davies EM (1987) Sexing monomorphic birds by vent measurements. *Auk* **104**, 779–783.

Broad S, Mulliken T, Roe D (2003) The nature and extent of legal and illegal trade in wildlife. In: *The trade in wildlife: regulation for conservation* (ed. Oldfield S), p. 210. Earthscan, London.

Brohede J, Primmer CR, Moller AP, Ellegren H (2002) Heterogeneity in the rate and pattern of germline mutation at individual microsatellite loci. *Nucleic Acids Research* **30(9)**, 1997 - 2003.

Brown AHD (1979) Enzyme polymorphism in plant populations. *Theoretical Population Biology* **15**, 1-42.

Article within a website: Brown S (2006) Record ivory cache traced to Zambia elephants, DNA shows. 18 Aug. 2006. 10 Nov. 2006 <http://news.nationalgeographic.com/news/2006/08/060817-ivory-dna.html>.

Brownstein M, Carpten J, Smith J (1996) Modulaton of non-templated nucleotide addition by *Taq* DNA polymerase: primer modifications that facilitate genotyping. *Biotechniques* **20**, 1004-1010.

Bruford MW, Hanotte O, Brookfield JFY, Burke T (1998) Multilocus and single-locus DNA fingerprinting. In: *Molecular genetic analysis of populations: a practical approach* (ed. Hoelzel AR). IRL Press, Oxford.

Burgener M, Snyman N, Hauck M (2001) *Towards a sustainable wildlife trade: an analysis of nature conservation legislation in South Africa with particular reference to the wildlife trade* Institute of Criminology, University of Cape Town, South Africa.

Burke T (1989) DNA fingerprinting and other methods for the study of mating success. *Trends in Ecology & Evolution* **4(5)**, 139-144.

Burke T, Bruford MW (1987) DNA fingerprinting in birds. *Nature* **327**, 149-151.

Burke T, Davies NB, Bruford MW, Hatchwell BJ (1989) Parental care and mating behaviour of polyandrous dunnocks (*Prunella modularis*) related to paternity by DNA fingerprinting. *Nature* **338**, 249-251.

Burt DW, Bumstead N, Bitgood JJ, De Leon FAP, Crittenden LB (1995) Chicken genome mapping: a new era in avian genetics. *Trends in Genetics* **11(5)**, 190-194.

Article within a website: CapeNature (2003) Man fined for illegal possession of endangered crane. 15 November 2004. 25 June 2006 <http://www.capenature.org.za/index.php?fArticleId=421>.

Catry P, Ruxton GD, Ratcliffe N, Hamer KC, Furness RW (1999) Short-lived repeatabilities on long-lived great skuas: implications for the study of individual quality. *Oikos* **84**, 473–479.

Chakraborty R, Hedrick P (1983) Paternity exclusion and the paternity index for two linked loci. *Human Heredity* **33**, 12-23.

Charlesworth B, Charlesworth D (1999) The genetic basis of inbreeding depression. *Genetic Research, Cambridge* **74**, 329-340.

Article within a website: CITES (2001) The CITES Secretariat. 18 Jun. 2001. 11 Nov. 2006 <http://www.cites.org/eng/disc/sec/index.shtml>.

Article within a website: CITES (2005) South Africa/Sudafrica/Afrique du Sud. Nov. 2005. Nov. 2006 http://www.cites.org/common/directy/s/south_africa.html.

Online CITES document: CITES (2006) Appendices I, II and III. 14 Jun. 2006. 1 Nov. 2006 http://www.cites.org/eng/app/appendices.pdf.

Coenye T, Vandamme P (2005) Characterization of mononucleotide repeats in sequenced prokaryotic genomes. *DNA Research* **12(4)**, 221-233.

Collins PJ, Hennessy LK, Leibelt CS, Roby RK, Reeder DJ, Foxall PA (2004) Developmental validation of a single-tube amplification of the 13 CODIS STR loci, D2S1338, D19S433, and amelogenin: the AmpF*l*STR identifiler PCR amplification kit. *Journal of Forensic Sciences* **49(6)**.

Congiu L, Chicca M, Cella R, Rossi R, Bernacchia G (2000) The use of random amplified polymorphic DNA (RAPD) markers to identify strawberry varieties: a forensic application. *Molecular Ecology* **9(2)**, 229-232.

Dahle G (1994) Minisatellite DNA fingerprints of Atlantic cod (*Gadus morhua*). *Journal of Fish Biology* **44(6)**, 1089-1092.

Dawson DA, Burke T, Hansson B, Pandhal J, Hale MC, Hinten GN, Slate J (2006) A predicted microsatellite map of the passerine genome based on chicken-passerine sequence similarity. *Molecular Ecology* **15(5)**, 1299-1320.

Dawson DA, Darby S, Hunter FM, Krupa AP, Jones IL, 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)**, 201-204.

Dawson DA, Pandhal J (2005a) *Precipitation of BigDye Terminator v1.1 cycle sequencing reactions*. Unpublished laboratory protocol. Sheffield Molecular Genetics Facility, University of Sheffield, UK.

Dawson DA, Pandhal J (2005b) *Preparation of an unenriched microsatellite library*. Unpublished laboratory protocol. Sheffield Molecular Genetics Facility, University of Sheffield, UK.

Dawson RJG, Gibbs HL, Hobson KA, Yezerinac SM (1997) Isolation of microsatellite DNA markers from a passerine bird, *Dendroica petechia* (the yellow warbler), and their use in population studies. *Heredity* **79**, 506-514.

DEAT (2004) National Environment Management: Biodiversity Act (Act 10 of 2004), p. 44. Department of Environmental Affairs and Tourism, South Africa.

DEFRA (2005) Wildlife crime: a guide to the use of forensic and specialist techniques in the investigation of wildlife crime, p. 91. Department of Environment Food and Rural Affairs, UK.

Derjusheva S, Kurganova A, Habermann F, Gaginskaya E (2004) High chromosome conservation detected by comparative chromosome painting in chicken, pigeon and passerine birds. *Chromosome research* **12**, 715-723.

Don R, Cox P, Wainwright B, Baker K, Mattick J (1991) 'Touchdown' PCR to circumvent spurious priming during gene amplification. *Nucleic Acids Research* **19(14)**, 4008.

Donohue KC, Dufty AM (2006) Sex determination of red-tailed hawks (*Buteo jamaicensis calurus*) using DNA analysis and morphometrics. *Journal of Field Ornithology* **77(1)**, 74-79.

Double M, Olsen P (1997) Simplified PCR-based sexing assists conservation of endangered owl, the Norfolk Island boobook (*Ninox novaeseelandiae undulata*). *Bird Conservation International* **7**, 283-286.

Edwards SV, Jennings WB, Shedlock AM (2004) Phylogenetics of modern birds in the era of genomics. *Proceedings of the Royal Society of London. Series B, Biological Sciences* **272**, 979-992.

Ellegren H (2004) Microsatellites: simple sequences with complex evolution. *Nature* **5**, 435-445.

Ellegren H, Fridolfsson AK (1997) Male-driven evolution of DNA sequences in birds. *Nature of Genetics* **17**, 182-184.

Falush D, Stephens M, Pritchard JK (2003) Inference of population structure using multilocus genotype data: linked loci and correlated allele frequencies. *Genetics* **164(4)**, 1567-1587.

Fernandez J, Toro MA, Caballero A (2004) Managing individual's contributions to maximise the allelic diversity maintained in small, conserved populations. *Conservation Biology* **18(5)**, 1358-1367.

Frankham R, Ballou JD, Briscoe DA (2002) *Introduction to conservation genetics* Cambridge University Press, Cambridge.

Frantz AC, Pope LC, Carpenter PJ, Roper TJ, Wilson GJ, Delahay RJ, Burke T (2003) Reliable microsatellite genotyping of the Eurasian badger (*Meles meles*) using faecal DNA. *Molecular Ecology* **12(6)**, 1649-1661.

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

Galbusera P, van Dongen S, Matthysem E (2000) Cross-species amplification of microsatellite primers in passerine birds. *Conservation Genetics* **1**, 163-168.

Gautschi B, Klug Arter M, Husi R, Wettstein W, Schmid B (2002) Isolation and characterisation of microsatellite loci in the globally endangered corncrake, (*Crex Crex* Linné). *Conservation Genetics* **3**, 451-453.

Giannelli PC (2006) Forensic science. *The Journal of Law, Medicine & Ethics* **34(2)**, 310-319.

Gibbs M, Dawson D, McCamley C, Wardle A, Armour J, Burke T (1997) Chicken microsatellite markers isolated from libraries enriched for simple tandem repeats. *Animal Genetics* **28**, 401 - 417.

Glenn TC (1997) *Genetic bottlenecks in long-lived vertebrates: mitochondrial and microsatellite DNA variation in American alligators and whooping cranes*. PhD thesis, University of Maryland, Maryland, USA.

Glenn TC, Ojerio RS, Stephan W, Braun MJ (1997) Microsatellite DNA loci for genetic studies of cranes. *Proceedings of the North American Crane Workshop* **7**, 36-45.

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

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

Groenen MAM, Cheng HH, Bumstead N, Benkel BF, Elwood Briles W, Burke T, Burt DW, Crittenden LB, Dodgson J, Hillel J, Lamont S, Ponce de Leon A, Soller M, Takahashi H, Vignal A (2000) A consensus linkage map of the chicken genome. *Genome Research* **10**, 137-147.

Gullberg A, Olsson M, Tegelstrom H (1999) Evolution in populations of Swedish sand lizards: genetic differentiation and loss of variability revealed by multilocus DNA fingerprinting. *Journal of Evolutionary Biology* **12(1)**, 17-26.

Hansson B, Akersson M, Slate J, Pemberton JM (2005) Linkage mapping reveals sex-dimorphic map distances in a passerine bird. *Proceedings of the Royal Society of London. Series B, Biological Sciences* **272**, 2289 - 2298.

Hardy ICW (2002) Sex ratios: concepts and research methods, p. 380. Cambridge University Press, Cambridge.

Harrison JA, Allan DG, Underhill LG, Herremans M, Tree AJ, Parker V, Brown CJ (1997) The atlas of southern African birds. Birdlife South Africa, Johannesburg.

Hasegawa O, Ishibashi Y, Abe S (2000) Isolation and characterisation of microsatellite loci in the red-crowned crane *Grus japonensis*. *Molecular Ecology* **9**, 1677-1678.

Hemley G (1994) International wildlife trade: a CITES sourcebook, p. 169. World Wildlife Fund and Island Press, Washington, D.C.

Hoelzel AR, Ford JKB, Dover GA (1991) A paternity test case for the killer whale (*Orcinus orca*) by DNA fingerprinting. *Marine Mammal Science* **7(1)**, 35-43.

Hoffman JI, Amos W (2005) Microsatellite genotyping errors: detection approaches, common sources and consequences for paternal exclusion. *Molecular Ecology* **14(2)**, 599-612.

Hudson V (2002) Crane mortalities through agrochemical misuse in South Africa. *Crane Link* **12**, 6-7.

Hughes AL, Hughes MK (1995) Small genomes for better flyers. *Nature* **377**, 391.

Huynen L, Millar CD, Lambert DM (2002) A DNA test to sex ratite birds. *Molecular Ecology* **11(4)**, 851-856.

Huyvaert KP, Anderson DJ, Jones TC, Duan W, Parker PC (2000) Extra-pair paternity in waved albatross. *Molecular Ecology* **9**, 1415-1419.

International Chicken Genome Sequencing Consortium (2004) Sequence and comparative analysis of the chicken genome provide unique perspectives on vertebrate evolution. *Nature* **432**, 695-716.

Article within a website International Crane Foundation (2006) Blue crane: *Anthropoides paradisea*. 22 Oct. 2006 <http://www.savingcranes.org/species/blue.cfm>.

Itoh Y, Arnold AP (2005) Chromosomal polymorphism and comparative painting analysis in the zebra finch. *Chromosome research* **13**, 47-56.

IUCN (2006) 2006 IUCN Red List of Threatened Species. Downloaded on 22 March 2007. 22 March 2007 <http://www.iucnredlist.org/>.

Jachmann H (2003) Elephant poaching and resource allocation for law enforcement. In: *The trade in wildlife: regulation for conservation* (ed. Oldfield S), p. 210. Earthscan, London.

Personal communication: Janse van Rensburg I (2005) National Zoological Gardens, South Africa. Department: Wildlife Breeding Resource Centre. e-mail: info@wbrc.org.za.

Jeffreys AJ, Wilson V, Thein SL (1985) Individual-specific 'fingerprints' of human DNA. *Nature* **316**, 76-79.

Jeffreys AJ, Wilson V, Thein SL (1985a) Hypervariable 'minisatellite' regions in human DNA. *Nature* **314**, 67-73.

Jehle R, Arntzen JW (2002) Review: microsatellite markers in amphibian conservation genetics. *Herpetological Journal* **12**, 1-9.

Jones AG, Ardren WR (2003) Methods of parentage analysis in natural populations. *Molecular Ecology* **12**, 2511-2523.

Article within a website: Jones K, Glenn T (1999) Screening the *Gamu7* microsatellite locus to determine the sex of captive whooping cranes. 08 Mar 2006. 11 May 2006 <http://www.promega.com/enotes/applications/ap0006_tabs.htm>.

Jones KL, Rodwell L, McCann K, Verdoorn GH (2006) Genetic conservation of South African wattled cranes. *Biological Conservation* **127**, 98-106.

Jones KL, T.C. Glenn, R.C. Lacy, J.R. Pierce, N. Unruh, C.M. Mirande, F.Chavez-Ramirez. (2002) Refining the whooping crane studbook by incorporating microsatellite DNA and leg-banding analysis. *Conservation Biology* **16(3)**, 789-799.

Kanz C, Aldebert P, Althorpe N, Baker W, Baldwin A, Bates K, Browne P, van den Broek A, Castro M, Cochrane G, Duggan K, Eberhardt R, Faruque N, Gamble J, Diez FG, Harte N, Kulikova T, Lin Q, Lombard V, Lopez R, Mancuso R, McHale M, Nardone F, Silventoinen V, Sobhany S, Stoehr P, Tuli MA, Tzouvara K, Vaughan R, Wu D, Zhu W, Apweiler R (2005) The EMBL nucleotide sequence database. *Nucleic Acids Research* **33(suppl. 1)**, 29-33.

Kayang BB, Vignal A, Inoue-Murayama M, Miwa M, Monvoisin JL, Ito S, Minvielle F (2004) A first-generation microsatellite linkage map of the Japanese quail. *Animal Genetics* **35(3)**, 195-200.

Komdeur J, Pen I (2002) Adaptive sex allocation in birds: the complexities of linking theory to practice. *Proceedings of the Royal Society of London. Series B, Biological Sciences* **357**, 373 - 380.

Krajewski C (1989) Phylogenetic relationships among cranes (Gruiformes: Gruidae) based on DNA hybridization. *The Auk* **106**, 603-618.

Krajewski C, Fetzner Jr JW (1994) Phylogeny of cranes (Gruiformes: Gruidae) based on cytochrome-B DNA sequences. *The Auk* **111(1)**, 351-365.

Kumar S, Tamura K, Nei M (2004) MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Briefings in Bioinformatics* **5**, 150-163.

Leviston A, Dawson DA (2004) *Using stand-alone nBLAST to check for duplicate sequences*. Unpublished protocol. University of Sheffield, Sheffield, UK.

Lillandt B, Bensch S, von Schantz T (2001) Parentage determination in kin-structured populations: microsatellite analysis in the Siberian jay (*Perisoreus infaustus*) during a 25-year population study. *Avian Science* **1(1)**, 3 -14.

Longmire JL (1997) Use of 'lysis buffer' in DNA isolation and its implication for museum collections. *Occassional Papers, Museum of Texas Tech University* **163**.

Longmire JL, Gee. GF, Hardekopf CL, Mark GA (1992) Establishing paternity in whooping cranes (*Grus americana*) by DNA analysis. *The Auk* **109(3)**, 522-529.

Luikart G, Cornuet J-M (1999) Estimating the effective number of breeders from heterozygote excess in progeny. *Genetics* **151**, 1211-1216.

Maclean GL (1985) *Roberts birds of southern Africa*, 6th edn. John Voelcker Bird Book Fund, Cape Town.

Manel S, Berthier P, Luikart G (2002) Detecting wildlife poaching: identifying the origin of individuals with Bayesian assignment tests and multilocus genotypes. *Conservation Biology* **16(3)**, 650-659.

Marshall TC, Slate J, Kruuk LEB, Pemberton JM (1998) Statistical confidence for likelihood-based paternity inference in natural populations. *Molecular Ecology* **7(5)**, 639 - 655.

McCann K, Morrison K, Byers A, Miller P, Friedman Y (2002) Blue crane (*Anthropoides paradiseus*). A population and habitat viability assessment workshop. Final

workshop report. Conservation Breeding Specialist Group (SSC/IUCN), Villiersdorp.

McInnes LM, Dadour IR, Stewart ME, Ditcham WGF, Mawson P, Spencer PB (2005) Characterisation of polymorphic microsatellite markers for Carnaby's cockatoo (*Calyptorhynchus latirostris*) and related black cockatoo species. *Molecular Ecology Notes* **5**, 504-506.

Meine C, Archibald GW (1996a) Ecology, status, and conservation. In: *Cranes: their biology, husbandry, and conservation* (ed. Ellis DH, G.F. Gee & C.M. Mirande), pp. 263-292. Hancock House Publishers, Blain, WA.

Meine CD, Archibald GW (1996b) The cranes: status survey and conservation action plan., p. 294. Canadian Museum of Nature, Gland.

Meine CD, Harris JT (1997) Putting the pieces together: international management of cranes and their habitats. In: *Principles of conservation biology* (eds. Meffe GK, Carroll CR), pp. 467 - 478. Sinauer Associates Inc., Massachusetts.

Millions DG, Swanson BJ (2006) An application of Manel's model: detecting bobcat poaching in Michigan. *Wildlife Society Bulletin* **34(1)**, 150-155.

Ministry of Agriculture and Forestry (2003) New Zealand's first Interpol conference: wildlife smuggling. *Primary Source: news from the Ministry of Agriculture and Forestry* **23**, 2.

Moretti TR, Baumstark AL, Defenbaugh D, A., Keys KM, Smerick JB, Budowle B (2001) Validation of short tandem repeats (STRs) for forensic usage: performance testing of fluorescent multiplex STR systems and analysis authentic and simulated forensic samples. *Journal of Forensic Sciences* **46**, 647-660.

Morrison K (2002) Illegal trade in cranes. *Crane Link* **12**, 9.

Mueller UG, Wolfenbarger LL (1999) AFLP genotyping and fingerprinting. *Trends in Ecology & Evolution* **14(10)**, 389-394.

Mundy NI, Winchell CS, Burr T, Woodruff D (1997) Microsatellite variation and microevolution in the critically endangered San Clemente Island loggerhead shrike (*Lanius ludovicianus mearnsi*). *Proceedings of the Royal Society of London. Series B, Biological Sciences* **264**, 869 - 875.

Nesje M, Roed KH, Lifjeld JT, Lindberg P, F. SO (2000) Genetic relationships in the peregrine falcon (*Falco peregrinus*) analysed by microsatellite DNA markers. *Molecular Ecology* **9**, 53 - 60.

Parker PG, Snow AG, Schug MD, Booton GC, Fuerst PA (1998) What molecules can tell us about populations: choosing and using an molecular marker. *Ecology* **79(2)**, 361-382.

Pierson B, Pearce JM, Talbot SL, Shields GF, Scribner KT (2000) Molecular genetic status of Aleutian Canada geese from Buldir and the Semidi Islands, Alaska. *The Condor* **102**, 172-180.

Pilastro A, Pezzo F, Olmastroni S, Callgarin C, Corsolini S, Focardi S (2001) Extrapair paternity in adélie penguin (*Pygoscelis adeliae*). *Ibis* **143**, 681-684.

Primmer CR (2005) Genetic characterisation of populations and its use in conservation decision making in fish. *The Role of Biotechnology, Turin, Italy*, 137-142.

Primmer CR, Møller AP, Ellegren H (1995) Resolving genetic relationships with microsatellite markers: a parentage testing system for the swallow (*Hirundo rustica*). *Molecular Ecology* **4**, 493-498.

Primmer CR, Møller AP, Ellegren H (1996) A wide-range survey of cross-species microsatellite amplification in birds. *Molecular Ecology* **5**, 365-378.

Primmer CR, Painter JN, Koskinen MT, Palo JU, Merilla J (2005) Factors affecting avian cross-species microsatellite amplification. *Journal of Avian Biology* **36**, 348 - 360.

Primmer CR, Raudsepp T, Chowdhary BP, Møller AP, Ellegren H (1997) Low frequency of microsatellites in the avian genome. *Genome Research* **7**, 471-482.

Ramey RR, Luikart G, Singer FJ (2000) Genetic bottlenecks resulting from restoration efforts: the case of bighorn sheep in Badlands National Park. *Restoration Ecology* **8(4)**, 85-90.

Raymond M, Rousset F (1995) GENEPOP (Version 1.2) population genetics software for exact tests and ecumenism. *Journal of Heredity* **86**, 248-249.

Rice WR (1989) Analyzing tables of statistical tests. *Evolution* **43(1)**, 223-225.

Richardson DS, Jury FL, Blaakmeer JK, Komdeur J, Burke T (2001) Parentage assignment and extra-group paternity in a cooperative breeder: the Seychelles warbler (*Acrocephalus sechellensis*). *Molecular Ecology* **10**, 2263-2273.

Rozen S, Skaletsky HJ (2000) PRIMER v3 on the WWW for general users and for biological programmers. In: *Bioinformatics methods and protocols: methods in molecular biology* (eds. Krawetz S, Misener S), pp. 365 - 386. Humana Press, Totowa, NJ.

SACWG (2000) National cane and habitat action plan, pp. 88-106. South African Crane Working Group, Parkview.

SACWG (2003) National guidelines for trade and keeping cranes in captivity, p. 23. Endangered Wildlife Trust, Wakkerstroom.

SACWG (2004) EWT/SACWG strategy workshop, p. 62. Endangered Wildlife Trust and the South African Crane Working Group, Wakkerstroom.

Sambrook J, Russel D (2000) *Molecular cloning: a laboratory manual*, 3rd edn. Cold Spring Harbour Laboratory Press, New York.

Schlötterer C (1998) Microsatellites. In: *Molecular genetic analysis of populations: a practical approach* (ed. Hoelzel AR). IRL Press, Oxford.

Sefc KM, Payne RB, Sorenson MD (2003) Microsatellite amplification from museum feather samples: effects of fragment size and template concentration on genotypic errors. *The Auk* **4**, 982-989.

Selkoe KA, Toonen RJ (2006) Microsatellites for ecologists: a practical guide to using and evaluating microsatellite markers. *Ecology Letters* **9(5)**, 615-629.

Sheldon B (1998) Recent studies of avian sex ratios. *Heredity* **80(4)**, 397-402.

Shetty S, Griffin DK, Marshal Graves JA (1999) Cooperative painting reveals strong chromosome homology over 80 million years of bird evolution. *Chromosome research* **7**, 289-295.

Sibley C, Ahlquist J (1990) *Phylogeny and classification of birds. A study in molecular evolution.* Yale University Press, New Haven.

Sobel E, Papp JC, Lange K (2002) Detection and Integration of Genotyping Errors in Statistical Genetics. *American Journal of Human Genetics* **70**, 496–508.

Storfer A (1999) Gene flow and endangered species translocations: a topic revisited. *Biological Conservation* **87**, 173-180.

Sunnucks P (2000) Efficient genetic markers for population biology. *Trends in Ecology & Evolution* **15(5)**, 199 - 203.

SWGDAM (2004) Revised validation guidelines. *Forensic Science Communications: standards and guidelines* **6(3)**.

Tarr CL, Ballou JD, Morin MP, Conant S (2000) Microsatellite variation in simulated and natural founder populations of the Laysan finch (*Telespiza cantans*). *Conservation Genetics* **1(2)**, 135-146.

TRAFFIC (2006a) Species fact sheet: illegal and unsustainable wildlife trade, p. 4. World Wildlife Fund, Switzerland.

Article within a website: TRAFFIC (2006b) What is wildlife trade worth financially? 30 Nov. 2006. 12 Nov. 2006 <http://www.traffic.org/wildlife/wild4.htm>.

UNEP (1998) Policy effectiveness and multilateral environmental agreements. Environment and Trade Series, UNEP Economics, Trade and Environment Unit, Geneva.

Personal communication: Vaez M (2006) Sheffield Molecular Genetics Facility, University of Sheffield, South Yorkshire, UK. Email: bop03mv@sheffield.ac.uk.

Vignal A, Milan D, SanCristobal M, Eggen A (2002) A review on SNP and other types of molecular markers and their use in animal genetics. *Genetics Selection Evolution* **34**, 275-305.

Wasser SK, Shedlock AM, Comstock K, Ostrander EA, Mutayoba B, Stephens M (2004) Assigning African elephant DNA to geographic region of origin: applications to the ivory trade. *Proceedings of the National Academy of Sciences* **101(41)**, 14847-14852.

Wetton JH, Carter RE, Parkin DT, Walters D (1987) Demographic study of a wild house sparrow population by DNA fingerprinting. *Nature* **327**, 147-149.

Zane L, Bargelloni L, Patarnello T (2002) Strategies for microsatellite isolation: a review. *Molecular Ecology* **11(1)**, 1-16.

Zhang D-X, Hewitt GM (2003) Nuclear DNA analyses in genetic studies of populations: practice, problems and prospects. *Molecular Ecology* **12(3)**, 563-584.

Zhang J (2003) Evolution by gene duplication: an update. *Trends in Ecology & Evolution* **18(6)**, 292-298.

Ziegle J, Su Y, Corcoran K, Nie L, Mayrand P, Hoff L, McBride L, Kronick M, Diehl S (1992) Application of automated DNA sizing technology for genotyping microsatellite loci. *Genomics* **14(4)**, 1026-1031.

APPENDICES

Appendix I Sample database

Below are three tables containing a database of sample information for the three South African crane species used in this study: blue crane *Grus paradisea* (Table 9.1a), wattled crane *G. carunculatus* (Table 9.1b), and grey-crowned crane *Balearica regulorum* (Table 9.1c) samples provided by SACWG and WBRC.

Note: age classes were defined as follows: chick, aged 10 weeks or less; juvenile, aged between 10 weeks and one year; adult, birds in full adult plumage. Although GPS co-ordinates were originally provided with most samples, they are not provided here for confidentiality.

Table 9.1 Database of crane samples

Table 9.1a Database of blue crane *Grus paradisea* blood and tissue samples

WBRC accession no.*	Other Id	Age †	Collection date	Sampler	Origin	Province
3193	9A20002	chick	11-Feb-02	K. Morrison	wild	Limpopo
3194	9A20006	chick	11-Feb-02	K. Morrison	wild	Limpopo
3195	9A20010	chick	11-Feb-02	K. Morrison	wild	Limpopo
3197	9A2007	Juvenile	07-Feb-03	K. Morrison	wild	Mpumalanga
3198	9A30099	chick	04-Jan-02	B. Coverdale	wild	Kwa Zulu Natal
3199	9A30101	chick	04-Jan-02	B. Coverdale	wild	Kwa Zulu Natal
3203	9A30109	chick	04-Jan-02	B. Coverdale	wild	Kwa Zulu Natal
3207	9A31051	chick	13-Feb-02	B. Gibbons	wild	Freestate
3208	9A31053	chick	13-Feb-02	B. Gibbons	wild	Freestate
3210	9A31080	chick	22-Feb-02	G. Ramke	wild	Mpumalanga
3211	9A31082	chick	06-Feb-03	G. Ramke	wild	Kwa Zulu Natal
3212	9A31083	chick	25-Jan-03	G. Ramke	wild	Mpumalanga
3213	9A31084	chick	06-Feb-03	G. Ramke	wild	Kwa Zulu Natal
3214	9A31087	chick	03-Feb-03	G. Ramke	wild	Mpumalanga
3222	9A31166	adult	09-Apr-01	J. Smallie	captive	Eastern Cape
3223	9A33287	chick	19-Feb-03	D. Jolliffe	wild	Northern Cape
3224	9A33291	chick	20-Feb-03	D. Jolliffe	wild	Northern Cape
3225	9A42254	chick	20-Feb-03	D. Jolliffe	wild	Northern Cape
3232	-	unknown	unknown	unknown	wild	unknown
3695	9A33299 R-leg,R-leg BWO,left R	juvenile	16-Feb-04	Cath,J.Venter	wild	Northern Cape
3696	9A42312 left leg,R-leg RGR,L-leg Red	juvenile	16-Feb-04	M.Anderson	wild	Northern Cape
3697	9-92129 R-leg,R-leg OWR,L-leg Red	juvenile	17-Feb-04	Cath,Jenny, J.Venter	wild	Northern Cape
3698	9A33297 R-leg,R-leg BWY, l-leg Red	juvenile	17-Feb-04	Cath,Jenny, J.Venter	wild	Northern Cape
3800	9A33300 r-leg,R-leg YRG,L-leg Red	juvenile	18-Feb-04	Cath,Jenny, J.Venter	wild	Northern Cape
3801	9-92124 R-leg,R-leg OYR,L-leg Red	chick	18-Feb-04	Cath,Jenny, J.Venter	wild	Northern Cape
3802	9A42251 R-leg,R-leg YWG,L-leg Red	chick	18-Feb-04	Cath,Jenny, J.Venter	wild	Northern Cape
3804	9A42259 left leg,R-leg WYB,L-leg Red	juvenile	19-Feb-04	Cath,Mark Anderson	wild	Northern Cape
3805	9-92118 left leg,R-leg BO,Left leg red	juvenile	19-Feb-04	Cath,Mark Anderson	wild	Northern Cape
3806	9-92121 left leg,R-leg Red,L-leg RB	juvenile	19-Feb-04	Cath,Mark Anderson	wild	Northern Cape
3807	9A42315 left leg,R-leg RYB,L-leg Red	unknown	19-Feb-04	Cath,Mark Anderson	wild	Northern Cape
3808	9A42260 left leg,R-leg WGW,L-leg Red	juvenile	19-Feb-04	Cath,Mark Anderson	wild	Northern Cape
3809	9-92120 left leg,R-leg YO,L-leg Red	juvenile	19-Feb-04	Cath,Mark Anderson	wild	Northern Cape
3810	9-92115 left leg,R-leg OB,L-leg red	juvenile	19-Feb-04	Cath,Mark Anderson	wild	Northern Cape
3811	9A42262 left leg,R-leg YRO,L-leg Red	juvenile	19-Feb-04	Cath,Mark Anderson	wild	Northern Cape
3812	9A42264 left leg,R-leg WBO,L-leg Red	juvenile	19-Feb-04	Cath,Mark Anderson	wild	Northern Cape
3813	9A42263 left leg,R-leg YWO,L-leg red	juvenile	19-Feb-04	Cath,Mark Anderson	wild	Northern Cape
3814	9A42314 R-leg,R-leg RGW,L-leg Red	chick	19-Feb-04	Jenny,J.Venter	wild	Northern Cape
3815	9A42316 R-leg,R-leg RYG,L leg Red	chick	19-Feb-04	Jenny, Kevin	wild	Northern Cape
3845	9A2009,R-leg Large green,L-Leg B,G	unknown	27-Jan-04	unknown	wild	unknown
3854	-	adult	01-Aug-02	J.O'Grady	wild - poisoned	Kwa Zulu Natal
4352	9A20012	chick	26-Apr-04	Kobus Pienaar	wild	Limpopo
4353	9A20008	chick	26-Apr-04	Kobus Pienaar	wild	Limpopo
4384	9A30178	chick	15-Jan-04	G. Ramke	wild	Mpumalanga
4385	9A42478	chick	11-Feb-04	G. Ramke	wild	Mpumalanga
4386	9A42479	chick	11-Feb-04	G. Ramke	wild	Mpumalanga
4387	9A42480	chick	11-Feb-04	G. Ramke	wild	Mpumalanga
4518	9A30114	juvenile	unknown	unknown	wild	Kwa Zulu Natal

Table 9.1a continued

WBRC accession no.*	Other Id	Age †	Collection date	Sampler	Notes	Province
4519	9A20005, rings - R-Green, L-White/red	chick	29-Apr-04	K. Painaar	wild	Gauteng
4530	9A31072	chick	24-Nov-04	G. Ramke	wild	Mpumalanga
4567	9A31089	chick	unknown	G. Ramke	wild	Kwa Zulu Natal
4576	9A30096	unknown	26-Aug-04	unknown	wild	Kwa Zulu Natal
4577	9A42810	unknown	08-Jan-03	unknown	wild	Kwa Zulu Natal
4581	-	unknown	unknown	unknown	unknown	unknown
4612	98 (yellow-left leg)	adult	24-Apr-02	H. King	captive	Kwa Zulu Natal
4613	457 (1530A)	unknown	24-Apr-02	H. King	captive	Kwa Zulu Natal
4614	Ring 252	unknown	24-Apr-02	H. King	captive	Kwa Zulu Natal
4615	39	adult	24-Apr-02	H. King	captive	Gauteng
4616	One wing C4C	unknown	24-Apr-02	H. King	captive	Gauteng
4618	Camp 56	adult	24-Apr-02	H. King	captive	Western Cape
4619	236	unknown	24-Apr-02	H. King	captive	Kwa Zulu Natal
4620	Camp 56	chick	24-Apr-02	H. King	captive	Western Cape
4626	Athena	chick	24-Apr-02	H. King	captive	Gauteng
4627	Ring 251	unknown	24-Apr-02	H. King	captive	Kwa Zulu Natal
4630	Ring 254	unknown	24-Apr-02	H. King	captive	Kwa Zulu Natal
4631	Ring 050	unknown	24-Apr-02	H. King	captive	Kwa Zulu Natal
4632	75 (white - right leg)	adult	24-Apr-02	H. King	captive	Kwa Zulu Natal
4633	Ring 049	unknown	24-Apr-02	H. King	captive	Kwa Zulu Natal
4634	Ring 258	unknown	24-Apr-02	H. King	captive	Kwa Zulu Natal
4635	Ring 257	unknown	24-Apr-02	H. King	captive	Kwa Zulu Natal
4636	40	adult	24-Apr-02	H. King	captive	Gauteng
4637	Ring 255	unknown	24-Apr-02	H. King	captive	Kwa Zulu Natal
5122	Ring 9A42273	unknown	22-Feb-05	unknown	wild	Northern Cape
5129	Ring 9A33273	unknown	17-Feb-05	M.Anderson	wild	Northern Cape
5130	Ring 9A33275	unknown	17-Feb-05	M.Anderson	wild	Northern Cape
5131	Ring 9A42279	unknown	14-Feb-05	R Visagie	wild	Northern Cape
5132	Ring 9A42280	unknown	14-Feb-05	R Visagie	wild	Northern Cape
5133	Ring 9A42282	unknown	15-Feb-05	R Visagie	wild	Northern Cape
5134	Ring 9A42288	unknown	14-Feb-05	R Visagie	wild	Northern Cape
5135	-	unknown	04-Jan-05	Kobus Pienaar	wild	Limpopo
5136	Ring 9A31156	unknown	16-Dec-04	G. Shaw	wild	Eastern Cape
5137	Ring 9A30306	unknown	unknown	G. Shaw	wild	Eastern Cape
5138	Ring 9A31170	unknown	10-Jan-05	G. Shaw	wild	Eastern Cape
5139	Ring 9A31200	unknown	15-Dec-04	G. Shaw	wild	Eastern Cape
5140	Ring 9A31151	unknown	10-Jan-05	G. Shaw	wild	Eastern Cape
5141	Ring 9A30899,r-leg large green, l-leg small white	unknown	08-Dec-04	Kobus Pienaar	wild	Limpopo
5142	Ring 9A30097	unknown	16-Jan-05	B. Coverdale	wild	Kwa Zulu Natal
5143	Ring 9A45811	unknown	17-Jan-05	Kobus Pienaar	wild	Limpopo
5144	-	unknown	04-Jan-05	Kobus Pienaar	wild	Limpopo
5145	Ring 9A30900	unknown	14-Jan-05	Kobus Pienaar	wild	Limpopo
5146	Ring 9A45851,r-leg green, l-leg green/yellow	unknown	08-Dec-04	Kobus Pienaar	wild	Limpopo
5147	Ring 9A42812	unknown	16-Jan-05	J. Wakelin	wild	Kwa Zulu Natal
none	J17701	chick	unknown	unknown	wild	Eastern Cape
none	J17718	chick	unknown	unknown	wild	Eastern Cape
none	Rhen 2	unknown	unknown	unknown	wild	Limpopo
none	Stoffie	chick	unknown	unknown	captive	Western Cape
none	J17715	unknown	unknown	unknown	wild	Eastern Cape
none	Orpen 1	unknown	unknown	unknown	wild	Eastern Cape
none	Esp. 1	chick	unknown	unknown	wild	Eastern Cape
none	Esp. 2	chick	unknown	unknown	wild	Eastern Cape
none	Orpen 2	chick	unknown	unknown	wild	Eastern Cape
none	Som. 1	chick	unknown	unknown	wild	Eastern Cape
none	Rhen d	unknown	unknown	unknown	wild	Limpopo

*all samples are owned by SACWG, and stored at the Wildlife Breeding Resource Centre, Pretoria

Table 9.1b Database of grey-crowned crane (*Balearica regulorum*) samples

WBRC accession no.*	Other Id	Age	Collection date	Sampler	Notes	Province
3200	9A30102	chick	18-Sep-02	K. McCann	Captive - released	Kwa Zulu Natal
3201	9A30103	chick	18-Sep-02	K. McCann	Captive - released	Kwa Zulu Natal
3202	9A30107	chick	18-Sep-02	K. McCann	Captive - released	Kwa Zulu Natal
3204	9A30303	chick	15-Feb-01	J. Smallie	Wild	Eastern Cape
3205	9A30308	chick	25-Feb-02	J. Smallie	Wild	Eastern Cape
3206	9A30309	chick	25-Feb-02	J. Smallie	Wild	Eastern Cape
3209	9A31073	chick	14-Mar-02	G. Ramke	Wild	Mpumalanga
3215	9A31094	juvenile	28-Mar-02	G. Ramke	Wild	Mpumalanga
3216	9A31095	juvenile	29-Mar-02	G. Ramke	Wild	Mpumalanga
4584	9A30089	juvenile	03-Dec-04	B. Coverdale	Wild	Kwa Zulu Natal

*all samples are owned by SACWG, and stored at the Wildlife Breeding Resource Centre, Pretoria

Table 9.1c Database of wattled crane (*G. carunculatus*) samples

WBRC accession no.*	Other Id	Age	Collection date	Sampler	Notes	Province
3226	J14952	chick	02-Dec-02	B. Coverdale	Wild	Kwa Zulu Natal
3227	J14953	juvenile	12-Aug-03	B. Coverdale	Wild	Kwa Zulu Natal
3228	J14956	juvenile	02-Oct-03	B. Coverdale	Wild	Kwa Zulu Natal
3229	J14957	juvenile	02-Oct-03	B. Coverdale	Wild	Kwa Zulu Natal
3230	J14959	juvenile	12-Aug-03	B. Coverdale	Wild	Kwa Zulu Natal
3231	J14961	juvenile	18-Oct-02	B. Coverdale	Wild	Kwa Zulu Natal
3233	J14963	juvenile	18-Nov-02	B. Coverdale	Wild	Kwa Zulu Natal
3234	J14967	juvenile	20-Aug-02	B. Coverdale	Wild	Kwa Zulu Natal
3235	J14968	juvenile	05-Jul-01	B. Coverdale	Wild	Kwa Zulu Natal
3237	J14970	chick	04-Aug-01	B. Coverdale	Wild	Kwa Zulu Natal

*all samples are owned by SACWG, and stored at the Wildlife Breeding Resource Centre, Pretoria

Appendix II Recipes for laboratory reagents

0.5 M EDTA 200 ml pH 8.0

Dissolve 37.2 g in 200 ml H_20

pH to 8.0 using NaOH pellets in order for EDTA to solubilise

Autoclave

1 M Tris-base 200 ml pH 8.0

Dissolve 24.22 g in 200 ml H_20

pH to 8.0

Autoclave

20 % SDS 100 ml

Wearing a mask, weigh 20 g SDS.

In a fume hood, dissolve 20 g SDS in 100 ml ddH20

Do not autoclave.

Digsol (Digestion solution) 200 ml pH 8.0

Final concentrations of each reagent required:

20 mM EDTA

50 mM Tris

120 mM NaCl

1 % SDS

Preparation:

8 ml of 0.5 M EDTA

10 ml of 1 M Tris-base

1.37 g NaCl

Add H_20 up to 190 ml. Mix and autoclave. Add 10 ml of 20 % SDS. pH to 8.0 with HCl

4 M Ammonium Acetate 100 ml pH 7.5

30.83 g Ammonium Acetate

Add H_20 up to 100 ml. Autoclave.

pH to 7.5 if necessary using Glacial Acetic Acid.

$T_{10}E_{0.1}$ 400 ml pH 7.5-8.0

Final concentrations of each reagent required:

10 mM Tris

0.1 mM EDTA

Preparation:

4 ml of 1 M Tris

80 µl of 0.5 M EDTA

Autoclave.

pH if necessary.

10 mg/ml Proteinase K

To a bottle containing 100 mg Proteinase K, add 10 ml of 'MilliQ' H_20.

Into 1.5 ml eppendorfs, aliquot 1 ml lots

Store at -20 °C.

Appendix III Blue crane sequences.

Provided below are the sequences generated in blue crane.

The loci '*AA-1*', '*AA/GC*', '*AT/AC-1*', all '*Gamu*'loci and '*SHC-AG-2*' were originally developed by Glenn (1997). '*Gj-M*' loci were originally developed by Hasegawa *et al.* (2000). Their respective locus names were used when generating the sequences provided here for the blue crane. Loci containing prefix *Gpa* denotes those developed for this study.

Before each sequence, the following information is provided: locus ID, clone name where available, EMBL accession number.

> *AA-1*, AM168498

CCCGTGGGGAATCCAGAGGGGGAAAGAAATACCTGAAACAATTAAGAAAAGAACAAAACACCAAAACAAAACA
AAAAAAAATCAAACCAAAACAAAAAAACACAAAAACAAAACAAAACAAGAAAAAGAGAAAGGTAGTAAATACA
AGGAGAACCAAAGGGGAGAAAGACATGCTGGAAAAAAAAAAAGGCTTTAGTATATCCATGCCAAAACCAATAG
A

> *AA/GC*, AM168499

CCATTTTATCTGTCTCTGTTCATAATTTGTCTGCGTGTGTATTTCTCAGTGTCTGTGTGCTCTTTATGTGAAG
GTTTGTCTTTGTTTTTAGGACCGTACTATTCCAATTTTTCAGCAAGCAAGCAAGCAATGAATTTTGCTTTTAC
TTTGCTGACTCCTTGAGGCTGTCAAATGTCTGTTCTTTAAGTAATCAGTGAAAGCCC

> *AT/AC-1*, AM168500

ATACTGGTAGTGCAGCTCCTAAAAATATATATATACACACACACACTGTCAGCACTACAAAGTTTCCAAGTGA
AAGACAGGTCAGCCATATGACAAGCAAAGACGGG

> *Gamu002*, AM168501

AACTTTTGCCCATACTTCTGTAGTAATTCTATACATCTCATCGGTTTTCCTTTCATACACATACACAGAGAAG
GAATTTCAGAACAATTCCTTATTTCTGATAACTCACATGCCAGGCCCGAACAATTCAGTTTAGGTTAAATAAC
AACAGAACACAGCAAAAGAAGCGGGAAC

> *Gamu003*, AM282934

ATCCCTGAAGCTAACAATAAACCGTCTGTGTGTGTGTGTGTGTGTGAGAGAGAGAGAGAGAGAGAAAGTGAGA
AGAGGGAGGGAGAGAAAGCAATACAACAGTCTGGCAATGTGAATCAC

> *Gamu004*, AM282935

CGATTAAGGGGGGATGCAAGAAGTCCAGCTGAGTTTGAGTTTGCAACTATTAGCAAAAAACACACACACACCC
CCCATTCCACCACCTCATGTGGTGAAAAACCATAAAAAAGAGGAAAAGAAGAAGAGTGATTAAAGATTGTCTGC
AGGCATGGCCCAAACCGAAAGCGTCCTTTGTGCTGGCTCGGGAATCAC

> *Gamu005*, AM168502

CGGATGTGTACTTGGCTCAGAAGCAGCATGAATCACGGCTTCAAAGAACAAAGCAACCTGTTTCATCACTAAA
TAGGGGAAATGAAAGCATCCAAAGGAGTTCCTGTTTCATCTGCATGCATGTGTGTGTGTTTGTGTGTGTGTAC
ACGTAGCCGACGCACTCACGCACACAACTAACCACAGGGAAA

> *Gamu006*, AM168503

CACCTTTTATTGCGTATGTATTTTCTATATCAATATTTAACACACACACACACACACACAAAAAGCTAGGTTG
TAATTTTGCAGAGTTTTGCATCAATGTGAAAAACAAAACCAAAACATAATCC

> *Gamu007*, AM168504

TAAAGGAGTGGCTGCTGCTGTGCAATCACACACCATTCATGGTGTCGCACATGCACACACACACACACACACA
CACACTTATTCCTGGGCACACTTCCTCCTCCTTCAGGCTTGACCCCAGGTTTCCCACAGCAGAGCCTCAG

> *Gamu010*, AM168505

AATAATTCTTTTAGATTCTTTTATGAAAGGATTAAGACTTCTGTAGAAAACAGACGTGGACACACACACACAT
ACACGACACTGTTGAGTGAATCTAATTCCTCATTCAAAAAAAC

> *Gamu011*, AM168506

CAATGGAGCGCCGTCACGTGGGGGACGGGCACGTCAAAGGTGCTGCTGCCGCTCCGCCACACAGCAGCCCTCC
TCCTCCTCCTCCTCCTCCTCGGCGCTCTCGGCTCGCTCACCGAAAACAGCGGCCAAGAAACTG

> *Gamu012*, AM168507

GCACGGAATGAATCTTTTCTGTGGAAGAACGAACGCGGTAAATTAAACGCGAATGTGTGTGTTCAGATCAGCG
GAGCTGCGAGCATCGGCATCAGTAGCATTAATTGGAGAGCAGATGGTTGGTC

> *Gamu013*, AM168508

AATAAGTTTGGAAATGTTTCTCATATGTGTCATATTTAGCTGAGTTTGAACATGTTCTTCAGATGTCTATTGT
GGGCTGGCATGTGCTTTACTGCTTTATTTTATCCCAGTTGTTTTAGGTTCATATACGTGGATGCATGTGTGTG
TGTGTGTGTGTGTGCCTTTAGTATTGACCAAGAAAA

> *Gamu*014, AM168509

CTTGTTCCACCGTTGTCAGCTAAGCAGGAACAGTTGTGTCTTTTGTATCTGTGTGTGTATATATATATATATA
TGTAACAGAGCTTCCTGTGAGCAGGAGTTCATTGCAGTCTTCTG

> *Gamu*015, AM168510

CACAAAACTCCCACTAACTTCAACGAAGATATATATGCACACACACACATACACATATATATACATGTGTATA
TACCACCTCATATAGACAGCCAGAATATTGGTTGCTAAATAGAGACTTGGTACAGAGACAGATTTGATGTTTT
AGTCTTATCCTCCTG

> *Gamu*017, AM282936

CGATTATTGCTGCTTACGAGACTCTGCTCCCAGTAGGTGGCTCTGCATCAGCCTGACTGCAGGTCACGCGCTT
TTTGTAGGCTTTTTTATTAAATATACTAAAAAGGCAATCAAGGTTTTGGGGAAAAGAAGTGTTTCTTTTTTTT
TACATATGTACGTGCGTGCGCGCGCGTGTGTGTGTGTATATATATACAGCTTCCACTGCTGTAGTATATGCAG
TAAAAGCATTAAAATTCATGGAACACTTACATTTGTACGCTGGTATAAGAAACAATCA

> *Gamu*018, AM168511

TAGCGAGGGTCTGAGGAGAACTAATGATGGTTAAGCACCATACCATGCTTGCCTGTGTGTTTGTGTGTGTGTG
TGTGTGTGTGTGTGTTTTTAAATACGGAGGGTAAGCAACCTCCCAGCCTGCGGAAAACCATAGAAGAGAACAA
AGATACCTTTCTGTAATATGAGTGGGAAGGATATAACGGTGT

> *Gamu*019, AM168512

TATGAGGAGAGGCTGAGAAACCTAGGGCTGTTTCATCTTCAGAAGAGAAGGCTTGGGGTGTGGGGGTGTGTAT
TGTCACTATGTATAAATCATAGCATCATAGAATATCTCAAGTTAGAAGGGACCCCATAAGAATCATCGAGTCC
AAC

> *Gamu*021, AM168515

CGTCTCAACACTGGCAAAATATTCCGAATATCTGGATATTTCTTCTGGAAAGCTTTTTTTTTTTGTGTGTGTG
TGTGAGGGGTTCTCCCCCCCCCTTTTTTTTTCCTGCTTGCCATTAGCCAGGTGCAGTCATTG

> *Gamu*022, AM168513

TAGGGGAAAGCAAGATGAGACGACATGCCGTTTGGGGAACATGCTGCCTTCTCCAACAGCTACGCGGCTGCTC
CACGGTGCTCAGTGTGATGGGCATTGCTTCCACTTTTTTGCCCATTTAGGTGTGTGTGTGTGTGTTTAAAAAG
TTTCATGTGCTCTCATAGAAAAGTCTACCATAAATGCAGTCCTT

> *Gamu*024, AM282937

CGATTGTTTCTTGCTGCCAGTACAGACCCTCTTACACACACACACACACATGCACTGACACACTAATGCACAC
GAGCTGTGCATCCCTCATGTAATTTGGCTTAGTCATGCAGTCCATGCAGGAGCTGGCCTGGGTCATTACTCTC
CCGTCCCTCCCACCACACTCCTGGAGACACGCACATACGCACGCCCACCCACCCACCCCAGGGAGTCCCTCAC
CAGGGAAAAGAGTTAGAAAGATGATTAGCAAGAAGAATCAC

> *Gamu*025, AM282938

TTAATAAAAATCCACAGTGAATTTCAAAATTGTTTCAGTTACTTATAAATACACACACACACACACATACACA
TCTCTCTATCCAAAGTTATTACAGTCCTGGTCTAGAAC

> *Gamu*101, AM168516

CAGTATAAAAACAAACAGGTGAGATTTTTATTTAAATAGCTGATTTTTTCAAACAGCTTCAACACCTCCCTCT
CATTAAACAATTTATTCCAAAGTTGCATGCGTGTTTAAGCTGGGTAGTAAGAAAAACATTATTATTATTATTA
TTGTTATTATTATTATTATAAAGCCTATGTTCTCCTGTACTTTTTTCA

> *Gamu*103, AM168517

TCAGCCCTTGGATGCGTCAGGATATGGGGGAGAGGAGGAGGAGGAGGAGGAAGGGGCTGGGGGGCCAGGG
AGCCCAGCGGAGGTGTGAACCTCCCGCCGGAGGGACCAGCCATCAGCGATGGGGACCCAGCTCTGCCCCTGGG
ACGTGCGGCCCCGTCAGCCGGCGGTGGGAGAGACCCCCCCGGCCAAACCGCCCGTGCCCGGGGAGAAGCTTCC
TTCCTCTTTGCTTTCCTGACTCCCA

> *SHC-AG-2*, AM168522

TCCAAGGGTGGTGATATCTTTTCTCTCTCTCTCTCTCTTTTCTTTCTCTCTTTCTTTTCCTCTCTATCACTCT
CTTTGTCTCTAACAATTTTCGGCACATTAGGGTAATATTGTCACACGTTTTGCTAAACGCTTACCTTTCATAA
TTCTGCTAAATTTTGAGCATTGTTATGACCTTTTCCAAGCCTCTATCTTTTGTAAT

> *Gj-M08*, AM168518

TCCGTCAAGCTTTTAGTCATGCATTTTTATATCTCTCTCTCTCATAGTATGTGTATGTGTGTATGTATATATA
TAAACTGTATTGCACCCACATTAACTGTA

> *Gj-M11a*, AM282939

CGATTTGGGGTGCAGTTCAAATAAGGGTTAATTGTGTTTTGCCAACAAAACAATAAAAAAACCCTATTAACAC
ACACACACACACAGTTTGGATGAAATTGAAATGTTTTGTAGGAATATATCAACTTTGGCATTTTTGGCAGGAA
GCAATCAGAATGCTTTGCATGTCCTTTTTGGATGCAGAAATCAC

> *Gj-M15*, AM168519

TCTACCAGATATCATCAGAGCTTGCTTCTTTTTTGTGTGTGCGTGCGTGTGTGTGTGTGTGTGTGTAATTT
TTCCCTTCTGGTGTCTTGGGGCCATCTGTTCATTCGCA

> *Gj-M34*, AM168520

TGCTCAACATTCATCAGGATTTGGGGACACACACACACACACTCTTGAGGAGCGCGGGGCTCCCTGCGCACCT
TGCAAGCAGGCCGAGAGGACAGACAGACAGTATTTTCAGCCAACACCAGAGGGA

> *Gj-M40*, AM168521

TGGGAGAATCCTGCAAATTCTGCTAAGAAGAACATGGCTAAGCTGCACACACACACACACACACTTGGTTTGC
TTGGTTTTCTGAAACAAGCATCGCTCATTCCTCA

> *Gj-M48b*, AM282940

CGATTGTTTCTTGGACCCTCCACCGAGAAGCCTGGGGGGTTTGGGGGGGTCAGGGGCAGCCAGAGACACACAC
ACACACACCCCCCCCCTTGCCCCGGGGGTCCCCGCGGTGCCATCAGCACTCGGACGTCGCCGGGACGCGCAGA
CCCCAGGGTGAGGGGCTGCAGGGACCCCAAAGCCCCCCCAAGACACAACAAAACCCCCAGGATCAATCAC

>*Gpa*01, clone 17E04, AM085152

GATCTCTCCATGAGGTAGAAGACAGAGCCAGCCTCTTCTCAGTGGTGCCTACTAACAGGACAACAGGCAATGG
GCACAAATAGAAATACAGGGAATAAAATAATCTTTTATTTTCACTATGACCAAGCACAGATACAGGTTGTTGC
CCACAAAGGTGGTGGAGTTTCCATCCTTGGAGATACTCAAAAGCCATCTGGACATGGTCCTGGGTAACTGGCT
GTAGGTGGACCTGCTTGAGCAGGGAAGTTGGACCAGATGCCCTCCAGAGATGCCTTCCAACCTCAGCCGTTCT
GGGATTCTGTGAAATACTTACTACACTTTAATAATCTTATGTTGTTACACAAATAGCTGCACTTTCCTCTCAA
AGTGAATCTGAGCTCTCTGTGTTACATCTTTACAGAGAGATTTTTCACAAGGAAAAGTTATGTACCGTAACTC
CATTAATTATGAGTCATGGGAACACTTATTTTGAAAGGTTTGTATTTTACCCTGTTTCAAAGTTCTCTTTACT
TTCAAATTACTTTTTGGTTGCCGTCAGAATTGTTTTAGGGTTTTTTCCTCAAATTGAAAATATGTTTATGTTA
TCAAAACAAATGTCATTGTGGGAAAAGAACCAAGCATTGCCAATGTTTTTTATAGCAGTAATATTTGATTTCG
TTTGTTGGGTGTCTGGTAAACCACTTGAGTAGAGAAAATGTCATTCTAACACATGCATGTACACACACACACA
CACACGCATATATTTGAATGCCCACGTATTTTGCCCCAAATTCAGGCTGTGTTAAATGTTCCTGCAGAACACA
CCCAGGTCAGATATGATC

>*Gpa*02, clone 41A02, AM282883

GATCGCTGACAGTTTTTTTCTTTTCTTCTTCCTTCCTTCCTTCCTTCCTTCCTTCCTTCCTTCCTTCCT
TCCTTCCTTCCTTCCTTCCTTCCTTCCTCTCTTCCTTCCTTCCTCTCTTCCTCTCTTCCTCTCTTCCTCTCTT
TTTCTCTCCAAAACTCTCTTTTTTTGACCCTCTCGAGTAATTGTCGTTACTCCTCACTAACACACAGAGAATT
AAGAAGCTAACCTCACCCTCCCCCACCGAGTGGGACGGGACAAATCCTGAAACCTCTCAACCGCATAAGAACG
GAGGACTTAAGTTATGCCAAGAGCCTTGGCTTCTCAGGGCATTTTGCATGTTAAGGAGCCCTCCGTGCCAAGT
TCCTGAACTGCAGAATCTGAAAGAAGATTGAACAGCCCTCTAGAGAAGTTGGCCAGCAGCATGTCTCCAAGTT
GCTGAACATGTTATTTCGTCCCACTCAAGAGATC

>*Gpa*03, clone 41A03, AM282884

GATCAGGTTGCAGGAGGGATTTCGACAGAGGCTAGAGCTGGCTGCTGGGAGATACAGGGCAGCTCCATTCGTC
TTGGCATTACTGAAGCCAATGGGATTTTGGCCATAGTCTTCAACAGGGCTAGGATTTCACTGAGCATGCCTAA
GAAAGCACCGTGTCTTACAGTTGTGTTGTCTGCCATGACATTTACTGCTAGTGAAACCAGTGTGCCCCCTGTC
CCACTTGTCTCCTTGTCTCTATCTTCATAGCTCTTCACCTGTGGCTTTTCCCTTCACCTACTGTCTTGTCTCT
ATCTATCTGTCTATCTATCTGTCTATCTATCTATCTATCTATCTATCTATCTATCTATTCTTTCTTCTG
TTTCTGTCTGATTGCTGCTTGCTGCAATTGCCCTCTCACGTACTCTGCATGGAATGTGCCCACAAGAGGTATT
TTTATTTGGCAGCCTCAGTAAGCTTTCTGTTTTGACCGCAGGTACTGAGATAAGCAGCCAATGGAGCTGAGAT
C

>*Gpa*04, clone 41A09, AM282885

GATCTGACCAAGCTGTTGATGCGAGTGCTCAGCCCAGCCCTGTCTCTCTTCTCTTTCCTGCCAAATGCCTTCT
TTCTCTTCCTTCCCTCCTTCACATTCCTAACTCACAGATTACTACATATTTTCTATCTTCTTTTCCAAATTAA
TCTTTTTTTCCAAAGTTAGAAAGTAAGATATTATTGTTATTATTTGATAAACATTAGAAGTGACAAAATTTAC
CAAGCAACATGATAGATAGATAGATAGATAGATAGATAGATAGACAGACAGACAGACAGACAGACAGACAGAC
AAACTATTTTTCTCTGTCATTCAAATGAGAAATAACCATGGATC

>*Gpa*05, clone 41B07, AM282886

GATCTCTGAGTCAGGGGCTTCCGTTATGGTTATACTGGCTGTGATTTAAGCAGATATACTTACCTGTATTGAA
AATTCCTAGCAAAGGAATATCTATCTATCTATCTATCTATCTATCTATCTATCTATCTATCTATCTGCCTATC
ATGTGCAATTCTTGAATTCTTCCAAAAGAGGGGTGCAAAGGAAAAGCCTTTGTGTTAAGACAGCTCAAAGATT
TGGTTCCGTACCAAGAGTTGTTCAGTAAATGATACTGATGATACAGTTTGATTTAGAGGACACTGGATAAAAT
AGATC

>*Gpa*06, clone 41D12, AM282887

GATCCTGAATGCTTCTGAGAGCATATTCAAAGTCTTTTGCTTTGGTGTGGGTTTTGTGGGTTGGTTTTTGGTT
TGTTTTTTTTTTTTTTGGGGGGGGGGCAGAGGCTCTATTTATATGTTTGTTTATCTATCTATCTATCTATCTA
TCTATCTATCTATCTATCAGTGGGCTGAGCCCCGCTATGAATTACGCTCCAGTGAGCCATGGCAGAGCTGGCC

AGTTCTCCCTGGGCTCGTGGAGTGAACCGTGGGAAGACGCTAAGAGGACTAACAGAACTCTGATGTAGATGAT
C

>*Gpa*07, clone 41F02, AM282888

GATCTTCCTGGAGTTCATGTCTACAAAGATACAGATGGAAATGAAAGTTCTGGTGGATTCTGGGGAGGTGAGG
ACTAACCATGGGACATCCACCATGGGTATGTGATGAGGGTAGAACAACTGTGGCAATAAATGCCAGTTAAAGG
GGAGAGAAGAGCATAGAAATATCATAAGTACAGATAGAATAACAGATAATCAGAGTGGGAAAGAGGTGTGGCA
AAGAGAGAAATATGAATTTTTAATAGATAGATACATAGGTAGGTACGTACGTAGGTAGATAGATAGATAGATA
GATAGATAGATAGATAGATAGATATCTCCCTCCTAGTATCTGACATACACAAACATCAAGGAAAAAAAATACT
GTTAGACTCCCAAGGCTGTTTCATGGAACAGAAGTATGAAACGAAGCAGCAAACAGTAAGAAAAGCTCATTCC
CAATACGAAATCAGCTTTGCTTTGCAGCCCTATAATGCAGAGATGTTTAAAAACATATTGGGAGAGCTTCAGA
GTGAATAGGCAGGTCCAGGGCTGTGATC

>*Gpa*08, clone 42C03, AM282889

GATCCCTATCGTTGGCCCTTGGGAAATGCAGACCTGGAGATGGTGCAAATCAGTGACCTTGATGAGATAGATA
GATAGATAGATAGATAGATAGATAGATAGATAGATAGAAAGAAAGAAAGAAAGAAAGAAAGAAAGAAAGAAAG
AAAGAAAGAAAACAAGCTTACATGTCTTCTTGAAGAGACCAATTCCTGTGCTGTGGTCATAGCAAAATGGAAA
GATGAAGATGGGATGAGGACGGGATGAGGACAAATGGCTTTATATGCTCCATCTGTCATCTTTATGTTCCTCT
ATGACAGAGAGATGTGAGTCGTCTATCTTTAGGACTGATGGGAAGAAAATAAATGCTTAAATATTGTTTATTT
CTCTCCCTTTCATTTGTTTGCTTTCTTTCTCGTCTTAGTTGGTTAAATCAAAGTGGGTCTGGGCATACAGACC
TCTACAAAGCAGATTTCCTAGATTCTGTACTTCGTTACACTGGCGTTTGAGCGATTCCTGCCTTGATC

>*Gpa*09, clone 43A08, AM282890

GATCCCTGCTTTAAAGCTAAACTTGGAGCCACATGCTGCTTTGTGTCCAGACAAAGAATGCTCTAGGACAGCA
GGATGCTGTAGACAATGATACCAGTGTTAAAGAAAAAAAAGAAAGAAAGAAAGAAAGAAAGAAAGAAAGAAAG
AAAGAAAGAAAGAAAGAAAGAAAGAAAGAAAGAAAGAAAGAAAGAAAGAAAGAAAGAAAGAAAGAAAGAAAGA
AAGAAAAAAAAAAAAGTAACAGAAAAAACCAGAATATGCTGACATATAGGCACCTATTAATAAAATATAATCTT
GCAACTGTGGGAGCAGAATTCGGCCCTTTAGCTGATGAATCCTTAGATAATAAAGAAGCTCCACCGGATACGG
CAATTTACTCTCTCCCTGCAGCTGTTGGAAGAAAAAATTT

>*Gpa*10, clone 43A10, AM282891

GATCCCCAAACAAGCATGGAGCATCTCCTTCAAAACTGCCTCTGGTAAAATTTTTCTGGAAACATGCCAGGGG
CAAAGTACTTGCCGTGTGTTTCATGTCCCATGCAGCATGTGAGATGGAGTTATTTGTTTGTTGCTGCTTTGCC
TTTGTTTGTGCAAACTGACGCCGCAGGGACTTCTTTTGAGGGCATTTTTCAGCCAAGAAGATTATCCCATATA
AATACAGTGAAATAAATAGATTTTTCACTTGAATGACTTCAGGCTGTTCAGTCAGTCAGTCAGTCAGTCAGTC
AGTCAGTCAGTCTATCTATCTATCTATCTATCTATCTATCTATCTATCTATCTATCTATCTAAATTCTGCTTG
CCTTTGTAGATAACAGAATAATAGAATTAAGTTCCTATCACTGTCTTAAACAGACACATTGGGTTTATCTTAT

TTTGGGTCTACACTGAAACTGTGAGCAAGAGCTGAGCTAATTAAATTGGGCAGATTTACCTGCCTCATGTACA
CCTTGGAGATACTGCTCACAGCTGATC

>*Gpa*11, clone 43B09, AM282892

GATCATTAATGTACTGTGAATCCATTTGTGGCAGCACATAAAATTCTCCCTCACTACCAGCATTTCCAGCTAT
TGTAATTTCCCCAAAGAGGTAGGAGATTTATTTTACACAGATACAGTACTTGAAAGATTTATCAGTGCTGGAC
ATTGTACAACTGCTAAATGACCCTCCTGGAATACATGACAAACCAAATCTTTTCTCCCTTCTTCTCTCTCTCT
CCCTTTCTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTC
TTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCT
TTCTTTTCTTTCTTTCTTTTCTTTCTTTTCTTTCTTTTCTTTCTTTCCCTCTCTCTCTCTTTCTGTATTCA
GTTTTTCCTTTCTTGTTGAGAATGACTTAAGAAAAATGGAAATGTATCAGAGTCTAAATAAATGTTTAAAGCA
TCACACGTGGCCATTACGTTCCTTCAGTGCTCCACTGTGTTGACATTCAGGTGCATATTGTCATTTACAATGC
ACTCAGGCATTTGGTGGATC

>*Gpa*12, clone 43C11, AM282893

GATCAATGCGAAGGATAGGGAGGTGGATGGAGAGATAGATAGATAGATAGATAGATAGATAGATAGATAGATA
GATAGACAGACTGATGGACAGGTAGATTGATAGATGGATGGATAGATAGATGGATAAATAGGTAGATGGATAA
ATAGGTAGATAGACTGATAGAGGTGAATGAATAGATGGATGGATAAATGGATATTGATGGGGCTGAGGCAAAT
AATAGATTGATGACATCGATGTGAAGGTGTATGTCTATCTATATGGGACCAGGTGTCTAGTAACCTATGGGAA
TAGCTGGACAGACTGATGGGTGACCAGAGAGCCATGTGCAGGTTGGATGGGTCAGGAGACAGATC

>*Gpa*12, clone 45E11

GATCAATGCGAAGGATAGGGAGGTGGATGGAGAGATAGATAGATAGATAGATAGATAGATAGATAGATAGATA
GATAGATAGACAGACTGATGGACAGGTAGATTGATAGATGGATGGATAGATAGATGGATAAATAGGTAGATGG
ATAAATAGGTAGATAGACTGATAGAGGTGAATGAATAGATGGATGGATAAATGGATATTGATGGGGCTGAGGC
AAATAATAGATTGATGACATCGATGTGAAGGTGTATGTCTATCTATATGGGACCAGGTGTCTAGTAACCTATG
GGAATAGCTGGACAGACTGATGGGTGACCAGAGAGCCATGTGCAGGTTGGATGGGTCAGGAGACAGATC

>*Gpa*13, clone 43F05, AM282895

GATCTGAACTCAGTTTGGAGGCAGATTATGAAAGGCCCTTGGAAGACAGATAGATAGATAGATAGATAGATAG
ATAGATAGATAGATAGATAGACAGATAGATAGATAGATAGATAAGGAGAGGGAAAAAGTTGCTTCAAAGGGAT
GAGGAACAGGATGTCCATATAAAAGAGTTTCCAGTGGGATC

>*Gpa*13, clone 41A05, AM282895

GATCTGAACTCAGTTTGGAGGCAGATTATGAAAGGCCCTTGGAAGACAGATAGATAGATAGATAGATAGATAG
ATAGATAGATAGATAGATAGACAGATAGATAGATAGATAGATAAGGAGAGGGAAAAAGTTGCTTCAAAGGGAT
GAGGAACAGGATGTCCATATAAAAGAGTTTCCAGTGGGATC

>*Gpa*14, clone 43F06, AM282896

GATCATACTTCAAGACTTTCCATTTGAAATTTGATGAAGATATAGCATGTGTTAATTAGATATCAGTAAAAAT
ATGGATGCAGAATTATGCCATTCATGCAGAGCAACCATCAGCTATTGCCACTGATGTAAGAAATTTTCATTTG
CACTTTTCGTACTCTGGTCATTGGATTTAATATGGAATTTTGAATTCCAGGATAAAAAGGAATTGCATGCTTT
TCAGTGGTGGGTTTTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTTCAT
TCTTTCTTTTCTTTCTTTTCTTTCTTTTCTTTTCTTTCTTTTCTTTCTTTTCTGCTGTTGTTCAACGTTTGCA
AGTTTTTAATTGTAAAAGATGCTGTAGATGGAGTTTTTCTTCTTAGCACTGCTGTCCTATTTTCCCATGATAT
CCCCTGGGGATC

>*Gpa*15, clone 44A04, AM282897

GATCTTCATCTGCTGTAGCAGGGATACTAATATCTGGGCTGGAAAGCAGAGCTTCCCATAAATATAATGGATA
TCAGAACAGGCCATGGATAGGTCTCAGCTTGCTCGGTGGAATACATAAGGAAAAAGGTACATAGATAGATAGA
TAGATAGATAGATAGATAGATAGATAGATAGATAGATGGGGGAGGTAGCCCCGGTCAGTTTATGTCATTACTC
TGAGCCCTCCAGCAGCGTCGTATAACTGGAAAGGAAGTTGAATGCCTAACTGCATGCACTACCTGAATCATTT
CTATGAAAGGAAGTTCATATTAAAGGGGCAAAATTCAGACTACTCATGATTTTAATTCTTTGCCATTTAAAAA
AAAACCACCAATGTTTAATTTAAAATGCCGGGTCCCAAAGCAAACTTAGCAGCTCAGATC

>*Gpa*16, clone 44A05, AM282898

GATCTACAGCTGGCCTAAACAAGAGATTTGTTTTGGACTGCAGCTGGCCTAATTGCAGCATGTAAAATAATGG
TATCACATAGAAAAAAATCATATGTAACATCTATCGGATAAAGGCATTAATCCTGCAGTGCAAGGCTGCCATA
CCCCAGTACAAGGGAAGGTTATAGCTAGACAGCAGTATGGAAGACACCTACACACAGGAAGGCTCGTGCTCCA
TATCTGCCTATTACCTTACTTGTGTCATATTGGCTATCAAAGGACTAGGGGAATTTTAATGAAGCTTTAGAGA
TAGATAGATAGATAGATAGATAGATAGATAGATAGATAGATAGATATAAACACTAGTATGAAAACAAGAATTT
CTCCTGCATTTTATTTCATTTGTCATAAGATAATTCCGTTTTGCTTAACACATTTAAATAAACTATACATTTC
CTAGGCTCTCCGTAAATGTAGCTATGCAATTGGATAACACTTTACAGAATTAATGGTTTTCCTTTCAAAATGA
AATAGTTGACAGATC

>*Gpa*17, clone 44A10, AM282899

GATCAGCCCACTACTTCATCTCAGTGAGTTGTTAGTACTGTGGTGCAATCCACATCTGCTGTATATGACAGCA
TACCACTGGTGGCTCAGTTGCATGTCAAGGTACATTTGTGTTAAGTACTGTAAGATACACCGTGAAGCTTAAG
CCATTGCTAAATGACACACATTATCTGTATGTATGTATGTATGCATGTATGTATGGATAGATAGATAGATAGA
TAGATAGACAGACAGACAGACAGACCAATCGATC

>*Gpa*18, clone 44B07, AM282900

GATCCCAGTCTGGAGAGCCATGTGATTCTGTTCACTTGAAGATATACTGAAAGAAAAAAGCCATGAGTCTCTT
CATTTATATTCATCTATCTATCTATCTATCTATCTATCTATCTATCTATCTATCTTTTAAAATTATTATTTTA
ATGGGGACATGTCACATTACAAATGCAGTGGGAGATAAAACTTCCAAACTTCAGCAGATAATTTTGTACAGGG
TTGCATGGCACTGTGCTCTGTTTTGTACTATGACAGATGGAAGAGCTTGTACCTTGTGTGTACTCCTTTGGTG
AAGTGAACAGGTATGTCTTAAATACTCAGAGCCATATAACATTTAGCCATGTCTCCTAACTTTAATCAGGCTA
GGTTTAGTCTGCAGGAGGGGACTGGAGGAGCTCTGATC

>*Gpa*19, clone 44C10, AM282901

GATCTAATAGACTTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTAGTTTCCTAA
GGCTAAAGAAGAACTCCTTTGCAAACAACAGACTTTCTAACCTACTTTTTGTTCAGCTATCTGACTGGGAAAT
CAATTATGTGGCACATATGTCTTTTTATTTCTCTTCTACTGCTAAATCAACCTTCCCCATTGTAGCTTGGTTT
TCAAGTGTATGTGTGATTTTCAAGCGTATGCAATAACCATGGGGAAAGGGTTAGGGCTGGACTGTGCTTGCGT
GGACTTGGACGTGCTTGCACACCACTCAGCTGGACTTCCTGAAGGGAATCCATAGCTGAGAGGCAAAAATGAG
ACTTCATCATGTCAAGGGTGTAGCACTGTCTTGGGTGGTGCTGCTTCTCTCTCGCTGGTGAGCAATTTAGCTC
ACGCAGTGGCTGTTGAAGGCAGAATAGGTCAGGCAGGGGAGCATGAGTATTTTACTTGCCACCTATGAGGAAA
CCTCTCTGATC

>*Gpa*20, clone 44G06, AM282902

GATCGATAGATAGATAGATAGATAGATAGATAGATAGATAGATAAACCTATACTGCACACAGCTCTGGTTTGG
TTTGCTGCGCTGCTGGGTAGGCTGTCATGAATGCGCTCTTAGTCCGTAGTCTGCTGCTGAACCTTCAGGTAAT
AGTAGAGTAAAAAGATACCACAAATGACAGTATTACTGGCTATTGACCAACACTCTTGAGTATCAAAAATGAG
AGACAAAAAAAAATGGACTTTTCTAGGAAGGAAAATGACATGTCATTCCAAGGCCACCATCATAAACATCATCC
TAAATGCCGTATCAGCGCTCTTGGGCCGAGATC

>*Gpa*21, clone 41C12, AM282933

GATCCCACTGTCCGTGTCGCCAACAAAGATGTTAAACAGCACCAGATGTGAAATTCTGTAACTTTCCTGAGTC
TGCCAAGCCCATTCTTCTCCCTCCGTCCCTCCATCCCTCCATCCCTCCCTCCAGCGATTTCTGCTCCAACTCA
TTGCATTAATCTTTAGATTAAAAATAAATAGATAGATAGATAGATAGATAGATAGATAGATAGATAAACA
GACAGATAAACAGCAATCCCTTTGAGCCCAGGTTGTATCCTTCTGAATGTCCTATGGCTGTGACCCTGGCAGG
AAATGCAGAGCATTGTGATC

>*Gpa*21, clone 44H05, AM282903

GATCCCACTGTCCGTGTCGCCAACAAAGATGTTAAACAGCACCAGATGTGAAATTCTGTAACTTTCCTGAGTC
TGCCAAGCCCATTCTTCTCCCTCCGTCCCTCCATCCCTCCATCCCTCCCTCCAGCGATTTCTGCTCCAACTCA
TTGCATTAATCTTTAGATTAAAAATAAATAGATAGATAGATAGATAGATAGATAGATAGATAGATAAACAGAC

AGATAAACAGCAATCCCTTTGAGCCCAGGTTGTATCCTTCTGAATGTCCTATGGCTGTGACCCTGGCAGGAAA
TGCAGAGCATTGTGATC

>*Gpa*22, clone 45A01, AM282905

GATCCTGTGATTAATTTATCTATCTATCTATCTATCTATCTATCTATCTATCTATCTATCTATATCTCAATAT
GTCATGCTGTTTGAGACTAGCTTCTTTAATATCAGTGGAGGGTGAGGTTTTTTGGTTGGGTTTGTTTATTGTG
TGGTTGTGGGGGTTTTGGGGGAATTTTTAAGTACTAAGAAGTGTCAGAAAACAGAGCTGATAGTTCAGAGACT
AATATAGTTACAGGATTCTGCTAACATCATAAATAGGTAACGTGCATTAAGATC

>*Gpa*22, clone 44H03, AM282906

GATCCTGTGATTAATTTATCTATCTATCTATCTATCTATCTATCTATCTATCTATCTATCTATCTATATCTCA
ATATGTCATGCTGTTTGAGACTAGCTTCTTTAATATCAGTGGAGGGTGAGGTTTTTTGGTTGGGTTTGTTTAT
TGTGTGGTTGTGGGGGTTTTGGGGGAATTTTTAAGTACTAAGAAGTGTCAGAAAACAGAGCTGATAGTTCAGA
GACTAATATAGTTACAGGATTCTGCTAACATCATAAATAGGTAACGTGCATTAAGATC

>*Gpa*23, clone 45A10, AM282907

GATCAAAACAGAGATAGATAGATAGATAGATAGATAGATAGATAGATAGATAGATAGATGTGGTATGTAGCAG
ATGTGAGAGACTTTTACATTAATGTTTGTTCTTTCTTCATCATCTGTTTGGATTAATTCATCCTGAAGATGGA
TAAGAAAACAAATTTTAATCACAATAATATTGGGAAATAATATTGAAATATATATTGAAACAGTATGAGTCAC
TTAAATACTAGGACACCAGTACTTTCTTTTCTATATGCAGATGTCTCCATTTGGCTAGATC

>*Gpa*24, clone 45A12, AM282908

GATCAGAGAGTCCCCTTCACAGGGGACATCCAGAAAAATGGGTTCAGGTTAGACCGTACTGAACCATGTAAGA
CTTTCCTTGTGACCATTTCTACTAAGCACATGCCCTTATTTCAGTGTGGATTTTTGAACAGAAACCAATGACT
TCAGCCTGTGCCTCGATATTCTGCTGTGTAAAGGTTTCCAGATGTGAACACACTTCAGAAAAATGCTGCACCA
GTGTTATTGCTTTAGTGGGGAACATCTTAAAAGAGGGAATCTAGCACGCTCCAAGTTAGCACAGTGAATAAAA
GCATCTTGTTCTGCTTCAGGTGTTTATTTCCCACCCCAAGATGGTCACAACCTCCCTTTTGGAGCCAGTTCTA
GCTCTCACCGTGGACCTGGTCTTTGGGCTGTTCAACTCTTCTGAGCTTTGATAGTTAGTTAGTTAGTTAGTTA
GTTATTGATAGTATTGTTGTTACTTTCCCCCTTACTTCATGGACACGATGCTTTGAGTTTAGAAGTGGGGAGG
TTGCTATACAGATGATC

>*Gpa*25, clone 45B05, AM282909

GATCTTTCTCAAAAGCAATAAATAGATAGATAGATAGATAGATAGATAGATAGATAGATAGATAGATAGATAA
AATAATTTGCAGGTGAATACTGATTTTCTTAGCTTTGTCCGATTTATGAGGTTTTTACTTCCACTTGAAGCAT
GAAGAAAATATTCTCTTTCTCATGTCCTTCTACCATAACCATTATTTTTTCCTTTGATGTTGGACAGAGTTTG
GCTTGGCACGATC

>*Gpa*25, clone 41B12, AM282910

GATCTTTCTCAAAAGCAATAAATAGATAGATAGATAGATAGATAGATAGATAGATAGATAGATAAAATAATTT
GCAGGTGAATACTGATTTTCTTAGCTTTGTCCGATTTATGAGGTTTTTACTTCCACTTGAAGCATGAAGAAAA
TATTCTCTTTCTCATGTCCTTCTACCATAACCATTATTTTTTCCTTTGATGTTGGACAGAGTTTGGCTTGGCA
CGATC

>*Gpa*25, clone 44G12, AM282909

GATCTTTCTCAAAAGCAATAAATAGATAGATAGATAGATAGATAGATAGATAGATAGATAGATAGATAGATAA
AATAATTTGCAGGTGAATACTGATTTTCTTAGCTTTGTCCGATTTATGAGGTTTTTACTTCCACTTGAAGCAT
GAAGAAAATATTCTCTTTCTCATGTCCTTCTACCATAACCATTATTTTTTCCTTTGATGTTGGACAGAGTTTG
GCTTGGCACGATC

>*Gpa*26, clone 45B07, AM282911

GATCCCCTATTAACTAGGTTAACAGATGCATTAACAGGTATAACACATAAAGATGGATAGATAGATAGATAGA
TAGATAGATAGATAGATAGATAGATAGATTTAACAGGTATCACACTTTCCCAGAAAGGCTTCCAGATTGCAGT
ATATTGTAACATGGGTCACTTCAGTGACAATTTAGTATTTTGGAATGGAAAGGCGTGTTAAGAGATACCGCTC
TTCCTATACCCGCCACTTCCTTCCACCATTCAACATGTGCAAGATTCTGTTAATGTTATGATC

>*Gpa*26, clone 45E05, AM282912

GATCCCCTATTAACTAGGTTAACAGATGCATTAACAGGTATAACACATAAAGATGGATAGATAGATAGATAGA
TAGATAGATAGATAGATAGATTTAACAGGTATCACACTTTCCCAGAAAGGCTTCCAGATTGCAGTATATTGTA
ACATGGGTCACTTCAGTGACAATTTAGTATTTTGGAATGGAAAGGCGTGTTAAGAGATACCGCTCTTCCTATA
CCCGCCACTTCCTTCCACCATTCAACATGTGCAAGATTCTGTTAATGTTATGATC

>*Gpa*27, clone 45B10, AM282913

GATCGATATAGATAGATAGATAGATAGATAGATAGATAGATAGATAGACGTAGGTAGAGATAGGTAGAGATAG
AAATACAGAGAGAGAGAGATAAAGATAGAGGTAGAGATAGAGATAGAGATGATAGAGATAGATAGAGATAGAG
AAGATAGAGATGAAGGGGATAGGTCTTGTCTACAACAGTCTTTTTGTAATACCTTTTTTCATCATAGTCTGGA
TTTCCCTAGCAAGAGAGAGACAGAGAGAGAGGGAAACCCCGACCTGACAGGAGGGTAACTTCAATCACATCTT
CCCAAAATTCCCAAGGGTGGGAAGAGAGATAGTAAAAGCAAATAAATGTCTAGGACCAGTGCTTAGGTTTCTC
AGATAAGATC

>*Gpa*28, clone 45D03, AM282914

GATCCATTTTGAACAGTCATTTGCACCAACTGGGTATGAAACTACTGTGAATCATCATGTTTCAGTGAGATTT
TGCAGTGAGATAGACAATGGGTTAAATTAAGGTACAATTCTCTTCATCATATGTAGATAGATAGATAGATAGA
TAGATAGATAGATAGATAGATAGATATACATACTGTATCTGCAAAGCAAAACCTAGACATAGGTCACAGGTTT

TATTCACATGAAGGAAGGGGCAAGTGCAGCTGAGATTTTTTTTTTCCCCCAGCAAGTGCCAGAGACTGTAGGA
AATCTGCCAGGCACGATGTAGGCATATTGATC

>*Gpa*29, clone 45C12, AM282915

GATCCCAAGCAGTTATAGATTCAAGCACGTATCCTGCCTTTGGTTACTGAGTTCAGACTGCTCTGCATGCTTC
AGTCCTTGTGCTGCCCTCAGGGTACTGCCCAGGTTGTGGTTTGCAGCCCTGGGCTGGTGGGTAGCGAGCAGCT
GCCACAGAGCCATTCCCACAGGCTGGGGAGAGTTAGAAACAGTCCAGGCAAGGGAGATAGCCCTCCCCTTCAA
TTTTACCATTTCTGTGGATTTTCATGTGGTCCAAATGGATGTGTCCTCAGCAATGCAAATGGCTGTGGCAGGA
CTCAGTTCTTCTCCTTTGCTCCCCTTCTGTAGAAGAGCATCTTGCTAGCTCTGAGATAGATAGATAGATAGAT
AGATAGATAGATAGATAGATAGATATATTCCTGTGCATGGCTTAGGGAACCTTCAGAGGTTCAGTGCCT
TAAATAGGTATGGATC

>*Gpa*30, clone 45F05, AM282894

GATCCATTAGCAGTGGAGAAGAAGTAAGAGATTTTAATTTACAGCCAAAGAACTAATTTATCTATCTATCTAT
CTATCTATCTATCTATCTATCTATCTATTTATTTTCAGAATCTGCACGTGTACTGAATTGTGCTCCTTG
ACACAGAAACAAATGTGGGGCTATGATTTAAAAAATAAAAAATCCTTAATTTCAACTCCCAAATAGTTCTGTT
AAAACTCTGGGGAAAAAAAATGGACAAGCTTTCAAACATGATGATC

>*Gpa*31, clone 45G08, AM282917

GATCGCAGTGGGCTGTGACCAGCATCTCCCCTGAGCTGGGATGCCTTTGGGGAAAATCTCCTTGAGGACCATC
TGCCGATGGAAGGCCAGGGTGAAGCCAGCCTCCTCAAGTCTCAACTATCACCCACTGAGGAATGCCGATGCGT
TGTATTTACTCTAGACTCGAAGAGGGAAATTTTAACTATAAGCTAGCGTCCTTCATCCACCTTCCTACCTATC
TATGTGTTTAAATATTCACATTTGCCTTCACAAGTGTGGGTGACTCATCACCGGGCCCAAGTATTTCTGTCTG
TGTGTATCTGTATGTTTTATGTTTATCTATCTATCTATCTATCTATCTATCTATCTATCTATCTACTTT
GATTTAAGATACCTTGAGGTAAGTTAGTGTGAATCTTGCCATCTTTGAATCTGTAATTAACTTGCTTTTTTGA
TC

>*Gpa*32, clone 45H08, AM282918

GATCAGTGCTAAAATCTGCTTTCTTTGCTGTTATTAGAGTTGCCCATAACCTAACCAAGCCAAGGAGACCTTT
TCACAGCCACGATGATGGTCTGCGAAACAAAGGTCCCAGCACACCGTGCATAAGACAGGCATGCAGCTGCAGA
AGTAGAAATACTGCAACTGGCCTGAAGAAAACACAAATGGAAGTGTGTGTGTGTGTGTGTGTGTGAAAGAGAG
GACTAGAAGAAGCAACAGAGCAAGGGTTAAAAGAATGGCCTGTACTGATAACCAAGGATGTGACCGACTGCAG
TAAAACTGGGTAAACGCTGACAAATGTGGTGTTTGCGACAAGCCCAGCAGCCTGCCCCCCGATAAGCCTCAAC
ACTGACGGCTCATCAGCTGATAAAGCCTCTGCGCAGATGACTCCTTAGGGAAAAAACTGTGCTCCAATAATAA
AAGGATGACAGCAAAGTCATAGCGCATCCCGTGTGCAAGTCGTCCTCCTCTTCCCCCAGCAGGAAGGCAGACT
GTGGGAGCTAAGGTACCCAAAATGGGGGGGAGGGGGGGAAAGCGACTAAGCAGCCACCTCTCCTAGGGCTCTC
TGAGGATGAGGTAAGCAAGATTTTGAGGCAAAGGGATC

>*Gpa*33, clone 45H10, AM282919

GATCTTTAATGAACAAACCAGGCAGAGAAATGGCTTAGAAATGGGATACAGTTGTATAAAAGATTGTCTTTCT
CTTTCTTTTTCTTTTTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTT
TCTCTCTTTCTCTCTTTCTCTCTTTCTCTCTTTCTCTCTTTCTCTCTTTCTCTCTTTCTCTCTTTCTCTCTTT
CTCTCTTTCTCTCTTTCTCTCTTTCTCTCTTTCTCTCTTTCTCTCTTTCTCTCTTTCTCTCTTTCTCTCTC
TTTCTCTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTCTTGCTTGGACGGTGGTGATC

>*Gpa*34, clone 49A09, AM282920

GATCTGCACAGTCTCAGACCTGCTGACTCCATCACTGCCTTGCACACAGTGAGGAGACCAGTGAGGTGACTCC
TGTCCTGCCTTCTGTGCTCTTTCTCTTTCTTCTCTTTCTCTTTCTCTTTCTCTTTCTCTTTCTCTTTCTCTTT
CTCTTTCTCTTTCTCTTTCTCTTTCTCTTTCTCTTTCTCTTTCTCTTTCTCTTTCTCTTTCTCTTTCTCTTTC
TCTTTCTCTTTCTCTTTCTCTTTCTTCTCTTTTTTTTCTTCTTTCTTCAGGGGCAGAGACACCTGCAGTTGTC
TTCCCACATCAAGGAGAGACTTGATC

>*Gpa*34, clone 47A04, AM282920

GATCTGCACAGTCTCAGACCTGCTGACTCCATCACTGCCTTGCACACAGTGAGGAGACCAGTGAGGTGACTCC
TGTCCTGCCTTCTGTGCTCTTTCTCTTTCTTCTCTTTCTCTTTCTCTTTCTCTTTCTCTTTCTCTTTCTCTTT
CTCTTTCTCTTTCTCTTTCTCTTTCTCTTTCTCTTTCTCTTTCTCTTTCTCTTTCTCTTTCTCTTTCTCTTTC
TCTTTCTCTTTCTCTTTCTCTTTCTTCTCTTTTTTTTCTTCTTTCTTCAGGGGCAGAGACACCTGCAGTTGTC
TTCCCACATCAAGGAGAGACTTGATC

>*Gpa*35, clone 47A05, AM282921

GATCCTAATTAGTAGTGCCAGTCAAATTAGTCATGAGAAAACTGCAAAGACCAGGGAATAATATTGTCTTCTC
CCCCGGCTTCTTTATTACATGCCAAAAGTGTTCAGTGCTTTAAGAATTCGCTTGAAGACATTAGACAGGGCTG
CTCCTGATTGCACATACCTGATTATTTATACGTATTTGCACTTTGTAGTTAGTGCCAGAGTATTATGAAAGGA
CAGATACATTTTATTTTTGCTGCAGATTTTTTCTTACAGGTAGCCAAAAACGGTTCTTTGAGAAATTATCGAT
TTACTGTGACAAATATGCTGAACAAATCCCAGTAACTTTTGTGCTTGGTAAGTACCGGTTACATGTGAAATTA
TTCTCCAATGCTGTCTCTTTTCAGGCGTTTTGTGAATGTCTGTGCATGGAAGCATTTCAGAGCTGAGGGGCAT
ATGGGAGGTTAGAGGCTCACAATAAAATCATCAGCTTCCAACAGGTCTCCTTAAATTCAAGTCTCTTTTGGCA
TTTATGGTCGTGTTCTTAATATACCAACAAAAGAGTAAAGTTTCCATACATACATATATGTGTGTGTGTGTGT
GTGTGTGTGTGTATATATAGGGCCCACAAACACTTATACATTGTGCCTGATC

>*Gpa*36, clone 47A12, AM282922

GATCAAGTTTAGAATGTAGAGCAACAGTGAACTTTGGAAAACATGGTGTGCTCCCATTTGTCAGATGTAGCGG
CATTGTATCTCTGATAGGTTTTTCTTTGTGCTATAGTGCAGAGCTGTCTGACAAAGAAAAAGGAGGGTTGTAT
GAGAACATTCATTTTCATAGACATATGCTTACCTGTTCTGGTCATTTGAAATAAAAAAAGTTTAAAGAAATAG

CAGACGTTTGCAAATTGGTCAGTGAATAATAATGCTTTATATCTGTCAATAAAAGGCCTCGAAAGGCTTTGAA
CTTCATGACAGCTCTGAAGCTAATATTTTGATATTCTTTTTGCTGATAGATAGATAGATAGATAGATAGATAG
ATAGATAGATAGATGGATGGATC

>*Gpa*37, clone 47D01, AM282923

GATCTCAATGAATATGGTGGGACATGATAGGCTCTAGGTAAACCAAACCAAACTGTGTATGTATGAACTTGAC
AGTCTTGACCCTTGCTAGTTTTGAACGTGTCTGATTTAAGGAATCTCCTCTTTCTTTCTTTCTTTCTTTCTTT
CTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTC
TTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCT
TTCTTCTTCAAAACCCAGGCTAGTGACTACTTTTTCACTTAAAGAGGCTCAAGTTTTCTTATGTTTTTGATGT
ATCACATGGACAAGTGGATC

>*Gpa*38, clone 47H05, AM282924

GATCTTCACTTGTTCAGATGAGGCCTGCCCTATGTGTGAAAATGAGTAGTGGGGCAGAAGCAAGTCTTTCATC
TGGTAACTTCTTGAACAGAAAGCAACTTCCACCTGTGTGTACACATCACATTACACAGAGGATATCTATCTAT
CTATCTATCTATCTATCTATCTATCTATCTATCTATCTATCTATTGAGTGGGAACTCTGTCACCATTCAAAAA
TAAAGTGCAACCAGCAAACATCTTCATTAATGATATGGATGATGGGGCAGAGTGTACCCTCAGCAAGTCTGCA
GATGATACCAAACCGGGAGGAGTGGCTGATACACCAGAAGGTCATTCTGTCACCCAAAGGAACCTCATCAGGC
TTGAGATATGGTCTGACAGGAACCTTATACAGTTCAACTAGAAGTTCAAAGTCCTGCACCTGGTGAAGAGCAG
TTCCATGTACCACTGTATACTGGGGTCTGCCCAGCTGGAAAGCAGCTTGGGAGAAGGACCTGGGGCTCCTGGT
GGACACCAGGTTGAACGTGAGCCAGCAATGTGCCCTTGCTGCAAAGGAGATGAATGGTATGCAGGGCTGCATT
AGGAGTGTGGCCGGCAGGTCAAGGGAGGTGATC

>*Gpa*39, clone 47H08, AM282925

GATCTGCCACAAAAGAAGAGGATTCGGCACCGTAAGTTATTAAGTGGTGTTGTAATAAAACCGTTCCCACGGA
GAATGATTCTCTGATTCTTTGAGAAGGGGAAAAAGTTACAGCAGTTTAGATGGGGAAGGTAAGCTCCGGTCCT
GAGGAAAGGGGACGAGGGCTCAGAGTGTGTCACTGGTTTCTCTGGAAAAACAGGTTGTGTGTGACTTAGCAAT
AAGGTGTTAACACGAATACGTGTACAGGATGAGGCGTAATGGCCTTTTATCTTTGCCTGCTGTCAGTATTCCT
GTCTTTACCTTCCACTTAAAGATGACCTCCTGTAAAATCTAGTCTCAGGAATACCTGCACAGGTTTGGCCAAG
AAGCTGGCTACTGAGAGATAGATAGATAGATAGATAGATAGATAGATAGATAGATAGATAGATAGATATAAAA
CCACACACCTTTAATTTCACTTTGGAAGATC

>*Gpa*40, clone 47H11, AM282926

GATCTTATACACAAAGTGTTTTGGTTAGGAGAGAAGAGGGGTGTGGTTTAGGGATGAGGACTGTAATAGATAC
TTTTCCATCATATATGAATCTTTTCTTCTTTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTT
CTTTCTTTCTTTCTTTCTTTTTTTTTTTTTTTTTTTCCCCAATAATTGCAGAGGCTGTCTATTCTATGGTCCGC
CAGGGACTGGAAAGACACTGGTTGCTCGTGCGCTTGCTAATGAATGCAGCCAAGGTGAGAGGAGAATAGCCTT

TTTTATGCGAAAAGGTGCCGACTGCCTGAGTAAATGGGCGGGGGAATCTGAACGACAGCTTCGGTTATTATTT
GATC

>*Gpa*40, clone 40G12, AM282927

GATCAAATAATAACCGAAGCTGTCGTTCAGATTCCCCCGCCCATTTACTCAGGCAGTCGGCACCTTTTCGCAT
AAAAAAGGCTATTCTCCTCTCACCTTGGCTGCATTCATTAGCAAGCGCACGAGCAACCAGTGTCTTTCCAGTC
CCTGGCGGACCATAGAATAGACAGCCTCTGCAATTATTAAAAAAAAAAAAAAAAAAAGAAAGAAAGAAAGAAAG
AAAGAAAGAAAGAGAAGAAAAGATTCATATATGATGGAAAAGTATCTATTACAGTCCTCATCCCTAAACCACA
CCCCTCTTCTCTCCTAACCAAAACACTTTGTGTATAAGATC

>*Gpa*40, clone 40H03, AM282928

GATCTTATACACAAAGTGTTTTGGTTAGGAGAGAAGAGGGGTGTGGTTTAGGGATGAGGACTGTAATAGATAC
TTTTCCATCATATATGAATCTTTTCTTCTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTTTTTT
TTTTTTTTTTAATAATTGCAGAGGCTGTCTATTCTACGGTCCGCCAGGGACCGGAAAGACACTGGTTGCTCGT
GCGCTTGCTAATGAATGCAGCCAAGGTGAGAGGAGAATAGCCTTTTTTATGCGAAAAGGTGCCGACTGCCTGA
GTAAATGGGCGGGGGAATCTGAACGACAGCTTCGGTTATTATTTGATC

>*Gpa*40, clone 40H08, AM282928

GATCAAATAATAACCGAAGCTGTCGTTCAGATTCCCCCGCCCATTTACTCAGGCAGTCGGCACCTTTTCGCAT
AAAAAAGGCTATTCTCCTCTCACCTTGGCTGCATTCATTAGCAAGCGCACGAGCAACCAGTGTCTTTCCGGTC
CCTGGCGGACCATAGAATAGACAGCCTCTGCAATTATTAAAAAAAAAAAAAAAAAAAGAAAGAAAGAAAGAAAGA
AAGAAAGAAAGAAAGAAAGAGAAGAAAAGATTCATATATGATGGAAAAGTATCTATTACAGTCCTCATCCCTA
AACCACACCCCTCTTCTCTCCTAACCAAAACACTTTGTGTATAAGATC

>*Gpa*40, clone 41B11, AM282929

GATCAAATAATAACCGAAGCTGTCGTTCAGATTCCCCCGCCCATTTACTCAGGCAGTCGGCACCTTTTCGCAG
AAAAAAGGCTATTCTCCTCTCACCTTGGCTGCATTCATTAGCAAGCGCACGAGCAACCAGTGTCTTTCCGGTC
CCTGGCGGACCATAAAACAGACAGCCTCTGCAATTATTAAAAAGAAAAAAAAAAAAAAAGAAAGAAAGAAAGA
AAGAAAGAAAGAAAGAAAGAAAGAAAAAGAAGAAAAGATTCATATATGATGGAAAAGTATCTATTACAGTCCT
CATCCCTAAACCACACCCCTCTTCTCTCCTAACCAAACCACTTTGTGTATAAGATC

>*Gpa*40, clone 42B06, AM282930

GATCTTATACACAAAGTGTTTTGGTTAGTAGAGAAGAGGGGTGTGGTTTAGGGATGAGGACTGTAATAGATAC
TTTTCCATCATATATGAATCTTTTCTTCTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTTCTTT
CTTTTTTTTTTTTTTTTTTTCCCCAATAATTGCAGAGGCTGTCTATTCTATGGTCCGCCAGGGACTGGAAAGA

CACTGGTTGCTCGTGCGCTTGCTAATGAATGCAGCCAAGGTGAGAGGAGAATAGCCTTTTTTATGCGAAAAGG
CGCCGACTGCCTGAGTAAATGGGCGGGGGAATCTGAACGACAGCTTCGGTTATTATTTGATC

>*Gpa*40, clone 45D05, AM282931

GATCAAATAATAACCGAAGCTGTCGTTCAGATTCCCCCGCCCATTTACTCAGGCAGTCGGCACCTTTTCGCAG
AAAAAAGGCTATTCTCCTCTCACCTTGGCTGCATTCATTAGCAAGCACACGAGCAACCAGTGTCTTTCCAGTC
CCTGGCGGACCATAGAATAGACAGCCTCTGCAATTATTGAAAAAAAAAAAAAAGAAAGAAAGAAAGAAAGAAAG
AAAGAAAGAAAGAAAGAAAGAGAAGAAAAGATTCATATATGATGGAAAAGTATCTATTACAGTCCTCATCCCT
AAACCACACCCCTCTTCTCTCCTAACCAAAACACTTTGTGTATAAGATC

>*Gpa*41, clone 49F10, AM282932

GATCAGTGTATATACTCGGGCTTTCTCCTCCTCAGCTGCTTTCAGTCAATAAGTTTTTGGAAAAGTTGTCTAG
CTAGGAGGCATCTCTATAGAGCAATTCAGTCTAATGTAAATGTCCAAGGCAGAAGTTAATCATCTTCTTGAGG
TATATTCTTTTCTTTCTTCCTTTCTTCCTTTCTTCCTTTCTTCCTTTCTTCCTTTCTTCCTCTCTCTCTCTTT
CTCTCTTTCTCTCTTTCTCTCTTTCTTTCTCTCTTTCTCTCTTTCTCTCTGTCTTCTCTGTCTTTTTCTGTCT
TTCTCTTTTTCTTCTCTTTCTTCTCTTTCTTTCCATTAATGATGGAGATC

>*Gpa*42, clone 41D11, AM282933

GATCTCTTGAGTGGGACGAAATAACTTGGAGACATGCTGCTGGCCAACTTCTTTAGAGGGCTGTTGAATCTTC
TTTCAGTTTCTGCAGTTCAGGAACTTGGCACGGAGGGCTCCTTAACATGCAAAATGCCCTGAGAAGCCAAGGC
TCTGGGCATAACTTAACTCCTCTGTTCTTATGTGGTTGAGAGGTTTCAGGACTTGTCCTGTCTCACTCAGTGG
GGGAGGGTGAGGTTAACTTCTTAATTCTCTGTGTGTTAGTGAGGAGTAACGACAATTACTCGAGAGGGTCAAA
AAAAGAGAGTTGTGGAGAGAAAAAGAGAGGAAGAGAGGAAGAGAGGAAGAGAGGAAGAGAGGAAGAGAGGAAG
AGAGGAAGAGAGGAAGAGAGGAAGAGAGGAAGGAAGGAAGGAAGGAAGGAAGGAAGGAAGGAAGGAAGGAAGG
AAGGAAGGAAGGAAGGAAGGGAGAAGAAAAGAAAAGAAAAAAACTGTCAGCACCCCTGGATC

>*Gpa*42, clone 49G12, AM282933

GATCTCTTGAGTGGGACGAAATAACTTGGAGACATGCTGCTGGCCAACTTCTTTAGAGGGCTGTTGAATCTTC
TTTCAGTTTCTGCAGTTCAGGAACTTGGCACGGAGGGCTCCTTAACATGCAAAATGCCCTGAGAAGCCAAGGC
TCTGGGCATAACTTAACTCCTCTGTTCTTATGTGGTTGAGAGGTTTCAGGACTTGTCCTGTCTCACTCAGTGG
GGGAGGGTGAGGTTAACTTCTTAATTCTCTGTGTGTTAGTGAGGAGTAACGACAATTACTCGAGAGGGTCAAA
AAAAGAGAGTTGTGGAGAGAAAAAGAGAGGAAGAGAGGAAGAGAGGAAGAGAGGAAGAGAGGAAGAGAGGAAG
AGAGGAAGAGAGGAAGAGAGGAAGAGAGGAAGGAAGGAAGGAAGGAAGGAAGGAAGGAAGGAAGGAAGGAAGG
AAGGAAGAAGGAAGGAATGTAGAAGAAAAGAAAAGACAAAAATTGTCAGCACTCCTGGATC

Appendix IV Genotype database

Individual genotypes for 103 blue crane (*Grus paradisea*) individuals (Table 9.2), 10 grey-crowned crane (*Balearica regulorum*) individuals (Table 9.3) and 10 wattled crane (*G. carunculatus*) individuals (Table 9.4) are provided.

Note: genders were considered as part of this study. Crane individuals whose samples were taken at the same GPS location were listed as related, and only unrelated individuals were used during the characterisation procedure. Grey-shading indicates the 16 loci chosen for inclusion in the forensic set of markers.

Table 9.2 Individual genotypes for 102 blue crane (*Grus paradisea*) individuals at 28 *Grus* loci and two universal sexing markers: P2/P8 (Griffiths *et al.* 1998) and 2550F/2718R (Fridolfsson *et al.* 1999). N, no; Y, yes; 0, could not be genotyped on the ABI 3730 DNA analyser (Applied Biosystems); -, not tested on the ABI 3730 DNA analyser (Applied Biosystems). Sexes of individuals not having genotypes for either of the sexing primers (e.g. crane sample 3212) were determined using 2550F/2718R primer products visualised on a 3 % agarose gel.

Crane sample ID.	Related to any other crane individual	Sex	P2/P8	2550F/ 2718R	*Gpa01*	*Gpa11*	*Gpa12*	*Gpa14*	*Gpa24*	*Gpa32*	*Gpa33*	*Gpa34*	*Gpa35*	*Gpa36*	*Gpa37*	*Gpa38*	*Gpa39*	*Gpa41*
3193	N	male	391 391	- -	297 297	212 214	228 236	298 308	247 251	189 191	239 239	267 267	174 176	232 232	210 210	184 184	118 126	237 253
3194	N	female	385 391	- -	297 297	220 220	216 232	290 306	247 255	183 183	225 243	261 273	176 178	232 232	198 198	188 192	122 126	245 261
3195	N	female	385 391	- -	297 297	270 270	228 232	302 308	247 251	185 189	239 239	285 285	172 176	228 232	226 226	180 184	122 142	0 0
3197	N	male	391 391	- -	291 297	256 256	232 236	294 316	247 251	189 191	243 303	255 267	166 172	224 236	206 218	188 188	122 122	245 253
3198	N	male	391 391	- -	291 293	218 218	232 236	290 306	247 255	191 193	243 281	255 267	172 172	224 240	206 218	188 188	122 122	245 245
3199	Y	female	385 391	- -	293 293	258 266	196 204	0 0	210 247	183 189	207 225	132 145	162 162	228 232	0 0	188 192	0 0	235 235
3203	N	male	391 391	- -	293 293	232 248	204 204	0 0	229 229	183 189	215 225	163 207	162 162	232 232	0 0	0 0	0 0	0 0
3207	N	male	391 391	- -	291 297	214 258	220 236	294 294	247 247	189 191	277 303	285 291	172 182	228 228	186 186	180 184	0 0	237 253
3208	Y	female	385 391	- -	293 293	270 304	0 0	0 0	229 231	183 189	207 207	139 145	162 162	232 236	0 0	184 184	0 0	223 239
3210	N	female	385 391	- -	297 297	272 350	216 236	302 312	251 251	189 191	235 249	279 365	172 172	228 240	230 318	188 192	122 126	237 245
3211	N	female	385 391	- -	297 297	210 284	212 236	0 0	247 251	185 187	243 253	139 145	172 182	228 228	198 214	188 188	118 126	245 245
3212	N	male	0 0	- -	297 297	280 364	228 228	302 312	251 251	183 189	235 249	279 371	172 176	232 232	230 354	192 192	118 122	245 245
3213	N	female	385 391	- -	293 297	196 252	224 228	294 298	247 251	183 183	235 243	261 297	174 176	228 232	230 238	184 196	118 126	253 253
3214	N	male	391 391	- -	293 293	232 258	196 196	0 0	221 251	183 189	207 207	151 163	160 162	232 236	0 0	188 192	0 0	239 239
3222	Y	male	391 391	- -	291 299	208 346	220 232	286 302	247 247	177 193	295 295	249 322	172 178	232 232	214 272	184 196	122 134	253 253
3223	N	male	391 391	- -	0 0	208 346	220 232	286 302	247 247	177 193	295 295	249 322	172 178	232 232	214 272	184 196	122 134	253 253
3224	N	female	385 391	- -	291 299	276 276	212 232	298 316	243 251	183 189	205 317	249 365	172 184	0 0	190 384	192 196	130 134	245 261
3225	Y	female	0 0	- -	297 297	276 346	212 232	298 316	247 251	183 189	205 417	249 267	172 184	236 236	222 222	192 196	118 126	245 253
3232	Y	female	385 391	- -	291 291	232 256	200 200	306 306	243 243	181 185	241 317	285 303	168 168	228 228	120 133	184 192	102 102	233 249
3695	N	male	391 391	- -	297 297	192 232	224 232	294 302	243 251	177 187	253 257	0 0	0 0	224 224	206 390	188 192	118 134	253 261
3696	N	female	385 391	- -	297 297	216 220	212 232	302 312	247 247	185 191	219 231	297 335	172 178	232 232	222 276	180 188	118 122	245 245
3697	N	male	391 391	- -	297 297	216 318	224 236	290 306	243 247	183 183	267 305	285 347	174 178	220 228	250 368	188 196	118 122	253 261
3698	Y	male	391 391	- -	293 297	315 318	220 224	302 306	239 247	183 183	305 391	347 381	178 186	228 228	250 364	188 188	122 126	251 253
3800	Y	male	0 0	- -	297 297	214 224	236 236	278 294	0 0	0 0	265 265	0 0	0 0	228 232	190 304	180 188	126 134	245 245
3801	N	female	385 391	- -	293 299	222 262	224 224	294 312	251 251	193 193	231 309	0 0	178 180	228 232	202 304	180 196	114 118	245 245
3802	Y	female	385 391	- -	297 299	212 212	224 232	290 294	243 251	185 193	231 309	249 249	178 180	228 232	236 236	188 196	118 118	245 245
3804	Y	female	385 391	- -	291 297	206 218	212 228	298 306	247 251	185 189	223 335	249 249	172 178	228 232	230 346	192 192	118 126	237 253
3805	N	female	385 391	- -	297 297	322 322	220 236	306 312	247 247	177 191	231 331	267 413	176 178	228 228	174 234	180 192	126 138	245 245
3806	Y	male	391 391	- -	297 297	302 322	220 228	306 312	247 251	177 191	261 273	267 413	172 178	228 228	174 234	180 192	118 138	245 245
3807	N	male	391 391	- -	297 299	222 252	224 232	298 306	247 247	191 191	0 0	279 279	0 0	228 232	202 238	184 192	118 126	237 253
3808	Y	male	391 391	- -	293 297	210 252	224 232	298 298	247 247	189 191	227 299	279 381	166 176	232 236	214 273	184 192	122 126	245 253
3809	N	male	391 391	- -	297 299	276 346	212 232	294 316	247 251	183 189	205 417	267 381	172 184	232 232	222 384	192 196	118 130	245 253
3810	Y	male	0 0	- -	297 299	326 346	212 232	298 312	243 251	189 189	205 205	267 267	172 180	236 236	190 190	192 196	118 130	253 261
3811	N	male	0 0	- -	291 297	218 220	220 224	286 298	247 247	185 189	305 339	335 381	172 182	232 232	214 214	180 192	118 122	245 253
3812	N	male	391 391	- -	297 297	218 326	224 224	278 306	247 251	183 185	249 345	261 353	172 180	232 236	218 222	180 200	118 138	245 261
3813	Y	male	0 0	- -	291 297	198 228	228 236	278 322	247 251	187 189	265 345	249 273	172 180	228 232	222 284	188 192	118 126	245 245
3814	N	male	391 391	- -	297 299	220 220	216 228	302 312	239 243	189 189	239 309	279 285	178 178	236 236	178 312	184 188	122 134	245 253
3815	N	female	385 391	- -	293 297	192 192	212 212	294 294	247 247	183 189	231 231	249 249	172 174	232 232	308 390	188 196	118 134	245 245

Table 9.2 cont.

Crane sample ID.	Related to any other crane individual	Sex	P2/P8	2550F/ 2718R	*Gpa01*	*Gpa11*	*Gpa12*	*Gpa14*	*Gpa24*	*Gpa32*	*Gpa33*	*Gpa34*	*Gpa35*	*Gpa36*	*Gpa37*	*Gpa38*	*Gpa39*	*Gpa41*
3845	N	male	391 391	- -	297 299	216 270	220 224	294 298	247 251	183 185	239 267	291 291	174 184	232 232	226 314	180 188	122 130	245 253
3854	N	male	391 391	- -	297 297	210 224	236 236	286 302	247 247	177 189	223 267	279 335	174 176	228 232	238 330	188 192	122 122	245 261
4352	N	female	385 391	- -	297 297	194 194	216 232	302 302	251 255	183 189	225 243	261 267	176 178	228 232	174 238	188 192	122 126	253 261
4353	Y	female	385 391	- -	297 297	220 220	232 236	290 302	247 255	183 191	221 225	0 0	176 178	228 232	198 383	188 192	122 126	245 261
4384	N	male	391 391	- -	291 297	220 258	224 232	302 316	247 251	189 191	291 291	249 249	172 172	232 236	214 222	180 188	122 134	245 245
4385	N	female	0 0	- -	297 297	208 208	212 232	294 324	251 251	189 189	235 249	291 341	176 178	232 232	190 334	192 192	126 134	245 245
4386	N	female	385 391	- -	293 299	224 228	212 236	282 290	243 247	187 187	235 295	285 387	178 178	228 228	354 396	188 192	114 118	229 245
4387	N	female	385 391	- -	293 297	222 270	220 232	280 290	243 247	189 191	225 383	267 279	176 176	228 232	152 226	196 196	118 118	237 245
4518	N	male	391 391	- -	297 297	214 220	236 236	282 298	247 251	189 189	239 311	285 335	178 186	228 228	230 368	180 192	122 126	245 245
4519	N	male	391 391	- -	297 297	224 232	228 240	298 302	247 247	185 187	221 299	297 335	178 180	224 236	202 230	180 196	114 126	237 253
4530	N	female	385 391	- -	291 291	224 244	232 236	294 306	251 251	183 185	225 235	341 341	172 178	232 232	218 234	196 196	118 126	245 245
4567	Y	male	391 391	- -	293 297	210 284	232 236	312 312	247 247	185 187	243 253	0 0	174 182	228 232	198 214	184 184	118 122	245 253
4576	Y	male	0 0	- -	297 297	214 220	236 236	298 306	247 247	183 189	239 311	285 401	172 176	228 236	148 250	180 192	118 126	245 245
4577	Y	female	0 0	- -	297 297	210 210	236 236	298 306	243 251	189 189	207 311	285 335	172 176	228 228	148 246	180 180	118 126	229 245
4581	Y	female	0 0	- -	297 297	194 194	0 0	290 306	247 247	189 191	225 243	261 273	172 178	228 232	174 198	192 192	122 126	245 253
4612	Y	male	0 0	- -	297 297	0 0	216 224	282 306	243 251	189 189	231 265	0 0	178 178	236 236	148 148	188 192	118 122	0 0
4613	Y	male	391 391	- -	297 297	318 318	224 236	306 306	247 251	185 189	209 239	267 297	176 178	228 228	399 420	188 192	126 126	245 261
4614	Y	male	391 391	- -	297 297	214 292	228 232	286 298	251 251	183 189	235 325	285 322	172 172	228 232	202 206	188 188	130 134	245 253
4615	Y	female	0 0	- -	291 293	198 220	232 236	286 324	247 247	187 189	209 239	267 375	172 176	228 228	226 226	188 188	114 130	245 245
4616	Y	female	385 391	- -	291 299	194 224	224 228	290 306	251 251	183 183	235 318	0 0	178 182	236 236	202 230	180 188	122 134	245 253
4618	Y	male	391 391	- -	291 291	266 266	224 236	294 312	243 247	189 189	221 239	0 0	176 178	228 228	202 206	188 192	118 126	245 245
4619	Y	male	391 391	- -	291 291	266 266	224 236	294 312	243 247	189 189	221 239	297 297	176 178	228 228	202 206	188 192	118 126	245 245
4620	Y	female	385 391	- -	297 297	206 248	216 220	282 294	243 251	177 189	249 265	267 329	178 178	232 236	148 218	192 192	118 122	229 245
4626	Y	male	391 391	- -	291 297	198 230	224 232	286 306	247 247	185 187	209 235	255 267	172 176	228 228	226 246	188 192	130 134	229 245
4627	Y	male	391 391	- -	297 297	214 288	236 236	290 298	247 251	183 189	235 325	303 322	172 176	0 0	238 295	188 192	122 134	245 253
4630	Y	male	391 391	- -	297 297	206 224	212 232	0 0	0 0	0 0	215 243	0 0	0 0	0 0	148 272	188 192	110 130	0 0
4631	Y	male	391 391	- -	293 297	220 284	232 232	306 324	247 251	187 191	0 0	255 255	172 172	220 228	222 226	188 200	118 130	245 245
4632	Y	female	385 391	- -	293 297	228 248	220 232	294 294	247 251	177 183	249 319	273 273	176 178	228 232	218 242	188 192	122 122	229 245
4633	Y	female	385 391	- -	293 297	220 230	232 236	286 298	247 251	185 189	209 235	237 267	172 176	228 228	226 246	188 200	114 118	229 245
4634	Y	female	385 391	- -	291 297	220 230	232 236	298 324	247 247	185 189	239 267	0 0	172 176	228 228	226 246	188 192	114 118	229 245
4635	Y	male	391 391	- -	291 291	198 236	216 232	278 304	247 247	183 187	209 239	267 375	174 176	228 232	226 246	184 188	114 130	245 245
4636	Y	male	0 0	- -	297 297	230 284	0 0	298 306	247 251	185 191	235 267	237 255	172 172	220 228	222 246	192 200	118 130	229 245
4637	Y	female	385 391	- -	297 297	224 241	232 236	294 298	247 251	189 189	305 352	267 267	178 180	228 232	226 242	184 188	122 122	253 261
5122	N	female	0 0	- -	297 299	202 224	228 232	316 322	243 255	177 187	239 325	255 267	172 174	224 236	198 288	188 188	134 134	245 261
5129	N	female	385 391	- -	293 297	186 224	232 232	298 308	247 251	183 185	253 311	341 353	172 180	228 232	214 296	188 192	122 122	245 261
5130	N	male	391 391	- -	297 297	206 206	212 212	298 306	247 247	183 189	301 311	249 353	172 180	228 232	230 346	188 196	122 122	245 245
5131	N	female	385 391	- -	293 297	230 236	220 232	298 298	247 251	185 187	219 223	291 371	174 178	224 232	186 194	188 192	118 126	245 245
5132	N	female	385 391	- -	297 297	206 218	216 220	302 324	247 251	189 191	225 305	303 335	180 180	228 232	0 0	180 192	118 130	245 261

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Table 9.2 cont.

Crane sample ID.	Related to any other crane individual	Sex	P2/P8	2550F/ 2718R	*Gpa01*	*Gpa11*	*Gpa12*	*Gpa14*	*Gpa24*	*Gpa32*	*Gpa33*	*Gpa34*	*Gpa35*	*Gpa36*	*Gpa37*	*Gpa38*	*Gpa39*	*Gpa41*
5133	N	male	391 391	- -	297 297	206 212	212 228	302 302	247 247	189 189	215 249	279 291	180 182	232 232	206 230	180 184	118 138	245 253
5134	Y	female	385 391	- -	293 297	218 220	212 224	278 298	247 251	189 191	225 305	276 297	180 180	228 228	222 346	184 192	122 130	245 261
5135	N	female	385 391	- -	291 297	220 296	220 224	302 302	247 251	181 189	231 243	285 395	172 178	232 232	230 276	180 184	118 134	245 245
5136	N	male	391 391	- -	297 297	248 262	212 232	290 316	247 247	183 189	215 373	249 279	172 176	228 232	238 252	184 184	122 126	245 253
5137	N	male	391 391	- -	291 297	202 262	220 232	286 294	247 247	187 189	205 339	279 335	172 172	232 232	242 376	180 192	118 130	245 245
5138	N	male	391 391	- -	293 297	216 236	212 224	290 302	251 251	185 189	243 387	0 0	172 178	232 236	194 362	188 188	122 134	245 245
5139	Y	male	391 391	- -	297 297	218 258	232 236	286 294	247 247	187 189	0 0	0 0	172 186	228 232	214 218	180 192	118 126	229 245
5140	N	male	391 391	- -	293 297	228 236	212 224	298 302	251 251	185 189	239 273	249 285	176 178	232 236	194 330	188 188	122 130	245 253
5141	N	male	391 391	- -	291 291	194 222	212 220	298 306	251 251	185 185	0 0	279 285	176 180	224 232	230 230	184 188	114 118	245 245
5142	N	male	0 0	- -	293 297	220 276	232 236	294 298	247 251	183 189	0 0	341 371	178 178	232 232	260 308	184 192	122 126	253 261
5143	N	female	385 391	- -	297 297	194 194	216 232	0 0	247 247	189 191	225 243	261 273	0 0	228 232	174 198	192 192	118 138	245 253
5144	Y	female	385 391	- -	297 297	0 0	220 236	302 302	247 251	185 189	215 215	285 395	176 176	232 232	246 318	180 184	122 134	229 245
5146	Y	male	391 391	- -	291 293	194 222	216 216	280 298	251 251	185 185	243 277	249 285	176 180	232 236	230 412	188 192	114 118	245 245
5147	Y	male	391 391	- -	293 297	218 224	232 236	298 302	247 251	183 183	361 379	285 371	178 178	228 232	226 380	184 192	126 130	253 261
30900	Y	male	391 391	642 642	297 299	196 198	220 224	302 302	247 251	177 183	233 267	341 387	172 172	232 232	218 222	180 188	122 122	245 245
Esp1	N	male	0 0	642 642	293 297	206 296	212 224	298 302	251 251	185 189	215 383	285 395	176 178	232 236	246 318	188 188	122 130	245 253
Esp2	Y	male	0 0	642 642	293 297	248 262	212 224	290 302	251 251	185 189	215 373	249 279	172 178	232 236	238 252	188 188	0 0	245 245
J17701	N	female	0 0	458 642	291 293	196 198	228 236	298 302	243 247	185 191	233 267	0 0	172 176	232 232	218 222	192 192	110 122	245 253
J17715	Y	male	0 0	642 642	291 297	228 236	224 236	304 324	247 247	183 189	239 273	249 285	172 180	228 228	194 194	188 188	126 126	0 0
J17718	Y	female	0 0	458 642	291 297	216 236	232 236	286 294	243 251	183 189	243 243	0 0	176 178	228 228	194 194	188 192	122 126	245 245
Orpen1	N	male	0 0	642 642	291 297	216 224	220 232	286 294	247 247	187 189	209 231	0 0	172 172	232 232	186 218	180 192	118 130	245 245
Orpen2	Y	male	0 0	642 642	297 297	220 220	232 236	286 294	247 247	187 189	285 291	279 297	172 172	228 232	222 238	180 192	118 126	245 245
Rhen2	N	female	385 391	- -	291 297	300 328	220 224	302 302	247 251	181 189	321 409	0 0	172 178	232 232	218 238	188 188	118 134	245 245
Rhend	Y	female	385 391	- -	297 297	202 262	220 236	302 302	247 251	185 189	205 339	285 285	176 176	232 232	242 376	180 184	122 134	229 245
Som1	N	male	391 391	642 642	297 297	218 258	212 232	290 316	247 247	183 189	205 339	249 279	172 176	228 232	214 218	180 184	122 126	245 253
Stoffie	N	female	0 0	458 642	291 297	228 272	220 228	0 0	0 0	0 0	225 277	0 0	0 0	228 232	226 226	184 192	120 120	245 253

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Table 9.2 cont.

Crane sample ID.	Related to any other crane individual	Sex	*Gamu 002*	*Gamu 003*	*Gamu 005*	*Gamu 006*	*Gamu 007*	*Gamu 011*	*Gamu 013*	*Gamu 014*	*Gamu 018*	*Gamu 024*	*Gamu 025*	*Gamu 101b*	*Gj-M15*	*Gj-M34*	*Gj-M48b*
3193	N	male	173 173	114 114	186 186	0 0	150 150	140 140	193 193	0 0	192 192	261 263	0 0	201 201	118 120	126 130	206 206
3194	N	female	173 173	112 114	186 186	123 125	150 150	137 140	191 193	122 122	192 194	251 269	113 113	201 201	106 112	124 130	0 0
3195	N	female	0 0	112 112	186 186	115 123	150 150	140 140	193 193	118 120	192 192	251 251	113 113	201 201	112 118	126 130	0 0
3197	N	male	173 173	114 114	186 186	125 127	152 152	140 146	191 193	120 122	184 194	251 261	113 113	201 201	112 120	126 130	206 210
3198	N	male	173 173	114 114	186 186	115 125	150 152	140 152	193 193	118 120	184 190	261 261	113 113	201 201	118 120	124 130	206 210
3199	Y	female	173 173	110 112	0 0	121 125	148 148	137 140	193 193	0 0	182 182	239 239	0 0	0 0	112 112	114 114	0 0
3203	N	male	171 171	110 114	176 176	121 125	148 148	137 140	195 195	0 0	182 182	239 239	109 109	186 186	112 112	114 114	0 0
3207	N	male	173 175	114 114	186 186	125 131	144 150	140 140	193 193	118 118	196 196	251 251	113 113	201 201	106 112	126 130	0 0
3208	Y	female	171 173	108 108	0 0	121 121	148 148	143 143	0 0	118 118	182 182	239 239	0 0	0 0	112 112	114 114	0 0
3210	N	female	173 175	114 114	186 186	125 125	150 150	140 146	193 193	118 120	192 192	269 269	113 113	0 0	106 106	124 126	204 206
3211	N	female	173 173	114 114	186 186	115 115	150 150	146 146	191 193	120 120	192 196	251 251	113 113	201 201	116 118	126 130	0 0
3212	N	male	173 175	114 114	186 186	123 125	150 150	140 146	193 193	120 120	0 0	251 269	113 113	201 201	106 106	124 126	206 208
3213	N	female	173 173	114 114	186 186	115 121	150 150	140 140	193 193	122 122	192 192	251 269	113 113	201 201	106 120	124 130	212 218
3214	N	male	171 171	110 118	176 176	121 121	148 148	143 143	0 0	0 0	182 182	239 239	109 109	186 201	112 112	114 114	0 0
3222	Y	male	173 175	114 114	- -	125 133	150 150	137 140	191 193	122 122	192 204	251 267	- -	- -	112 118	126 130	206 208
3223	N	male	173 175	114 114	- -	125 133	150 150	137 140	191 193	122 122	192 204	251 267	- -	- -	112 118	126 130	206 208
3224	N	female	173 173	114 114	- -	125 133	150 150	146 146	193 193	120 122	192 196	251 269	- -	- -	110 118	124 126	0 0
3225	Y	female	173 173	114 114	- -	121 125	150 150	140 146	193 193	118 124	190 190	269 269	- -	- -	110 118	124 126	0 0
3232	Y	female	175 175	110 118	- -	119 123	144 144	137 137	193 193	0 0	0 0	255 255	- -	- -	110 112	122 122	214 214
3695	N	male	0 0	114 114	- -	115 125	0 0	140 146	193 195	0 0	192 192	0 0	- -	- -	118 118	124 124	0 0
3696	N	female	173 173	114 114	- -	121 123	144 144	140 140	191 193	120 122	192 192	261 269	- -	- -	106 110	124 124	0 0
3697	N	male	173 175	114 114	- -	121 123	0 0	140 140	191 193	120 122	0 0	251 267	- -	- -	106 118	124 124	0 0
3698	Y	male	175 175	114 116	- -	121 123	144 144	140 146	191 193	120 122	0 0	269 269	- -	- -	118 118	124 124	0 0
3800	Y	male	173 173	0 0	- -	115 125	0 0	134 140	193 193	0 0	194 196	0 0	- -	- -	106 118	126 126	0 0
3801	N	female	173 175	114 114	- -	121 123	144 144	149 149	193 193	124 124	194 196	0 0	- -	- -	118 118	124 126	0 0
3802	Y	female	173 175	114 114	- -	121 123	152 152	140 149	191 193	120 122	184 194	0 0	- -	- -	106 118	124 126	0 0
3804	Y	female	173 173	114 114	- -	115 123	150 150	140 140	191 193	120 120	190 196	251 251	- -	- -	106 116	124 130	0 0
3805	N	female	173 173	114 114	- -	115 125	146 146	146 146	193 193	118 122	190 192	273 273	- -	- -	106 112	124 130	206 216
3806	Y	male	173 173	114 114	- -	115 117	146 146	137 140	193 193	118 122	192 196	273 273	- -	- -	106 112	124 124	0 0
3807	N	male	175 175	114 114	- -	115 133	144 150	134 137	193 195	120 120	192 192	251 251	- -	- -	112 116	124 130	0 0
3808	Y	male	175 175	114 114	- -	115 115	150 150	134 149	193 195	120 120	192 192	273 273	- -	- -	112 118	124 130	206 214
3809	N	male	173 173	114 114	- -	115 121	146 150	140 152	193 193	118 120	0 0	251 251	- -	- -	116 118	124 126	208 212
3810	Y	male	173 173	114 114	- -	115 133	146 150	140 152	193 193	118 124	0 0	0 0	- -	- -	110 118	124 126	0 0
3811	N	male	173 175	114 114	- -	125 125	144 150	137 140	193 193	0 0	192 192	267 269	- -	- -	106 116	124 130	0 0
3812	N	male	173 173	114 114	- -	115 115	146 150	137 146	191 193	122 122	0 0	251 261	- -	- -	112 118	124 126	206 208
3813	Y	male	173 173	114 114	- -	115 125	150 150	140 146	191 193	0 0	192 192	261 261	- -	- -	112 118	124 126	0 0
3814	N	male	173 173	114 114	- -	123 123	144 150	137 140	191 193	122 122	192 194	251 269	- -	- -	110 112	124 130	208 208
3815	N	female	173 173	114 114	- -	115 125	150 150	140 149	193 193	0 0	192 192	267 269	- -	- -	106 116	126 130	0 0

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Table 9.2 cont.

Crane sample ID.	Related to any other crane individual	Sex	*Gamu 002*	*Gamu 003*	*Gamu 005*	*Gamu 006*	*Gamu 007*	*Gamu 011*	*Gamu 013*	*Gamu 014*	*Gamu 018*	*Gamu 024*	*Gamu 025*	*Gamu 101b*	*Gj-M15*	*Gj-M34*	*Gj-M48b*
3845	N	male	175 175	114 114	- -	117 125	150 150	140 140	191 191	122 122	192 192	273 273	- -	- -	116 118	124 126	206 208
3854	N	male	173 173	114 114	- -	125 125	150 150	140 149	193 193	118 120	192 192	267 269	- -	- -	118 118	124 130	208 208
4352	N	female	173 173	112 114	- -	115 125	150 150	140 140	191 191	122 122	192 194	251 269	- -	- -	112 112	124 130	208 208
4353	Y	female	173 173	114 114	- -	123 125	150 150	137 146	191 191	122 122	0 0	269 273	- -	- -	112 112	124 126	206 208
4384	N	male	0 0	0 0	- -	125 125	150 150	140 146	193 193	0 0	192 194	0 0	- -	- -	118 118	124 130	0 0
4385	N	female	173 175	114 114	- -	125 125	144 144	140 140	193 193	120 120	192 194	251 251	- -	- -	116 118	124 130	206 208
4386	N	female	173 173	114 114	- -	115 125	150 150	140 140	193 193	118 120	190 192	267 267	- -	- -	106 118	124 130	206 206
4387	N	female	173 173	114 114	- -	115 115	150 150	137 140	191 193	118 120	192 192	251 273	- -	- -	112 120	124 126	206 210
4518	N	male	173 173	114 114	- -	125 125	150 150	140 140	193 193	122 122	184 192	261 273	- -	- -	112 112	124 126	208 208
4519	N	male	173 175	114 114	- -	115 125	148 150	140 146	193 193	120 122	192 196	251 269	- -	- -	106 112	124 124	208 218
4530	N	female	175 175	114 114	- -	115 127	150 150	137 146	191 193	118 120	192 196	267 273	- -	- -	106 116	124 130	204 210
4567	Y	male	173 173	114 114	- -	115 125	150 150	146 152	193 193	122 122	184 192	251 261	- -	- -	106 116	124 130	0 0
4576	Y	male	173 175	114 114	- -	121 123	150 150	140 146	193 193	118 122	192 192	261 273	- -	- -	106 112	124 130	206 208
4577	Y	female	173 173	114 114	- -	121 125	150 150	140 140	191 193	122 122	0 0	261 267	- -	- -	112 118	124 126	206 208
4581	Y	female	173 173	100 102	- -	115 123	146 146	137 146	191 193	122 122	188 192	0 0	- -	- -	106 106	126 130	206 206
4612	Y	male	0 0	114 114	- -	125 125	0 0	140 140	193 193	122 122	192 192	0 0	- -	- -	106 118	130 130	0 0
4613	Y	male	173 173	112 114	- -	115 125	146 150	140 140	191 193	120 120	188 196	251 251	- -	- -	118 118	124 126	0 0
4614	Y	male	173 173	114 114	- -	121 131	150 150	137 146	191 193	122 122	192 196	251 261	- -	- -	118 118	126 130	208 218
4615	Y	female	0 0	0 0	0 0	115 125	0 0	140 149	191 193	0 0	0 0	0 0	0 0	0 0	106 106	124 124	0 0
4616	Y	female	173 173	114 114	- -	115 125	150 150	140 140	193 193	120 120	192 192	269 269	- -	- -	118 118	126 130	0 0
4618	Y	male	173 173	114 114	0 0	115 123	146 150	137 140	191 193	118 124	192 192	261 263	0 0	0 0	118 120	124 124	206 206
4619	Y	male	173 173	114 114	- -	115 123	146 150	137 140	191 193	116 122	192 192	261 263	- -	- -	118 120	124 124	0 0
4620	Y	female	173 173	112 114	0 0	117 125	150 150	134 140	193 193	122 122	192 192	261 261	0 0	0 0	118 118	130 130	204 206
4626	Y	male	173 173	114 114	0 0	115 115	144 150	140 146	191 193	122 122	188 196	251 261	0 0	0 0	106 106	124 130	0 0
4627	Y	male	173 175	114 114	- -	121 131	150 150	137 146	191 193	118 122	0 0	251 261	- -	- -	112 118	126 126	206 208
4630	Y	male	173 175	112 112	- -	121 125	0 0	146 149	191 193	0 0	192 196	0 0	- -	- -	106 112	126 126	0 0
4631	Y	male	173 175	114 114	- -	115 123	0 0	146 149	191 193	0 0	192 196	251 251	- -	- -	106 116	124 124	0 0
4632	Y	female	173 175	112 114	- -	115 117	144 144	134 146	191 193	122 122	192 192	261 267	- -	- -	112 118	124 130	0 0
4633	Y	female	173 173	100 100	0 0	115 115	144 144	140 149	193 193	122 122	192 196	251 273	113 113	200 200	106 116	124 130	206 218
4634	Y	female	173 175	116 116	- -	125 125	150 150	140 140	193 193	122 122	0 0	0 0	- -	- -	106 106	124 124	0 0
4635	Y	male	0 0	0 0	- -	115 125	146 150	149 149	191 193	124 124	192 196	251 273	- -	- -	106 106	124 124	206 208
4636	Y	male	173 173	114 114	186 186	115 123	0 0	140 146	193 193	0 0	192 194	0 0	0 0	0 0	106 116	124 130	206 206
4637	Y	female	173 175	112 112	- -	115 125	150 150	137 140	191 193	118 120	196 196	261 269	- -	- -	106 118	130 130	208 210
5122	N	female	173 173	114 114	- -	115 127	150 150	140 149	193 195	122 122	188 196	251 269	- -	- -	112 120	124 130	206 210
5129	N	female	173 175	112 112	- -	115 125	150 150	140 140	191 193	120 120	192 194	251 275	- -	- -	110 116	124 126	206 206
5130	N	male	173 173	114 114	- -	115 123	144 150	140 140	191 193	118 120	184 196	251 269	- -	- -	106 116	124 130	206 208
5131	N	female	173 173	114 114	- -	125 125	144 144	140 146	193 193	120 120	192 196	251 269	- -	- -	112 118	124 130	206 210
5132	N	female	173 173	100 100	- -	117 125	150 150	134 152	191 193	120 120	0 0	251 273	- -	- -	106 116	124 130	208 218

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