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Conditions for Rapid Sex Determination in 47 Avian Species by PCR of Genomic DNA From Blood, Shell-Membrane Blood Vessels, and Feathers

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The ability to rapidly and reliably determine the sex of birds is very important for successful captive-bird breeding programs, as well as for field research. Visual inspection of adult birds is sufficient for sexually dimorphic species, but nestlings and monomorphic species are difficult, if not impossible, to sex by sight only. A method for rapid extraction of gDNA from blood, shell-membrane blood vessels, and fully grown feathers, using Chelex, and the PCR conditions for determination of sex-specific bands in 47 species (39 genera, 21 families, and 10 orders) are described. The PCR primers used amplify a length of DNA spanning an intron in the CHD-1 gene, which is present on both the W and Z chromosomes. The intron differs in size between the two sex chromosomes, resulting in PCR products that separate into two bands for females and a single band for males in most avian species (except ratites). Because this simple technique uses Chelex, a rapid gDNA isolation protocol, and sets of PCR primers independent of restriction enzyme digestion, birds can be accurately sexed within 5 hr of sample collection. Zoo Biol 22:561–571, 2003. © 2003 Wiley-Liss, Inc.

Key words: molecular bird sexing; CHD gene; Chelex DNA isolation

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INTRODUCTION

A fast and reliable method for determining the sex of monomorphic birds (which comprise an estimated 50% of all birds) and young chicks is necessary in successful breeding programs, as well as in investigations of population-sex ratios in nestling and adult free-ranging populations.

Several authors have reported the use of genetics-based techniques to establish sex ratios for fledgling and adult birds in both field and captive breeding projects. Projects involving the sexing of flamingos [Bertault et al., 1999]; whooping cranes [Duan and Fuerst, 2001]; black-eared miners [Ewen et al., 2001]; Tengmalm's owl [Hornfeldt et al., 2000]; Hawaiian honeycreepers [Jarvi and Banko, 2000]; macaws, parrots, toucans, and curassows [Miyaki et al., 1992, 1998; Griffiths and Tiwari, 1995; Russello and Amato, 2001]; and Old World vultures [Wink et al., 1998] have emphasized the importance of this technique in conservation projects.

Several groups compared the results obtained with molecular sexing techniques to those derived from morphometric measurements such as weight and tail length [Martin et al., 2000], size and plumage [Baker and Piersma 1999], sex-specific behavior, and head plus bill length [Jodice et al., 2000]. These studies demonstrated that molecular sexing requires less handling time than morphometrics, especially if feather sexing is used. It is critically important to minimize handling-induced stress when sexing chicks, fragile individuals, or endangered species.

Sex in birds is assigned genetically: the female is the heterogametic sex (ZW) and the male is the homogametic sex (ZZ). Therefore, it can be assumed that some genetic sequences occur in females but not in males [Smith and Sinclair, 2001]. The gene-encoding chromo-helicase-DNA binding protein 1 (CHD-1) has been found on the Z and W chromosomes; however, the alleles are not identical, which provides an opportunity to differentiate between males and females. The CHD-1 gene has at least one intron of different sizes on the Z and W alleles [Griffiths and Korn, 1997; Ellegren, 1996; Griffiths and Tiwari, 1993]. Multiple primers have been designed to take advantage of the CHD-1 gene [Griffiths et al., 1996, 1998; Kahn et al., 1998], two of which do not rely on post-PCR restriction digests, the P8/P2 and the 1237L/1272H primers. Other genomic sequences that differ between the Z and W chromosomes have also been used to genetically sex birds [Petitte and Kegelmeyer, 1995; De Mattos et al., 1998; Kahn et al., 1998]. The use of multiple PCR primer pairs to verify the sex of an as-yet-unstudied species may be beneficial, as some primers appear to work better in some species than in others.

Trefil et al. [1999] and Bello et al. [2001] demonstrated that genomic DNA (gDNA) suitable for sexing and paternity testing can be isolated from bird feathers and blood. Furthermore, Russello and Amato [2001] demonstrated the usefulness of Chelex (Sigma, St. Louis, MO) for rapid sex determination in the St. Vincent Amazon parrot. In this work we report a fast, reliable method for the extraction of high-quality gDNA from blood, feather, or eggshell membrane, which obviates the need for ultralow freezers, hazardous chemicals, and extensive precipitation steps. We describe the protocol for Chelex, a technique that is currently used by forensic science to purify gDNA from very small blood samples [Walsh et al., 1991]. PCR conditions and expected sizes of CHD-Z and CHD-W bands are reported for 47 species (39 genera, 21 families, and 10 orders). The protocols described allow birds to

be accurately sexed within 5 hr of sample collection, with minimal or no handling of the birds.

METHODS

Sample Collection

Blood was collected during routine annual veterinary exams or during clinical case investigations by Zoological Society of San Diego (ZSSD) staff veterinarians. Eggshell membranes were collected from hatched chicks at the Avian Propagation Center of the ZSSD, and feathers were collected from adult birds by keepers. All of the birds used in the study were of known sex, as determined by laparoscopy, behavior, morphology, or Zoogen® sexing. The sizes of both the male and female bands are shown in Table 1.

DNA Isolation and Polymerase Chain Reaction (PCR) Amplification

Unless otherwise noted, all chemicals were purchased from Fisher (Pittsburg, PA). DNA was isolated from red blood cells by Chelex 100 resin (Sigma, St. Louis, MO), as previously described [Walsh et al., 1991]. Briefly, approximately 1–3 μL of packed red blood cells were suspended in 200 μL 6% Chelex (Sigma), and incubated at 56°C for 30 min. They were then briefly vortexed and centrifuged at 7,000 *g* for 1 min, boiled for 8 min, and stored at –20°C until they were used in the PCR reactions. Samples purified by the phenol-chloroform method were homogenized by piston in 500 μL homogenization buffer (0.1 M NaCl, 0.01 M EDTA, 0.3 M Tris-HCl pH 8.0, and 0.2 M sucrose), followed by the addition of 31.25 μL 10% sodium dodecyl sulfate (SDS; Sigma), and incubation at 65°C for 30 min. Following the addition of 87.5 μL 8 M potassium acetate, and incubation on ice for 60 min, the samples were centrifuged at 5,000 *g* for 10 min. The supernatant was added 1:1 to phenol : chloroform : isoamyl alcohol (25:24:1) and then vortexed and centrifuged at 6,000 *g* for 5 min. The aqueous phase was added 1:1 to chloroform : IAA (24:1) and centrifuged at 6,000 *g* for 5 min. The aqueous phase was then precipitated in 100% ethanol at –70°C overnight. The samples were centrifuged at 7,800 *g* for 30 min, followed by three 75% ethanol washes and resuspension in 25 μL sterile water. Shell-membrane blood vessels and feathers were treated as described above (200 μL 6% Chelex). A minimum of 10 mm of shell-membrane blood vessels, and one to four feather calami (depending on feather size) were used for each reaction.

The primers used were P8 (5′-CTCCCAAGGATGAGRAAYTG-3′) and P2 (5′-TCTGCATCGCTAAATCCTTT-3′), as described by Griffiths et al. [1998], or 1237L (5′-GAGAAACTGTGCAAAACAG-3′) and 1272H (5′-TCCAGAA-TATCTTCTGCTCC-3′) primers, as described by Kahn et al. [1998]. Both pairs of primers produce a single Z-band in males, and Z- and W-bands in females. PCR amplifications were carried out in 25-μL reactions containing 1 × reaction buffer (16 mM $(NH_4)_2SO_4$, 67 mM tris-Hcl pH 8.8, 0.01% Tween-20; Bioline, Randolph, MA), variable concentrations of $MgCl_2$ (4–6.7 mM), 1 μM dNTP mix (Bioline), 2 μM each primer, 0.2 U Taq (Bioline), and 2 μL of DNA from the Chelex reaction. The annealing temperatures and $MgCl_2$ concentrations were varied to optimize PCR conditions for each bird species to yield a single or double band in males and females, respectively, in the 200–500 bp range. PCR reactions were performed on a

TABLE 1. PCR conditions for 47 species representing 10 avian orders of birds

Order	Family	Genus	Species	Common name	Z band (bp)	W band (bp)	Primer pair	anneal (°C)	(MgCl) mM
Anseriformes	Anatidae	*Dendrocygna*	*viduata*	White-faced Whistling Duck	320	240	P8/P2	52	3.5
Galliformes	Phasianidae	*Bambusicola*	*fytchii fytchiii*	Mountain Bamboo Partridge	250	260	1272H/ 1237L	56	6.7
Galliformes	Phasianidae	*Tragopan*	*caboti*	Cabot's Tragopan	320	380	P8/P2	49	6.7
Galliformes	Phasianidae	*Tragopan*	*temminckii*	Temminck's Tragopan	250	270	1272H/ 1237L	56	6.7
Gruiformes	Rallidae	*Gallirallus*	*owstoni*	Guam Rail	300	290	1272H/ 1237L	56	6.7
Gruiformes	Rhynochetidae	*Rhynochetos*	*jubatus*	Kagu	390	440	P8/P2	48	4
Gruiformes	Rhynochetidae	*Rhynochetos*	*jubatus*	Kagu	270	320	1272H/ 1237L	54	4
Gruiformes	Eurypgidae	*Eurypyga*	*helias major*	Greater Sunbittern	350	380	P8/P2	51	4
Gruiformes	Gruidae	*Grus*	*japonensis*	Red-crowned Crane	350	400	P8/P2	52	3.5
Charadriiformes	Alcidae	*Brachyramphus*	*marmoratus*	Marbled Murrelet	370	310	P8/P2	54	4
Columbiformes	Columbidae	*Caloenas*	*nicobarica nicobarica*	Common Nicobar Pigeon	400	390	P8/P2	50	6.7
Columbiformes	Columbidae	*Caloenas*	*nicobarica nicobarica*	Common Nicobar Pigeon	280	270	1272H/ 1237L	56	6.7
Columbiformes	Columbidae	*Columba*	*livia domestica*	Domestic Pigeon	360	320	P8/P2	52	3.5
Columbiformes	Columbidae	*Ducula*	*forsteni*	White-bellied Imperial Pigeon	420	390	P8/P?	50	6.7
Columbiformes	Columbidae	*Ptilinopus*	*magnificus Puella*	Northern Magnificent Fruit Dove	380	350	P8/P2	52	3.5
Psittaciformes	Psittacidae	*Ara*	*ararauna*	Blue and Gold Macaw	390	410	P8/P2	50	6.7
Psittaciformes	Psittacidae	*Ara*	*ararauna*	Blue and Gold Macaw	290	310	1272H/ 1237L	54	4
Psittaciformes	Psittacidae	*Ara*	*macao cyanoptera*	Northern Scarlet macaw	380	410	P8/P2	50	6.7
Psittaciformes	Psittacidae	*Ara*	*ambiqua ambiqua*	Great Green Macaw	360	380	P8/P2	50	6.7
Psittaciformes	Psittacidae	*Eclectus*	*roratus roratus*	Grand Eclectus Parrot	380	410	P8/P2	52	4
Psittaciformes	Psittacidae	*Eunymphicus*	*cornutus*	Horned Parakeet	400	390	P8/P2	52	3.5

Psittaciformes	Psittacidae	*Psittacula*	*cyanocephala*	Plum-headed Parakeet	370	410	P8/P2	52	3.5
Psittaciformes	Psittacidae	*Psittacula*	*cyanocephala*	Plum-headed Parakeet	260	300	1272H/1237L	56	6.7
Psittaciformes	Psittacidae	*Psittacula*	*longicauda*	Long-tailed Parakeet	350	380	P8/P2	52	3.5
Psittaciformes	Psittacidae	*Psittaculirostris*	*edwardsii*	Edward's Fig Parrot	400	410	P8/P2	52	3.5
Psittaciformes	Psittacidae	*Psittrichas*	*fulgidus*	Pesquet's Parrot	370	410	P8/P2	50	6.7
Psittaciformes	Loriidae	*Charmosyna*	*papou goliathina*	Mount Goliath Lorikeet	340	370	P8/P2	52	3.5
Psittaciformes	Loriidae	*Eos*	*semilarvata*	Blue-eared Lory	370	410	P8/P2	52	3.5
Psittaciformes	Loriidae	*Lorius*	*lory erythrothorax*	Geelvink Black-capped Lory	390	410	P8/P2	52	3.5
Psittaciformes	Loriidae	*Lorius*	*domicella*	Purple-Naped Lory	350	380	P8/P2	52	3.5
Psittaciformes	Loriidae	*Phigys*	*solitarius*	Collared Lory	370	410	P8/P2	52	4
Psittaciformes	Loriidae	*Psitteuteles*	*goldiei*	Goldie's Lorikeet	370	410	P8/P2	51	4
Psittaciformes	Loriidae	*Psitteuteles*	*iris iris*	Western Iris Lorikeet	360	410	P8/P2	52	3.5
Psittaciformes	Loriidae	*Trichoglossus*	*haematodus mitchellii*	Mithcell's Lorikeet	360	400	P8/P2	52	3.5
Psittaciformes	Loriidae	*Trichoglossus*	*johnstoniae*	Mount Apo Lorikeet	350	390	P8/P2	52	3.5
Psittaciformes	Loriidae	*Vini*	*australis*	Blue-crowned Lory	370	400	P8/P2	52	3.5
Musophagiformes	Musoohagidae	*Corythaeola*	*cristata*	Great Blue Turaco	380	310	P8/P2	48	4 to 5
Musophagiformes	Musoohagidae	*Corythaeola*	*cristata*	Great Blue Turaco	280	210	1272H/1237L	55	4
Trochiliformes	Trochilidae	*Colibri*	*coruscans coruscans*	Western Sparkling Violet-eared Hummingbird	390	410	P8/P2	48	6.7
Trochiliformes	Trochilidae	*Colibri*	*coruscans coruscans*	Western Sparkling Violet-eared Hummingbird	290	310	1272H/1237L	54	4
Coraciiformes	Alcedinidae	*Todirhamphus*	*cinnamominus*	Micronesioan Kingfisher	350	400	P8/P2	52	4
Coraciiformes	Coraciidae	*Coracias*	*cyanogaster*	Blue-bellied Roller	370	330	P8/P2	52	3.5
Coraciiformes	Coraciidae	*Coracias*	*benghalensis indicus*	South Indian Roller	380	350	P8/P2	49	4
Coraciiformes	Coraciidae	*Coracias*	*benghalensis indicus*	South Indian Roller	280	250	1272H/1237L	56	4
Passeriformes	Eurvlaimldae	*Calyptomena*	*viridis continentis*	Northern Lesser Green Broadbill	340	390	P8/P2	52	4
Passeriformes	Corvidae	*Corvus*	*hawaiiensis*	Hawaiian Crow (Alala)	330	380	P8/P2	52	3.5
Passeriformes	Corvidae	*Nucifraga*	*caryocatactes caryocatactaes*	Northern Spotted Nutcracker	350	390	P8/P2	51	4

TABLE 1. (continued)

Order	Family	Genus	Species	Common name	Z band (bp)	W band (bp)	Primer pair	anneal (°C)	(MgCl) mM
Passeriformes	Paradisaeidae	*Paradisaea*	*raggiana augustaev*	Empress of Germany Bird of Paradise	360	410	P8/P2	50	6.7
Passeriformes	Paradisaeidae	*Paradisaea*	*raggiana augustaev*	Empress of Germany Bird of Paradise	250	300	1272H/ 1237L	56	6.7
Passeriformes	Laniidae	Lonius	*ludovicianus mearnsi*	San Clemente Island Loggerhead Shrike	390	320	P8/P2	52	3.5
Passeriformes	Muscicapidae	*Copsychus*	*malabaricus*	Indochinese white-rumped shama	350	400	P8/P2	52	3.5
Passeriformes	Muscicapidae	*Cochoa*	*azurea*	Javan Cochoa	360	390	P8/P2	50	4
Passeriformes	Muscicapidae	*Cochoa*	azurea	Javan Cochoa	260	290	1272H/ 1237L	56	4
Passeriformes	Pycnonotide	*Pycnonotus*	*melanlcterus*	Javan Ruby-throated Bulbul	320	400	P8/P2	52	4
Passeriformes	Timaliidae	*Garrulax*	*galbanus simaoensis*	Yunnan Yellow Throated Laughingthrush	330	370	P8/P2	52	3.5
Passeriformes	Timaliidae	*Minla*	*cyanouroptera*	Eastern Blue-winged Sivia	350	400	P8/P2	52	4

PCRexpress gradient thermocycler (Hybaid, Franklin, MA) in three steps: 1) denaturization at 95°C for 5 min; 2) amplification for 35 cycles at 95°C for 45 sec, $T_{annealing}$ for 45 sec, 72°C for 1 min; and 3) a final extension at 72°C for 7 min, with a max ramp speed of 3°C/sec. PCR products were separated on a 2–3.3% agarose gel, and visualized on a Kodak DS (electrophoresis documentation and analysis system 120; Kodak, Rochester, NY). Species with small differences in the Z- and W-bands were electrophoretically separated on high percent gel to facilitate separation of close bands.

Statistical and Phylogenetic Analysis

The estimated size difference in PCR product length was analyzed for significance using analysis of variance (ANOVA) on StatView (Abacus Concepts®, Berkeley, CA). The estimated size differences were used to construct phylogenetic trees, using PAUP (Sinauer Associates, Inc., Sunderland, MA).

RESULTS

The amount and quality of gDNA isolated from shell-membrane blood vessels, blood, and feathers by both standard DNA isolation (Fig. 1A) and Chelex (Fig. 1B) protocols were sufficient for sexing by PCR. Figure 1C shows gDNA that was isolated from shell-membrane blood vessels (after storage for 1 month at room temperature in a sealed, sterile sample bag) and blood (after storage for 13 months at –20°C). There was only limited degradation of the gDNA.

Table 1 shows the PCR conditions, including preferred primer pair, annealing temperature, and $MgCl_2$ concentration, for 47 species of birds. The estimated sizes of the CHD-W and CHD-Z PCR products, as determined by agarose gel electrophoresis, are also shown.

The 1237L/1272H and P8/P2 primer pairs span the same intron, yielding PCR products with identical size differences between the Z- and W-bands (Fig. 2). The length of the Z- and W-introns differed between species of birds (Fig. 3 and Table 1); however, some families of birds consistently exhibited a larger Z-intron than W-intron. The W-intron was larger than the Z-intron in 18 of 19 species of Psittaciformes, all three Galliformes and the single Trochiliformes species tested. All Passeriformes (n = 10), Columbiformes (n=4), Charadriformes (n=1), Anseriformes (n = 1), and Musophagiformes (n=1) exhibited a larger Z-intron, whereas two of three Coraciiformes, and one of four Gruiformes exhibited a larger Z-intron. The difference in size between the introns amplified from the Z- and W-alleles were significantly larger in the Passerine group than in the non-Passerine group ($P > 0.0097$). When compared at the level of Order, the size differences between Z- and W-introns remained significantly greater in Passeriformes than in Galliformes ($P < 0.03$), Columbiformes ($P < 0.007$), and Psittaciformes ($P < 0.0008$), and tended to be greater than in Gruiformes ($P < 0.06$.). This is not surprising, considering the high level of diversity within the Order Gruiformes. In phylogenetic analysis, using the difference in intron length as a trait, Passeriformes and Coraciiformes were grouped together, with Gruiformes, Columbiformes, Psittaciformes, and Galliformes (in that order) progressively further away on the tree (data not shown).

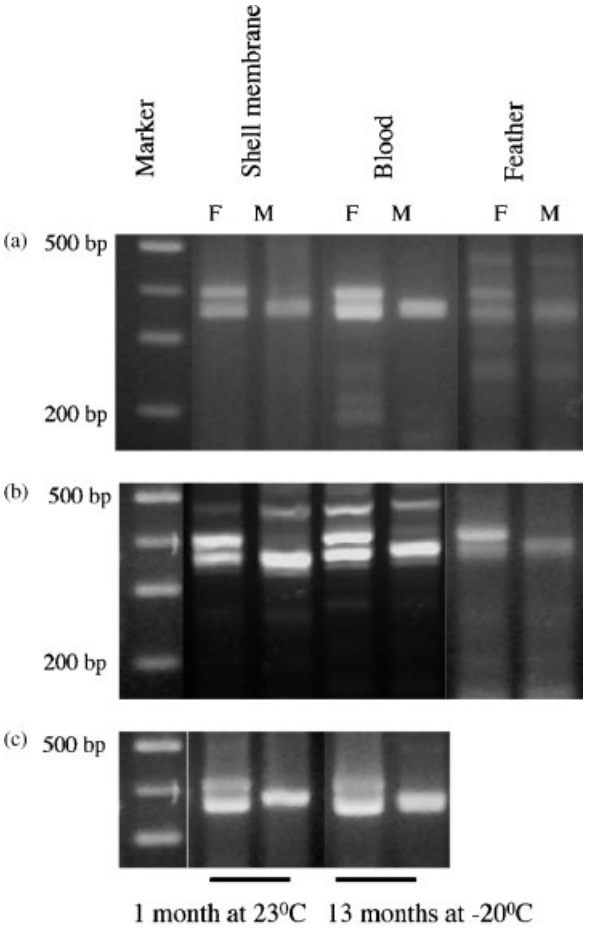

Fig. 1. Comparison of PCR products of gDNA isolated from fresh Pesquet's parrot shell membrane, blood, and feather. PCR products were separated on a 3% agarose gel. **A:** PCR products from gDNA isolated by DNA lysis-buffer followed by phenol/chloroform/IAA purification, with no further purification. **B:** PCR products from gDNA isolated by the Chelex method. **C:** PCR products from shell membrane and blood stored 1 month at room temperature and 13 months at –20°C, respectively, prior to DNA isolation by Chelex.

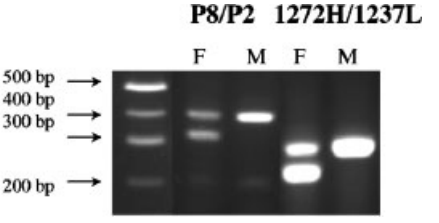

Fig. 2. Great Blue turaco sexing PCR products derived from primer pairs P8/P2 and 1237L/1272H. Although the size of the products differ, the size difference between the Z- and W-bands is consistent, as both primer pairs span the same intron.

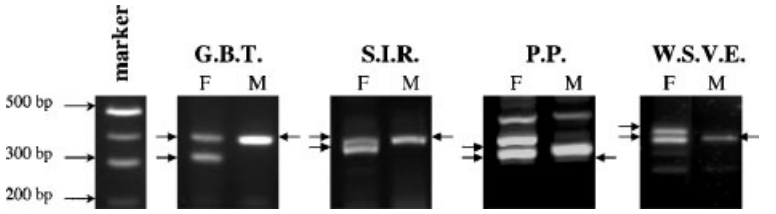

Fig. 3. The differences in length between the Z- and W-CHD intron spanned by the P8/P2 and H/L primers, and whether the Z- or the W-intron is longer, are species-specific. G.B.T., Great Blue turaco (Musophagidae); S.I.R., South Indian roller (Coraciidae); P.P., Pesquet's parrot (Psittacidae); W.S.V.E., Western sparkling violet eared hummingbird (Trochilidae).

DISCUSSION

Both the 1237L/1272H and the P8/P2 primer pair can be used to verify sexing results because products from both primers span the same intron. The primers anneal at two different sites, but both span the same intron and therefore yield the same difference in size between the Z- and W-bands. This is especially useful for verifying the sex of a species that has not previously been examined.

Changing the annealing temperature of the PCR by a few degrees and/or changing the $MgCl_2$ concentration allowed most species to be sexed using the P8/P2 primer pair. However, differences in PCR machines and laboratories may necessitate slight modifications of the parameters described here. The 1237L/1272H primer pair yielded more nonspecific bands (compared to the P8/P2 primer pair) on most species of birds tested, and thus obscured the Z- and W-bands. This may be a result of a lack of degeneracy in the 1237L/1272H PCR primer, or greater variability in the genetic sequence between species in the region of the 1237L/1272H primer pair.

The size differences between the Z- and W-products were significantly greater in passerine birds than in non-passerine birds ($P > 0.0097$). At the family level, only Galliformes ($P < 0.03$), Columbiformes ($P < 0.007$), and Psittaciformes ($P < 0.0008$) differed significantly from Passeriformes. However, because of the small sample size and the fact that we only estimated intron size in this study, it was difficult to ascertain whether Passeriformes, the newest and most successful branch on the tree, derive some evolutionary advantage from an increase in intron size. The difference in intron size for most species was 30–50 bp, but the range was 10–80 bp. With few exceptions, the identity (Z or W) of the longest band was consistent within the orders studied. Phylogeny analyses of the difference in intron size showed relationships consistent with the ANOVA test of variance, and grouped the Gruiformes, Columbiformes, Psittaciformes, and Galliformes progressively further away from the Passeriformes and Coraciiformes on the tree. The evolutionary or functional significance of this finding remains to be elucidated.

Because feathers yield a small amount of gDNA, nonspecific background bands can often complicate interpretation. Therefore, the routine protocol in our laboratory is to perform two independent isolations of feather gDNA with PCR to confirm results. Although in this study less gDNA was purified from feathers than from blood, all of the sources yielded sufficient quantities for correct sexing. The use

of shell-membrane blood vessels allows newly hatched chicks to be sexed without being handled. Likewise, feather sexing of adults minimizes handling compared to what is required in collecting blood. Minimal handling of birds in a breeding situation decreases stress and thus increases the chances of successful breeding.

Although the isolation of gDNA by standard DNA lysis-buffer and phenol/chloroform/IAA protocols yields very pure gDNA, it is time-consuming, requires expensive equipment, and generates hazardous phenol waste. gDNA isolated by Chelex is less pure, but the process can be completed in about 1 hr, with simpler equipment and no hazardous waste. These advantages make the Chelex technique available to a much larger number of facilities. Compared to phenol/chloroform extraction, Chelex isolation of gDNA may produce more nonspecific bands. However, these bands do not significantly interfere with identification of ZW-specific bands.

CONCLUSIONS

1. The use of Chelex yielded fast isolation of gDNA of sufficient quality to be used for sexing by PCR.

2. The isolation of gDNA from feathers or shell-membrane blood vessels yielded PCR products of similar quality to those derived from blood samples.

3. The storage of shell-membrane blood vessels at room temperature in sealed sample bags for short time periods, and blood at –20°C did not significantly affect our ability to correctly sex the individual.

4. The difference in size between the Z- and W-alleles of the CHD intron amplified by the P8/P2 primers was significantly ($P > 0.0097$) larger in Passeriformes than in non-Passeriformes. In addition, whether the Z- or the W-allele was larger in size was consistent within order and family (with a few exceptions), which may indicate an evolutionary selection pressure on intron size.

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