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Feasibility of Non-Invasive Molecular Method for Sexing of Parrots

Jasna Bosnjak[1]*, Marija Stevanov-Pavlovic[1], Milos Vucicevic[2], Jevrosima Stevanovic[1], Predrag Simeunovic[1], Radmila Resanovic[2] and Zoran Stanimirovic[1]

[1]Department of Biology, Faculty of Veterinary Medicine, University of Belgrade, Blvd. oslobodjenja 18, 11000 Belgrade, Serbia

[2] Department of Equine, Small Animal, Poultry and Wild Animal Diseases, Faculty of Veterinary Medicine, University of Belgrade, Blvd. Oslobodjenja 18, 11000 Belgrade, Serbia

Abstract. - The aim of this study was to test the feasibility and efficacy of a non-invasive molecular method for gender identification of parrots, using different types of samples and the Chromo Helicase DNA-binding (CHD) gene as a molecular marker. DNA was isolated primarily from feathers and the amplification of the CHD gene was performed using 2550F/2718R primers. In order to compare the reliability of different sources of DNA, we used buccal swab, blood and feces. All sample types exerted successful sexing results with the exception of feces samples where the success rate was 25%. Sexing was successfully determined in 239 birds belonging to 32 species of parrots. In 6 species (*Amazona finschi, A. leucocephala, Aratinga aurea, Barnardius zonarius, Coracopsis nigra* and *Nymphicus hollandicus*), 2550F/2718R primers proved to work well for the first time. Species used in this study are on the IUCN red list of threatened species. Furthermore, *Amazona finschi, A. leucocephala, Cacatua moluccensis* and *C. sulphurea* are on the list of CITES Apendix I. Since the failure in reproduction is one of the main causes of illegal trafficking of parrots, the non-invasive and universal molecular sexing method we tested may be a very useful tool in the preservation of endangered parrot species.

Key words: Psittaciformes, conservation, molecular sexing, CHD gene, 2550F/2718R.

INTRODUCTION

Avian order Psittaciformes (parrots) accounts for around 375 species in 86 genera (BirdLife International, 2013) They are commonly held in captivity, with over 20 000 parrots housed in Zoological Gardens and other animal holding facilities and millions more held in private hands (Young *et al*., 2012). Therefore, trapping wild parrots for pet trade, as well as hunting, habitat loss and competition from invasive species led to the decrease of wild parrot populations, making them more exploited than any other group of birds (Snyder *et al*., 2000). In fact, nineteen parrot species are extinct, 15 are critically endangered, 34 endangered, 48 vulnerable and 42 near threatened (International Union for Conservation of Nature - IUCN 2012). In globally threatened species, the sex ratio distortion seems to be often larger than in non-threatened species, suggesting that their extinction risk could be higher than currently estimated (Donald, 2007). A number of recent studies have been focused on the development of efficient molecular methods for sex identification, which are gaining undivided attention as an aid in research and conservation of many bird species (Cerit and Avanus, 2007).

According to Jensen *et al*. (2003) it is critically important to minimize handling-induced stress when sexing chicks, fragile individuals, or endangered species. That implies selection of samples for DNA extraction without risk for survival of birds. In light of that, the following types of samples were proposed as favourable: blood (Hoysak and Weatherhead, 1991; Lanctot, 1994; Gaunt and Oring, 1999; Goymann and Wingfield 2004), feathers (Bello *et al*., 2006), buccal swab (Handel *et al*., 2006) and feces (Idaghdour *et al*., 2003). Although blood sampling has no significant effect on the short-term and long-term survival of developing birds (Sheldon *et al*., 2008), Brown and Brown (2009) suggested caution in collecting blood from wild birds.

This study was based on Chromo Helicase DNA-binding genes (CHDW/CHDZ genes) (Ellegren and Sheldon, 1997) due to their proven usage in almost all bird species, with the exception of ratites (Griffiths *et al*., 1996). Due to its high

* Corresponding author: bosnjakjasn@gmail.com
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degree of conservation, Ellegren (1996) suggested that the CHD gene is an interesting marker which provides an opportunity for developing a universal method for molecular Psittaciformes sexing. Generally, PCR amplification of the CHD gene with primers used in this study produces a single Z-band in male and two bands (Z and W) in female birds due to the difference in intron size. However, errors can occur due to allelic dropout, where females are miss-sexed as males (Arnold *et al*., 2003; Robertson and Gemmell, 2006) or due to the formation of heteroduplex DNA molecules, where males can be misidentified as females (Casey *et al.*, 2009).

Taking all given facts into account, the aim of this study was to test the feasibility and efficacy of molecular sex identification in Psittaciformes using different types of samples for DNA extraction (blood, feathers, buccal swab and feces) and applying the protocol that had proven to work well in our previous research (Vucicevic *et al*., 2012).

MATERIALS AND METHODS

Sampling

A total of 239 individuals were sampled for gender identification. Sampled birds belong to 32 parrot species. Among a total of 398 samples, there were 239 feather samples, 15 blood samples, 72 buccal samples and 72 feces samples (Table 1). Samples were provided by Zoological Gardens and private breeders.

Blood was collected with a sterile cotton swab after clipping a toenail; one to three thoracic feathers were sampled by plucking and quills were cut into 2-5 mm long pieces; sterile cotton swabs were used to collect buccal and feces samples.

Isolation of DNA

DNA was isolated by using the KAPA Express Extract kit (KAPA Biosystems Cat. No. KK7152), following the kit protocol. The incubation step lasted 20 min at 75°C and additional 5 min at 95°C. Fifty µL of the obtained DNA isolate was added to 200 µl 1x TE buffer (Serva, Cat. No. 39799.01). Ten µL of the obtained dilution of DNA isolate were used in the PCR reaction.

PCR amplification

The following set of primers was used for the amplification of the CHD gene:

2550F (5´-GTTACTGATTCGTCTACGAGA-3´) and
2718R (5´-ATTGAAATGATCCAGTGCTTG-3´)

published by Fridolfosson and Ellegren (1999).

The amplification was carried out in 25 µL reaction volume containing 12.5 µL of KAPA2G Robust HotStart ReadyMix (Cat. No. KK7152, Kapa Biosystems), 1.25 µL volume of 1µM final concentration of each primer from 2550F/2718R primer set and 10 µL DNA sample.

The recommended thermal protocol of KAPA2G Robust HotStart ReadyMix was used: 3 min of initial denaturation at 95°C, followed by 45 cycles of denaturation (15 sec at 95°C), primer annealing (15 sec at 52°C), and extension (15 sec at 72°C). The final extension step at 72°C lasted 8 min.

Visualization of PCR products

The PCR products were visualised with UV light after staining the 2% agarose gel with ethidium bromide. A commercial O'RangeRuler 50 bp DNA Ladder (Fermentas) and Nippon 100bp DNA Ladder were used as the size markers to determine Z- and W-bands.

RESULTS

Gender of sampled birds (N=239) was successfully identified using feather samples. The results were additionally confirmed using other types of samples: 72 buccal swabs, 15 blood samples and 72 feces samples. All types of samples from a single individual gave the same result.

However, when feces were used as a sample, gender identification had a success rate of 25% using the same KAPA2G Robust HotStart ReadyMix and 2550F/2718R primer set. After electrophoresis of PCR products, parrots' gender was determined by visualization on agarose gel as two bands in females (Z and W) or one band in males (Z) with the difference range of 150 to 250 bp between Z- and W-bands, which made separation and visualization simple and reliable (Fig.1).

Table I.- Success rate of four different sample types (feather, blood, buccal swab and feces) used for sex determination of 32 parrot species.

Scientific name	Common name	Status by IUCN	M/F	Number and % of success rate of sample type used in this study			
				Feather	Blood	Bucal swab	Feces
Amazona aestiva	Blue-fronted Amazon	Least Concern	9/9	18(100%)	2(100%)	8(100%)	6(33,33%)
Amazona albifrons	White-fronted Amazon	Least Concern	0/1	1(100%)	/	/	/
Amazona amazonica	Orange-winged Amazon	Least Concern	4/6	10(100%)	/	4	4(0%)
Amazona finschi	Lilac-crowned Amazon	Vurneable	0/1	1(100%)	/	/	/
Amazona leucocephala	Cuban Amazon parrot	Neat threatened	1/0	1(100%)	/	/	/
Amazona ochrocephala	Yellow-crowned Amazon	Least Concern	2/4	6(100%)	/	2(100%)	2(0%)
Aprosmictus erythropterus	Red-winged Parrot	Least Concern	4/0	4(100%)	/	/	/
Ara ararauna	Blue-and-gold Macaw	Least Concern	26/18	44(100%)	1(100%)	28(100%)	28(35.7%)
Ara chloropterus	Green-winged Macaw	Least Concern	4/3	7(100%)	/	4(100%)	2(0%)
Ara severus	Chestnut-fronted Macaw	Least Concern	7/5	12(100%)	/	4(100%)	5(20%)
Aratinga acuticaudata	Blue-crowned Conure	Least Concern	4/1	5(100%)	2(100%)	2(100%)	/
Aratinga aurea	Peach-fronted Conure	Least Concern	1/0	1(100%)	/	/	/
Aratinga erythrogenys	Red-masked Conure	Neat threatened	6/3	9(100%)	/	4(100%)	4(25%)
Aratinga jandaya	Jandaya Parakeet	Least Concern	1/2	3(100%)	/	/	/
Aratinga solstitialis	Sun Parakeet	Endagered	4/4	8(100%)	/	/	/
Aprosmictus erythropterus	Red-winged Parrot	Least Concern	0/4	4(100%)	/	/	/
Barnardius zonarius	Australian Ringneck	Least Concern	2/2	4(100%)	/	/	/
Cacatua alba	White Cockatoo	Vurneable	1/4	5(100%)	/	2(100%)	2(0%)
Cacatua moluccensis	Salmon-crested Cockatoo	Vurneable	1/0	1(100%)	/	/	/
Cacatua sulphurea	Yellow-crested Cockatoo	Critically endagered	1/0	1(100%)	/	/	/
Cacatua galerita (eleonora)	(medium) Sulphur-crested Cockatoo	Least Concern	1/1	2(100%)	/	/	/
Coracopsis nigra	Black Parrot	Least Concern	0/2	2(100%)	/	/	/
Cyanoliseus patagonus	Burrowing Parrot	Least Concern	0/2	2(100%)	/	/	/
Lorius lory	Black-capped Lory	Least Concern	3/5	8(100%)	/	/	/
Nymphicus hollandicus	Cockatiel	Least Concern	5/3	8(100%)	8(100%)	/	/
Pionites melanocephala	Black-headed Parrot	Least Concern	1/1	2(100%)	/	/	/
Psittacula eupatria	Alexandrine Parakeet	Least Concern	0/1	1(100%)	/	/	/
Psittacus erithacus	African Grey Parrot	Vurneable	34/25	59(100%)	2(100%)	13(100%)	18(22,22%)
Poicephalus senegalus	Senegal Parrot	Least Concern	0/1	1(100%)	/	/	/
Probosciger aterrimus	Palm Cockatoo	Least Concern	3/0	3(100%)	/	/	/
Pyrrhura molinae	Green-cheeked Parakeet	Least Concern	0/2	2(100%)	/	/	/
Trichoglossus haematodus	Rainbow Lorikeet	Least Concern	1/3	4(100%)	/	1(100%)	1(0%)

M /F-number of individuals studied of each sex: M, male; F, female.

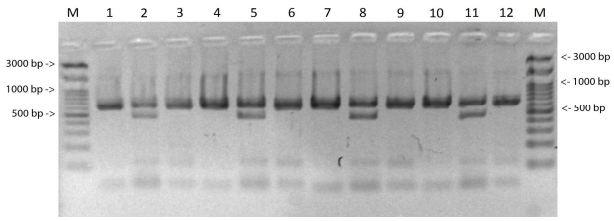

Fig. 1. PCR products of DNA isolated from four different types of samples (blood, faeces, buccal swab and feather samples) originating from a two individuals of *Ara ararauna* species and one from *Ara chloroptera* parrot. Males have a single band visualized at approximately 650 bp whereas females have two bands sized around 400 and 650 bp.; M, Nippon 100bp DNA Ladder; 1, *Ara ararauna* (♂), feather; 2, *A. ararauna* (♀), feather; 3, *Ara chloroptera* (♂), feather; 4, *Ara ararauna* (♂), buccal swab; 5, *Ara ararauna* (♀), buccal swab; 6, *Ara chloroptera* (♂), buccal swab; 7, *Ara ararauna* (♂), blood; 8, *Ara ararauna* (♀), blood; 9, *Ara chloroptera* (♂), blood; 10, *Ara ararauna* (♂), feces; 11, *Ara ararauna* (♀), feces; 12, *Ara chloroptera* (♂), feces; M, Nippon 100bp DNA Ladder.

Primers 2550F/2718R were for the first time successfully used for amplification of CHD gene of *Amazona finschi*, *A. leucocephala*, *Aratinga aurea*, *Barnardius zonarius*, *Coracopsis nigra* and *Nymphicus hollandicus*.

All species sampled in this study are on the IUCN red list of threatened species (Table I). Four species (*Amazona finschi*, *A. leucocephala*, *Cacatua moluccensis* and *C. sulphurea*) are on the list of CITES Appendix 1 (the Convention on International Trade in Endangered Species of Wild Fauna and Flora).

DISCUSSION

Parrots are considered to be amongst the most heavily traded birds on Earth (Collar, 2000). In countries where parrot export and import are illegal, the time spent for the reproduction process, consequently causes very significant financial losses (Cahill *et al.*, 2006).

Conservation techniques such as enhanced education, generation of public awareness and support, habitat protection, and law enforcement initiatives are highly effective and economical for dealing with a problems posed by the bird trade (Snyder *et al.*, 2000; Mahboob and Nisa, 2009). Snyder *et al*. (2000) indicated that for management of vulnerable and endangered species (as the majority of parrots), potential bias in sex ratio is of great concern. Same group of authors gave general recommendations for parrots' reintroduction programs, where it is advised to determine the sex of individuals who are about to be released into the wild. Additionally, numerous bird protection programs aimed at preservation of various species through intensive bird breeding imply that the sex of individuals is accurately identified (Ito *et al.*, 2003). Still, captive breeding as a conservation technique used for increasing extant populations, correcting sex-ratio imbalances, re-establishing extirpated populations, and/or establishing new populations is considered to be expensive (Wilson *et al.*, 1994).

Therefore, economical, non-invasive and precise method for the sexing of parrots is indispensable. In that regard, the aim of this study was to assess the effectiveness of a molecular method for sex determination in parrots and samples were taken from species with a relatively wide distribution throughout the Psittaciformes order. In previous studies (Miyaki *et al.*, 1998; Taylor and Parkin, 2008), sexing various species of parrots was mostly conducted with P2/P8 primer set described by Griffits *et al.* (1998). Ong and Vellayan (2008) indicated that 2550F/2718R primer set, which was used in this research, provides a higher confidence level of establishing the sex of birds even without the use of polyacrylamide gels as is required in some bird species with the use of primers P2/P8 and 1237L/1272H (Kahn *et al.*, 1998). In this study, primers of 2550F/2718R for the first time enabled successful sex determination in *Amazona finschi*, *Aratinga aurea*, *Barnardius zonarius* and *Coracopsis nigra*, but also in *Amazona ochrocephala* and *Nymphicus hollandicus* where previous attempts with the same primers failed (Wang *et al.*, 2007). Nevertheless, gender of *Nymphicus hollandicus* (Cerit and Avanus, 2007) and *Amazona ochrocephala* (Taylor and Parkin, 2008) was previously successfully determined with P2/P8 primer set.

In birds, feathers represent the most common sample for DNA isolation since sampling is easy and causes minimum pain if plucked or no pain at all if collected after molting (Seki, 2006; Leekaew *et al.*, 2008). For that reason, we used feathers in this study as source of DNA for gender identification and proved its reliability in all sampled individuals. In addition, alternative sources of DNA, such as blood (*Bush et al.*, 2000) or buccal swab (Seki, 2003) also gave reliable results (Table 1). In our study feces exerted only 25% success rate in sex determination, probably due to PCR inhibitors, such as pigments (Baignet *et al*., 2005), dead cells, RNA (Nielsen *et al*., 2000), or various microorganisms. Having in mind that Robertson *et al*. (1999) suggested avian feces as is good source of DNA when ecological questions need to be resolved in rapid and non-invasive manner, further research should be carried out to improve and simplify sexing of parrots when feces is sampled.

In this study both feathers, buccal swab and blood proved to be very reliable sources of genomic DNA with a 100% success rate. Still, for all sampling procedures of biological material, except for sampling feces and molted feathers, bird handling is necessary. Invasiveness of a procedure can be judged based on the degree of pain, tissue injury and duration of a procedure. Plucking feathers takes less time than the other two methods, and thus discomfort of individuals is considerably smaller (Directive 2010/63/EU). Thus, sampling of feathers is favored by several ornithological societies (McDonald and Griffith, 2011). Taking into account all above mentioned, it would be recommended that feathers are used as DNA source in all cases where possible. The method for determining sex in parrots presented in this study is a reliable, economical, fast, simple and does not include aggressive sampling for DNA extraction, a fact highly important when dealing with bird species that are endangered or on the CITES list.

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